NOTE TO USERS

Transport of Proteins Across The Nuclear Envelope: The Ran GTPase System And Nuclear Import Of Heat Shock Proteins

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ABSTRACT

Nucleocytoplasmic trafficking of macromolecules and heat shock proteins are key elements of the stress response; the analysis of nuclear transport and hsp70s will therefore contribute significantly to our understanding of cell physiology.

To study the formation of the Ran/Gsp1p nucleocytoplasmic gradient, an essential component of many nuclear transport reactions, I have used mutant strains of the yeast *Saccharomyces cerevisiae*. My experiments have identified the nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p and the enzyme acetyl-CoA carboxylase (*MTR7*) as proteins that regulate the distribution and cellular concentration of Ran/Gsp1p. Moreover, I have defined the mechanisms that underlie the control of the Ran/Gsp1p gradient formation by these factors.

Using HeLa cells as a model system, I have demonstrated that the C-terminal part of mammalian hsc73 contains a unique targeting signal that is necessary and sufficient for stress-induced nuclear accumulation. This C-terminal segment promotes shuttling between nucleus and cytoplasm and has unique requirements for stress-dependent nuclear import.

Words: 152

RÉSUMÉ

Le trafic nucléo-cytoplasmique des macromolécules et des protéines de choc thermique sont des éléments clés de la réponse au stress; l'analyse du transport nucléaire et des hsp70s contribueront donc significativement à notre compréhension de la physiologie cellulaire.

Afin d'étudier la formation du gradient nucléo-cytoplasmique de Ran/Gsp1p, un composant essentiel de nombreuses réactions de transport nucléaire, j'ai utilisé des lignées de mutants de la levure *Saccharomyces cerevisiae*. Mes expériences ont identifié les nucléoporines Nup133p, Rat2p/Nup120p, Nup85p, Nic96p et l'enzyme acetyl-CoA carboxylase (*MTR7*) comme protéines qui régulent la distribution et la concentration cellulaire de Ran/Gsp1p. De plus, j'ai défini les mécanismes qui controlent le gradient de formation de Ran/Gsp1p par ces facteurs.

En utilisant les cellules Hela comme modèle, j'ai démontré que la partie C-terminale de hsc73 de mammifère contient un signal d'adressage unique, nécessaire et suffissant à l'accumulation nucléaire induite par le stress. Ce segment est responsable du transport nucléo-cytoplasmique et utilise une voie de transduction unique pour l'importation nucléaire dépendante du stress.

Words: 162

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CONTRIBUTION TO PUBLICATIONS

All experiments were designed and supervised by Dr. Ursula Stochaj.

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Figure 2.1-2.2 and table 2.3 were done by N. Sumanaweera, Figure 2.6-2.7 and Table 2.4 were performed by U. Stochaj. All other experiments were done by H. Gao.

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C. Sarmento, generated some of the constructs in Figure 3.1. H. Gao, did all other experiments.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CytB	Cytochalasin B
Crm1	Export receptor for the leucine-rich nuclear export signal
DAPI	4', 6-diamidino-2-phenylindole
GFP	*Aequorea victoria* fluorescent protein
Gsp1p	Ran homologue in *Sacharomyces cerevisiae*
hnRNP	Heterogeneous nuclear ribonucleoprotein
HSP	Heat shock protein
Hsc73	Heat shock protein 73
IBB	Importin-beta-binding domain of importin-alpha
NE	Nuclear envelope
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NTF2	Nuclear transport factor 2
Nup	Nucleoporin
PBS	Phosphate buffer saline
PK	Pyruvate kinase
RanBP	Ran-binding protein
RanGAP	Ran GTPase-activating protein
RCC1	Guanine nucleotide exchange factor in higher eukaryotes
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV40-NLS	Nuclear localization signal of SV40 T-antigen

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CHAPTER 1

INTRODUCTION

1. Nucleocytoplasmic transport

1.1 Transport across the nuclear envelope

The division of eukaryotic cells into different compartments gives rise to a need for intercompartmental transport of macromolecules. Nuclear transport is required, and both import and export occurs across the nuclear envelope (NE). All nuclear proteins, such as histones and transcription factors are synthesized in the cytoplasm, they must be imported into the nucleus, while messenger RNA (mRNA), ribosomal RNA (rRNA), and tRNA are synthesized in the nucleus and need to be exported to the cytoplasm. Moreover, many proteins shuttle continuously between the nucleus and cytoplasm. In total, this gives rise to an enormous level of nucleocytoplasmic traffic. In eukaryotic cells, nuclear transport is also an integral part of many regulated processes, such as gene expression, signal transduction and cell-cycle progression.

1.2 The Nuclear Pore Complex

Nucleocytoplasmic exchange of macromolecules occurs through a proteinaceous structure in the NE called nuclear pore complexes (NPCs). They are multiprotein assemblies that display an eight-fold rotational symmetry (Fig. 1.1). On the cytoplasmic face, there are eight flexible filaments that extend into the cytoplasm. The nucleoplasmic face also has eight filaments joint at their tips and forming a structure known as the nuclear basket. The cytoplasmic filaments and the nuclear basket contain docking sites for factors essential for nucleocytoplasmic trafficking. NPCs are large protein assemblies of approximately 125 MDa in mammalian cells and approximately 60 MDa in yeast. The NPC is composed of between 30-60 distinct polypeptides that are called nucleoporins

(Nups), some of which contain clusters of phenylalanine-glycine (FG) repeats. The NPC forms aqueous channels, which allow the diffusion of small molecules such as metabolites. In its closed position, the channel is ~9 nm in diameter, but it can expand to a diameter of ~26 nm during active translocation of cargo. When closed, the NPC mediates passive exchange of ions, small molecules and small proteins (<40 KDa), but restricts the passage of macromolecules to only those bearing appropriate signals [1-4].

1.3 Nuclear transport signals

Macromolecular cargo exceeding the diffusion radius of the NPC carries a nuclear localization signal (NLS), or a nuclear export signal (NES) to be able to either enter or exit the nucleus. The first identified NLSs, referred to as classical NLSs, fall into two categories: a single cluster of several basic residues (monopartite NLS), or two clusters of basic residues separated by a linker of 10-12 residues (bipartite NLS). The prototype of the monopartite NLS is present in the SV40 large T antigen, whereas nucleoplasmin contains a bipartite signal [5]. Both monopartite and bipartite NLSs bind in an extended conformation to the ARM-repeat domain of importin α [6, 7]. For export, leucine-rich NESs have been identified in a variety of cellular and viral proteins [8].

Besides the classical basic-type NLS, several other sequence motifs have been reported to mediate the nuclear import of proteins. None of them, however, appear to be as frequently encountered as the classical motif. A well-known example of a non-classical NLS is the so-called M9 sequence, a 38 amino acid –long fragment from hnRNPA1 and A2 proteins [9]. The M9 sequence is recognized by transportin, a member of the importin β family of receptors (see below). Other examples of non-classical signal are the KNS

sequence from hnRNP K, the HNS sequence of HuR protein and ankyrin repeats in IκBα [10-12]. In contrast to the conserved basic character of the cNLS, other NLS sequences are heterogeneous in nature. This opens the possibility that the diversity of signals remaining to be discovered may still be extensive. A common feature of all transport signals is that they are recognized by import or export receptors.

1.4 Receptors for nuclear targeting signals

Most transport events through the NPC are mediated by soluble receptors that specifically recognize their cargoes and facilitate the passage of receptor-substrate complexes in and out of the nucleus. As expected from the large variety of transport substrates, multiple classes of nuclear transport receptors exist. Most transport receptors are members of the importin β family, homologous proteins known as importins and exportins [13-15].

1.4.1 Importin α

Importin α is an adaptor protein that binds proteins containing classical NLSs and importin β1. The complete yeast genome reveals that *Saccaromyces cerevisiae* has a single gene for importin α, called Srp1. In contrast, in mammals, it has been demonstrated that importin α constitutes a multigene family and that this family can be classified into three distinct subgroups. Moreover, it is now clear that these distinct importin α molecules have a functional divergence and are differentially expressed in various tissues [16, 17]. Importin α is divided into three structural units: a central NLS-binding domain with ten armadillo (ARM) repeats, a small hydrophilic C-terminal domain of unknown function

and a positively charged, autoinhibitory N-terminal domain that can bind either the ARM domain or importin β. Importin α recognizes a variety of classical NLSs, such as the basic monopartite SV40 T antigen NLS (PKKKRKV), the more hydrophobic monopartite c-myc NLS (PAAKRVKLD) and the bipartite nucleoplasmin NLS (V**KR**PAATKKAQA**KKKK**LD) [18]. The domain of importin α that binds to canonical NLSs is a helical repeat built by the regular stacking of 10 ARM repeats. ARM repeats consist of approximately 40 amino acids that fold into three α-helices (H1, H2 and H3) arranged with an approximately triangular cross-section. The central ARM-repeat domain of importin α is flanked by a short C-terminus and an N-terminal importin β binding (IBB) domain. In the absence of importin β or an NLS, the IBB domain may function as an autoinhibitory segment by binding in an extended conformation at the NLS-binding pockets of importin α [19].

1.4.2 Importin β family

The importin β family of transport receptors includes fourteen proteins in yeast and at least 22 proteins in humans. The relative molecular masses of members of importin β proteins vary between 90 KDa and 130 KDa, but all are characterized by an acidic isoelectric point. The overall sequence similarity between various transport receptors is low (less than 20% amino acid identity) and, in many cases, restricted to the N-terminal domain. Nuclear transport receptors of the importin β family have distinct binding specificities but share a similar structural framework. They consist entirely of tandemly repeated modules known as HEAT motifs. These are sequences of approximately 40 residues characterized by a loose consensus pattern of conserved hydrophobic amino acids

folded into a bent helix (A) and a straight helix (B) [20-22]. Work mainly on importin β1 has demonstrated that these receptors bind RanGTP via the N-terminal domain and cargo (importin α) via the C-terminal domain [21, 23]. However, many importin β substrates bind to importin β through a mode distinct from that of importin α. For example, cyclin B1 and PTHrP have been shown to bind the N-terminus and central region (residues 380-642) of importin β1, respectively [24, 25]. These studies suggest that the importin β family may contain multiple distinct substrate-binding sites and, therefore, each importin β may also recognize more than one type of NLS. It appears that the variety of NLSs that bind each importin β may be quite large. In many importin β transport pathways, difficulty in identifying linear sequences as NLSs, coupled with the lack of sequence similarity of substrates within individual pathways, suggests that many of the unidentified signals may be defined by three-dimensional epitopes, rather than by linear amino acid sequences.

1.5 The Ran GTPase system

The small GTPase, Ran, which belongs to the Ras superfamily, and the proteins that regulate its GTP binding and hydrolysis have a well-defined role in nuclear transport [13, 14]. RCC1, the nucleotide exchange factor of Ran, is localized in the nucleus on chromatin, where it generates RanGTP. RanGAP, the GTPase-activating protein, in conjunction with either RanBP1 or RanBP2, stimulates the low intrinsic GTPase activity of Ran. RanGAP, RanBP1, and RanBP2 are all excluded from the nucleus [26]. Cytoplasmic Ran is found mainly in the GDP-bound form, while nuclear Ran is primarily in a GTP-bound state. As a result of this distribution there is a concentration gradient of RanGTP across the NPC: low in the cytoplasm and high in the nucleus. In addition, it has

clearly been shown that although Ran is sufficiently small to diffuse passively into the nucleus, the nuclear import of Ran is mediated by NTF2 [27].

Ran and its regulators have been proposed to function in numerous nuclear events, e.g. maintenance of the nuclear structure, nuclear import, cell cycle control, DNA replication, transcription, RNA processing and export [28, 29]. Importantly, RanGTP interacts with members of the importin β family and this causes dissociation of the import complexes inside the nucleus and generation of RanGTP/importin β complexes. The RanGTP gradient across the NPC controls the formation of import complexes: assembly occurs in the cytoplasm (low RanGTP) and disassembly in the nucleus (high RanGTP). For nuclear export, the presence of RanGTP in the nucleus permits the formation of the cargo/export receptor/RanGTP complexes. All importin β-like carriers involved in nuclear export bind their specific cargoes preferentially in the presence of RanGTP. The export complexes are translocated through the NPC to the cytoplasm, where GTP hydrolysis through the combined action of RanGAP and Ran BP1 results in dissociation of Ran from the complexes. As a consequence, the export receptor will release its cargo [30].

The importin β family members exit the nucleus as complexes with RanGTP, and therefore constantly deplete Ran from the nucleus. Ran is actively reimported into the nucleus via the transport factor NTF2, which binds specifically to RanGDP [31]. NTF2 is a 15 KDa protein conserved from yeast to higher eukaryotes. It also binds directly to FXFG-containing nucleoporins and mediates the translocation of Ran through the NPC. The release of Ran from NTF2 in the nucleus probably involves nucleotide exchange to generate RanGTP, for which NTF2 has no detectable affinity.

1.6 Classical nuclear transport pathway

Details of the classical NLS-mediated nuclear transport pathway are well understood. The key players in the import pathway are importin α, importin β, and the Ran GTPase system. The NLS of the cargo is recognized by importin α, which forms a complex with importin β. Importin β accounts for the interaction with the NPC and mediates the translocation of the cargo-importin complex through the NPC into the nucleus. The translocation is terminated by direct binding of nuclear RanGTP to importin β, which releases the complex from the NPC, dissociates importin α from importin β, and liberates the cargo into the nucleus. The RanGTP/importin β complex can directly exit into the cytoplasm. On the other hand, importin α, after releasing the cargo is recycled back into the cytoplasm by a specialized exportin, CAS. In the cytoplasm, both the RanGTP/importin β complex and the importin α/CAS/RanGTP complex are disassembeled before the importins can recombine for another round of import (Fig. 1.2 Stochaj and Rother 1998).

1.7 Models for the translocation through the NPC

Recent studies of the importin β-nucleoporin interactions and of the dynamic behavior of FG-repeats have led to several detailed models of translocation [32]. The Brownian affinity gating model proposes that the central NPC channel is gated at both ends by dynamic, dense filamentous FG nucleoporins that block the passive diffusion of all macromolecules except transport complexes that bind specifically to the nucleoporins [33]. Beyond the Brownian gate of the central channel, the asymmetric distribution of nucleoporins and soluble transport factors provides directionality for movement of the

transport complexes. A similar model, known as the selective phase hypothesis, has also been proposed to explain the translocation process. This model views the electron-dense central channel of the NPC as a network or mesh of hydrophobic interactions among FG repeats that allow the passage of small molecules, but present a barrier for larger ones [34]. Transport complexes bind FG repeats and become part of the meshwork to pass through the barrier. A third model for translocation is named the affinity gradient mechanism [35]. This model is based on evidence for a progressive increase in the affinity of importin β1 for the nucleoporins Nup358 (NPC cytoplasmic fibers), the p62 complex (central channel) and Nup153 (nuclear basket).

2. Heat Shock Proteins

2.1 Introduction

Cells respond to heat shock and a variety of stresses by the rapid synthesis of a highly conserved set of polypeptides known as heat shock proteins (Hsp). They are important modifying factors in cellular responses to a variety of physiological conditions such as exercise, hyperthermia, metabolic challenges, and aging [36]. Hsps comes in different forms and are classified into families based on their molecular weight. Heat shock protein 70s (Hsp70s) are a highly conserved family of proteins. They can be found in all prokaryotes and in different cellular compartments of eukaryotes. Hsp70s function as chaperons, they are important to maintain cellular functions during exposure to stress. Stress may result in damaged and misfolded proteins that require the assistance of molecular chaperones for refolding. Hsp70s can recognize reversibly and irreversibly damaged proteins, repair misfolded proteins and help to prevent inter- or intramolecular

aggregation. In these processes, Hsp70s bind short hydrophobic segments in partially folded polypeptides, thereby preventing aggregation and arresting the folding process [37, 38].

The mechanism for the induction of Hsp expression is through the heat shock transcription factors (HSFs). HSFs have a NLS that is both necessary for the transition of HSFs from inactive to active state and for nuclear import. Depending on the stress stimulus, several proteins kinases have been shown to phosphorylate HSF1, which is considered to be the main HSF. Upon stress, HSF1 is translocated to the nucleus and binds to the promoter of stress-inducible Hsp genes to induce their transcription [39].

Hsp70s consist of three functionally coupled domains, which have been crystallized separately [40]. The 44 KDa N-terminal domain mediated ATP binding followed by a 18 KDa polypeptide-binding domain that binds the substrate polypeptide and a variable C-terminal domain. The ATPase domain transmits conformational changes to the substrate-binding domain depending on the presence of ATP.

2.2 Nuclear transport of Hsp70s

Hsc70 is localized in the cytoplasm and the nucleus and normally shuttles between these compartments [41]. Hsc70s contain a single basic amino acid sequence, KRKHKKDAISENKRAVRR, which extends from position 246 to 262 [94, 95]. However, inactivation of this signal by deletion of the first six amino acids had no effect on Hsc70 import [96], suggesting that an alternative signal can be used to import Hsc70s into nuclei. Chu *et al.* indicated that heat-induced nuclear concentration of Hsc70 proteins depends on cell density. Low-density cultures efficiently imported Hsc70s into nuclei when exposed

to heat, whereas high density cultures failed to do so. Furthermore, inhibitors of Ser/Thr phosphatases and Tyr phosphatases abolished the nuclear concentration of Hsc70 proteins [93]. Shulga *et al.* showed that the C-terminal domain of Ssb1p, a cytoplasmic Hsc70 in *Saccharomyces cerevisiae*, contains a leucine-rich nuclear export signal and this signal is necessary and sufficient to direct nuclear export [103]. Unpublished data of our laboratory show that Hsc70 export into the cytoplasm is insensitive to leptomycin B, a specific inhibitor of Crm1/exportin1. However, latrunculin B and cytochalasin B, drugs that affect actin filament formation, prevent Hsc70 nuclear export.

Hsc70s have been implicated in classical nuclear import, but the mechanism is still not understood. Inhibition experiments involving either microinjection of anti-Hsc70 antibodies into cells or their addition *in vitro* assays provided evidence for a role of Hsc70 in NLS-protein import [42, 43]. Imamato *et al.* found that BSA-SV40 large T NLS conjugates were unable to enter the nuclei of cultures embryonic lung cells when coinjected with anti-Hsc70 antibodies [43]. More persuasively, depletion of Hsc70s from cytosolic extracts abolished nuclear import *in vitro*. This effect could be reversed by addition of recombinant Hsc70. More recent experiments in yeast showed that overexpression of Hsc70 increases import rates [44]. Data obtained by Yang and DeFranco showed that Hsc70, although required for the nuclear import of BSA-large T NLS conjugates, is not necessary for the uptake of the glucocorticoid receptor; thus, the requirement for Hsc70 might not be universal, but limited to a subset of karyophilic proteins [45]. Although it is difficult to rule out nonspecific effects of the Hsc70s in all of these experiments, i.e. a general stabilization of the active conformation of import factors, these results may indicate a direct role for Hsp70s in import.

3. Objectives and rationale of the project

3.1 Objectives

Living organisms are constantly exposed to stress. Therefore, appropriate cellular responses need to ensure the survival and repair of stress-induced damage. My projects focus on the Ran GTPase system and Hsc70 nuclear import upon heat stress. The following aspects were investigated:

(1) The role of nucleoporins in the generation of the Ran/Gsp1p nucleocytoplasimc gradient.

(2) The mechanisms underlying the nuclear transport of hsc73 in response to heat shock.

3.2 Rationales

In chapter 2, we determine the role of nucleoporins in the generation of the nucleocytoplasmic Gsp1p (the Ran GTPase in yeast) concentration gradient in the yeast *Saccharomyces cerevisiae.* Previous studies have revealed that several FXF repeat nucleoporins participate in nuclear trafficking. We have now identified several non-repeat containing nucleoporins and the enzyme acetyl-CoA carboxylase as components that play a role in the nuclear accumulation of Gsp1p, thereby regulating trafficking across the NPC.

In chapter 3, we investigated the mechanisms underlying heat shock proteins' nuclear accumulation upon stress in HeLa cells. Proteins of the Hsc70 family relocate to the nucleus upon heat stress when other nuclear transport reactions, such as classical nuclear protein import, are inhibited [46, 47]. We propose that the nuclear transport of Hsc70s follows a unique pathway. I have identified a novel shuttling sequence in the

C-terminal domain of hsc73, and I have defined the requirements for stress-induced nuclear accumulation of this domain.

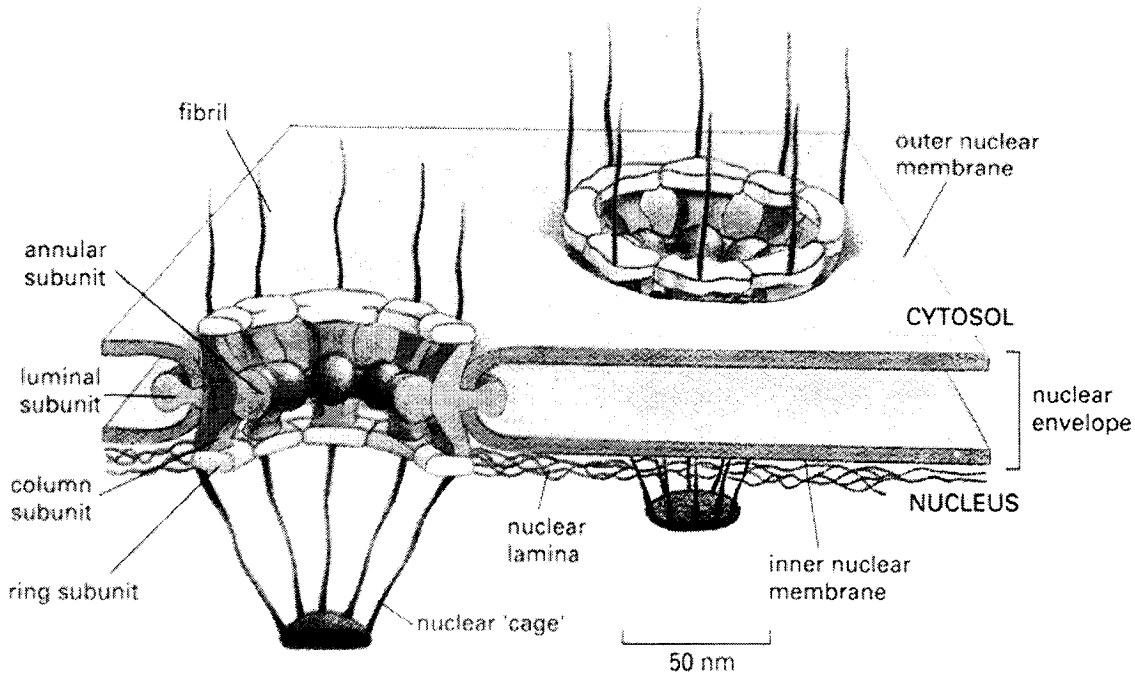


Fig. 1.1 The nuclear pore complex.

A schematic three-dimensional representation of the nuclear pore complex.

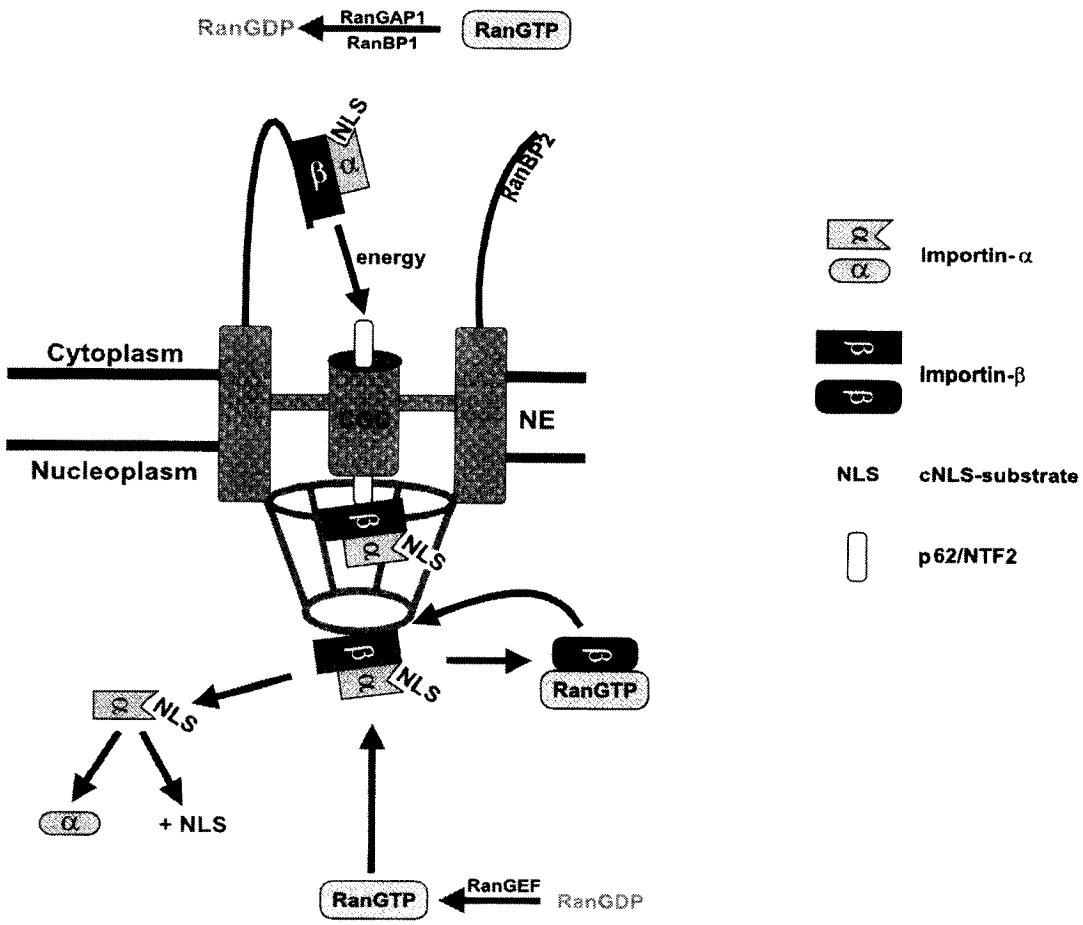


Fig. 1.2 Classical nuclear import.

A schematic representation of nuclear import of proteins with classical NLSs. For each import cycle, the NLS of the cargo is recognized by importin α, which forms a complex with importin β1, which interacts with the NPC and mediates the translocation of the cargo-importin complex through the central gated channel (CGC) of the NPC into the nucleus. RanBP2 can provide an initial docking site by binding to importin β1. Translocation is terminated in the nucleus by direct binding of nuclear RanGTP to importin β1, which releases the complex from the NPC, dissociates importin α from the importin β1, and releases the cargo into the nucleus. The RanGTP/importin β complex can directly exit into the cytoplasm. Upon reaching the cytoplasm, RanGTP is converted to RanGDP, this reaction is stimulated by RanGAP and RanBP1. Ran GDP is then taken by NTF2 into the nucleus, where the RanGEF RCC1 promotes the conversion of RanGDP to RanGTP. Please see text for more details.

CHAPTER 2:

Nuclear accumulation of the small GTPase Gsp1p depends on nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p and the acetyl-CoA carboxylase Acc1p.

Preface

This chapter of my thesis is based on the following paper:

Gao, H.*, Sumanaweera, N.*, Bailer, S.M., and Stochaj, U. (2002) **Nuclear accumulation of the small GTPase Gsp1p depends on nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p and the acetyl-CoA carboxylase Acc1p.** (* Both authors contributed equally to this work) *J. Biol. Chem.* **278**: 25331-25340

Contribution to the study:

The study presented in this chapter was designed and supervised by Dr. U. Stochaj. Experiments in Figure 2.1 and Figure 2.2 as well as Table 2.3 were carried out by N. Sumanaweera, results in Figure 2.6, Figure 2.7 and Table 2.4 were obtained by U. Stochaj. All other experiments were done by H. Gao.

Abstract

The small GTPase Ran/Gsp1p plays an essential role in nuclear trafficking of macromolecules, as Ran/Gsp1p regulates many transport processes across the nuclear pore complex (NPC). To determine the role of nucleoporins in the generation of the nucleocytoplasmic Gsp1p concentration gradient, mutations in various nucleoporin genes were analyzed in the yeast *Saccharomyces cerevisiae.* We show that the nucleoporins Nup133p, Rat2p/Nup120p, Nup85p, Nic96p, and the enzyme acetyl-CoA carboxylase (*MTR7*) control the distribution and cellular concentration of Gsp1p. At the restrictive temperature the reporter protein GFP-Gsp1p, which is too large to diffuse across the nuclear envelope, fails to concentrate in nuclei of *nup133Δ, rat2-1, nup85Δ, nic96ΔC,* and *mtr7-1* cells, demonstrating that GFP-Gsp1p nuclear import is deficient. In addition, the concentration of Gsp1p is severely reduced in mutants *nup133Δ*and *mtr7-1* under these conditions. We have now identified the molecular mechanisms that contribute to the dissipation of the Gsp1p concentration gradient in these mutants. Loss of the Gsp1p gradient in *nup133Δ*and *rat2-1* can be explained by reduced binding of the Gsp1p nuclear carrier Ntf2p to NPCs. Likewise, *nup85Δ* cells that mislocalize GFP-Gsp1p at the permissive as well as non-permissive temperature have a diminished association of Ntf2p-GFP with nuclear envelopes under both conditions. Moreover, under restrictive conditions Prp20p, the guanine nucleotide exchange factor for Gsp1p, mislocalizes to the cytoplasm in *nup85Δ, nic96ΔC*, and *mtr7-1* cells, thereby contributing to a collapse of the Gsp1p gradient. Taken together, components of the NPC subcomplex containing Rat2p/Nup120p, Nup133p, and Nup85p, in addition to proteins Nic96p and Mtr7p, are shown to be crucial for the formation of a nucleocytoplasmic Gsp1p gradient.

Introduction

Transport of macromolecules across the nuclear envelope is mediated by nuclear pore complexes (NPCs) and requires soluble factors as well as nucleoporins, the components of the NPC [48, 49]. Although yeast NPCs are less complex than their mammalian counterparts [50] the mechanisms of nucleocytoplasmic trafficking are conserved among eukaryotes. The key component believed to control the directionality of transport between nucleus and cytoplasm is the small GTPase Ran in higher eukaryotes or its yeast homolog Gsp1p [48, 49, 51]. Under normal growth conditions cells display a Ran/Gsp1p concentration gradient, with high levels in the nucleus and low concentration in the cytoplasm. Ran/Gsp1p occurs in two different forms in the cell, predominantly bound to GTP in the nucleus or associated with GDP in the cytoplasm. The different nucleotide-bound states are generated by the unequal distribution of GTPase modulating factors; the GTPase-activating protein Rna1p (or the higher eukaryotic homolog RanGAP1) is preferentially located in the cytoplasm, where it generates Gsp1p-GDP. In contrast, the GTP/GDP exchange factor Prp20p (RCC1 in higher eukaryotes) is concentrated in the nucleus, where it binds to chromatin [52]. Prp20p catalyzes the production of nuclear Gsp1p-GTP, which is believed to associate with nuclear anchors. The association with anchors would then prevent Gsp1p diffusion into the cytoplasm and help maintain high Gsp1p concentrations in the nucleus. Members of the importin family of transporters and other factors may retain Ran/Gsp1p in the nucleus [47, 53]. Furthermore, Mog1p, a nuclear binding protein for Gsp1p-GTP, is required for the generation or maintenance of a nucleocytoplasmic Gsp1p gradient [47]. It is believed that the asymmetric distribution of Ran/Gsp1p-GTP and Ran/Gsp1p-GDP fuels protein import

as well as nuclear export of proteins and some RNAs.

In higher eukaryotes, translocation of Ran across the nuclear envelope requires Ntf2, a small protein that specifically binds to Ran-GDP [53, 54]. Ntf2 transports Ran-GDP into the nucleus where RCC1 converts it to Ran-GTP, thereby dissociating the Ran-Ntf2 interaction. The nucleoporin p62 is likely to play an important role for the translocation of Ran across the NPC, as both Ran and Ntf2 bind to p62. In particular, the F*X*F repeats present in the N-terminal domain of p62 are involved in these binding reactions [55]. Similarly, the yeast nucleoporin Nsp1p, a functional homolog of p62, binds to Ran and Ntf2 [55]. As well, the repeat domain of Nsp1p interacts with Gsp1p *in vitro* [56], indicating that the interactions Ran-p62 and Nsp1p-Gsp1p have been conserved and Gsp1p accumulates in yeast nuclei by the same mechanism as in metazoan cells. This idea is further supported by our recent results, which show that mutations in the yeast *NTF2* gene interfere with Gsp1p nuclear accumulation.

In addition to soluble factors, nuclear trafficking requires nucleoporins. With about 30 different nucleoporins, the NPC overall organization in yeast is less complex but similar to higher eukaryotes [33]. Various yeast nucleoporins have been shown to be involved in nuclear transport of proteins, RNA, or both types of macromolecules. For instance, Nup49p and Nsp1p play a role in protein import as well as mRNA export [58-63]. For *RAT2/NUP120, NUP82,* and *NUP159*, only mutations affecting the nuclear export of mRNA have been reported so far [64-68]. Recent studies on nuclear export of 60 S ribosomal subunits showed that yeast strains carrying mutations in *NSP1, NUP49,* or *NIC96* accumulate 60 S subunits, whereas *NUP84* or *NUP85* deletion mutants were not impaired [69]. Moreover, mutations in *NSP1* and *NUP159* interfere with nuclear export of

the precursor of signal recognition particle [70]. With respect to nucleocytoplasmic transport, the nucleoporin Nsp1p is particularly interesting as it assembles into biochemically distinct protein complexes [70-72]. Nsp1p is associated with Nup49p, Nup57p, and Nic96p both on the cytoplasmic and nuclear side of the NPC [64,70,72]. In contrast, the Nup82p-Nsp1p-Nup159p subcomplex is found on the cytoplasmic side only [64,65,67,68,72,73], where it interacts with Nup116p-Gle2p, an association likely to be required for mRNA export [74,75]. Despite the pivotal role of Nsp1p in nucleocytoplasmic trafficking, only its C-terminal domain, which does not contain F*X*F repeats, is essential for viability. Furthermore, mutant *nsp1-ts18* fails to incorporate the Nsp1p-Nup57p-Nup49p complex into NPCs. Thus, NPCs in *nsp1-ts18* are lacking the repeats provided by this core complex. Although impaired in growth, the *nsp1-ts18* mutant is viable. These observations are consistent with the idea that members of the Nsp1p complex perform a function that is redundant with other nucleoporins.

Previous *in vitro* analyses revealed that the middle FG repeat domain of Nsp1p directly interacts with Gsp1p and also with Ran, the mammalian homolog of Gsp1p. By contrast, binding to the essential C-terminal domain was not observed. Various mutations located in subdomains coil 1–4 of the essential C-terminal part of Nsp1p show distinct defects in nucleocytoplasmic trafficking. As such, strain *nsp1-5* (coil 2) fails to accumulate MatΔ2 and Pho2p in nuclei at the non-permissive temperature. Likewise, *nsp1-5* and *nsp1-ala6* (coil 2) are impaired in export of the 60 S ribosomal subunit [69,76] Furthermore, mutant L640 S (coil 1) [77], shows mRNA export defects, whereas L697P (coil 2), ts18 (coils 3 and 4), and W644C (coil 1) are deficient in nuclear import of GFP-Npl3p and classical nuclear import under nonpermissive conditions. Together with

Nsp1p and other nucleoporins Nic96p forms an NPC subcomplex [73]. Nic96p is organized into three domains, with heptad repeats in the N-terminal portion and stretches of uncharged amino acid residues in the central domain. Deletion of Nic96p heptad repeats and point mutations in the central portion, such as *nic96-1*, impair nuclear protein trafficking [64]. By contrast, RNA export defects have not been observed in these mutants [64]. Like nsp1p, mutant nic96p can alter the export of 40 S and 60 S ribosomal subunits from the nucleus [78]. Likewise, Nic96p is required for classical nuclear protein import [64].

Synthetic lethal screens with mutant *nsp1* alleles identified *NUP84* and *NUP85* as components genetically interacting with *NSP1* [79]. Nup84p, Nup85p, and Rat2p/Nup120p are part of the same NPC subcomplex consisting of several distinct proteins [79,80] and cells carrying a deletion of *NUP84, NUP85/RAT9,* or *RAT2/NUP120* show defects in poly(A)$^+$ mRNA export [79-83]. Moreover, disruption of the *NUP84* or the *NUP85* gene causes abnormalities of the NPC and nuclear envelope organization [79,80]. In addition, mutant alleles of *RAT2/NUP120* or deletions of nucleoporin genes like *NUP133* induce clustering of NPCs [81,83]. However, cluster formation does not correlate with transport defects, and both mutants show clustering even under permissive conditions. Mutations in Nup133p are synthetic lethal with *nup85, rat2/nup120,* and *nsp1* [74] and two-hybrid screens have identified Nup84p as a component that interacts with Nup133p [75]. Furthermore, Nup133p associates with the Nup84p complex *in vitro* and *in vivo* [84,85].

Previous studies have revealed that several nucleoporins, in particular Nsp1p, participate in nuclear trafficking. This prompted us to determine their role in the nuclear

accumulation of Gsp1p, a GTPase implicated in various nuclear transport reactions. Although the interaction of Gsp1p/Ran and Ntf2 with F*X*F repeats of nucleoporins is well established, other components of the NPC involved in Gsp1p nuclear transport have yet to be defined. For instance, zinc finger-containing nucleoporins that bind Ran-GDP in mammalian cells have not been found in *Saccharomyces cerevisiae*. We have now identified several non-repeat yeast nucleoporins that play a role in the nuclear accumulation of Gsp1p, thereby regulating trafficking across the NPC.

Methods and Materials

Yeast Strains and Growth Conditions

Yeast strains used in this study are listed in Table 2.1. Cells growing logarithmically in YEPD at room temperature were exposed to 37°C for the times indicated in the figure. Standard procedures were used for yeast transformation and selection of transformants.

Plasmids

To monitor classical nuclear transport, the gene encoding SV40-GFP [44] was transferred into a centromeric plasmid carrying the *URA3* marker. Expression of SV40-GFP is controlled by the *ADH1* promoter. Nuclear accumulation of SV40-GFP requires classical nuclear import to be constitutively active as described previously [47]. A centromeric plasmid carrying the *GFP-GSP1* gene and a *LEU2* marker was kindly provided by D. Lau and E. Hurt (Heidelberg, Germany). To allow expression in mutant strains that are Leu^+, we have transferred the *ADH1* promoter and the *GFP-GSP1* coding sequence into a centromeric plasmid containing the *URA3* gene. To generate *NTF2-GFP,* the complete coding sequence of *NTF2* was fused in-frame to *GFP,* and the correctness of the construct was verified by DNA sequencing. For regulated gene expression in yeast, *NTF2-GFP* was cloned into centromeric plasmids containing the *GAL1* promoter and the *URA3* or *LEU2* marker. Expression of *NTF2-GFP* was induced by overnight growth at room temperature in selective medium supplemented with 2% galactose.

Generation and Affinity Purification of Antibodies

His_6-tagged Gsp1p was affinity-purified to raise antibodies in mice [47]. Polyclonal

antibodies were generated against purified His_6-tagged Rna1p essentially as described. For immunofluorescence studies antibodies were preadsorbed to the His_6-tag and affinity-purified with immobilized His_6-Rna1p.

Fluorescence Microscopy

Fixing of yeast cells, generation of sphero-plasts, and incubation with various antibodies have been described previously [47]. Affinity-purified secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Molecular Probes, Eugene, OR) were diluted 1:1000 (Cy3-coupled anti-mouse IgG) or 1:250 (TRITC-labeled anti-rabbit IgG). Cells were incubated with secondary antibodies for 60 min at room temperature and washed three times in PBS/bovine serum albumin. DNA was stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI), and slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA). To visualize GFP-containing reporter proteins, cells were fixed for 10 min in 3.7% formaldehyde, collected by centrifugation, and resuspended in 0.1 M potassium phosphate, pH 6.5, 1.1 M sorbitol. Cells were immobilized on polylysine-coated multiwell slides and stained with 1 μg/ml DAPI in PBS/bovine serum albumin for 2 min at room temperature. Slides were then mounted as described above. Cells were inspected with a Nikon Optiphot at x 1000 magnification and photographed with TMAX 400 films. Negatives were scanned and processed with Adobe Photoshop 5.5.

Western Blotting

Western blot analysis was carried out essentially as described [86]. In brief, equal amounts of protein from unstressed and stressed cells were separated by SDS-PAGE and blotted to

nitrocellulose. Filters were blocked with 5% skimmed milk in PBS, 0.1% Tween 20 and incubated with primary antibodies in PBS/Tween 20/milk overnight at 4°C. To detect anti-Gsp1p antibodies, filters were incubated with horseradish peroxidase-coupled secondary antibodies in PBS/Tween 20/milk for 1 h at room temperature. Filters were washed, and immunoreactive material was visualized with an ECL system (PerkinElmer Life Sciences).

Results

Heat Stress Transiently Collapses the Nucleocytoplasmic Gradient of Gsp1p

We have demonstrated recently that heat stress affects the Gsp1p concentration gradient across the nuclear envelope. Heat shock at 37°C for 10 min transiently increases the cytoplasmic concentration of Gsp1p (Fig. 2.1D). However, as cells adapt to heat, the Gsp1p gradient is regenerated in wild type cells, and Gsp1p again becomes concentrated in nuclei. In various wild type strains the Gsp1p gradient was rebuilt after about 1 h of exposure to 37°C and remained stable when cells were kept at 37°C (Fig. 2.1F, H, J). When wild type cells were incubated in the presence of cycloheximide for 6 h, the Gsp1p gradient collapsed, even at room temperature (Fig. 2.1L). With the assay described in figure 2.1, we have shown previously that mutations in *NTF2, PRP20,* and *MOG1* alter the nucleocytoplasmic distribution of Gsp1p. We have now used this protocol to determine whether mutations in nucleoporins or the *MTR7* gene, which is involved in nuclear envelope organization, affect the Gsp1p gradient formation when cells are treated with heat. As long periods of exposure to non-permissive conditions may have secondary effects on the Gsp1p gradient, we have exposed cells for a maximum of 6 h to elevated temperatures. Therefore, our studies have identified only mutants that display a rapid collapse of the Gsp1p concentration gradient under non-permissive conditions.

The Nucleoporin Mutants *Nup133Δ, rat2-1,* and *nup85Δ* Mislocalize Gsp1p upon Heat Stress

When the clustering strain *nup133*Δ was incubated for 3 h at 37°C, the non-permissive temperature, cytoplasmic levels of Gsp1p increased as compared with cells kept at room

temperature (Table 2.2). Moreover, after 6 h at 37°C the Gsp1p concentration gradient had collapsed in most cells (Fig. 2.2D and Table 2.2). Likewise, in the clustering mutant *rat2-1* the Gsp1p nucleocytoplasmic gradient dissipated at the restrictive temperature (Fig. 2.2H) Double immunofluorescence labeling with antibodies against nucleoporins and Gsp1p demonstrated that the GTPase associated with NPC clusters in both mutants under permissive conditions (data not shown). To characterize further the defect of *nup133*Δ and *rat2-1* in maintaining a nucleocytoplasmic gradient of Gsp1p at 37°C, we compared levels of the GTPase in control and heat-treated cells by Western blot analysis. When equal amounts of protein from unstressed and heat-shocked cells were analyzed in parallel, Gsp1p levels were reduced in *nup133*Δ, but no drastic changes were observed for *rat2-1* (Fig. 2.3 and Table 2.2) Additional members of the Nup84p-Nup133p NPC module were studied for Gsp1p distribution. As such, 6 h of heat exposure abolished the gradient formation in *nup85*Δ cells but did not alter the Gsp1p concentration gradient in *nup84*⁻ cells, which carry a complete disruption of the *NUP84* gene. Although the deletion of *NUP85* prevented Gsp1p gradient formation at elevated temperatures, concentrations of the GTPase were similar to unstressed cells (Fig. 2.3). Taken together, our results demonstrate that three members of the Nup84p-Nup133p NPC subcomplex which *in vitro* are essential for its assembly are involved in concentrating Gsp1p in nuclei of stressed cells.

The Nucleocytoplasmic Gradient of Gsp1p Collapses in the mRNA Transport Mutant *mtr7-1* under Restrictive Conditions

Yeast cells carrying the *mtr7-1* mutation, also called *acc1-7-1*, fail to synthesize very long

chain fatty acids under non-permissive conditions. At the restrictive temperature NPCs appear as "spots," and increased cytoplasmic concentrations of nucleoporins are detected (data not shown and see Ref. 87). Furthermore, the integrity of the nuclear envelope and nuclear export of mRNAs are defective at 37°C [87]. Thus, the *mtr7-1* allele has a more general effect on nuclear envelope organization that could also alter nucleocytoplasmic trafficking and retention of Gsp1p. In line with this idea, we found elevated cytoplasmic concentrations of the GTPase upon shift to 37°C for 3 h (Table 2.2). After 6 h at 37°C, Gsp1p was no longer accumulated in nuclei. Furthermore, the total concentration of the GTPase was reduced (Fig. 2.3).

Mutations in the Essential C-terminal Domain of NSP1 Have Only Minor Effects on the Distribution of Gsp1p

We have analyzed the role of several *NSP1* mutations (see Introduction) in establishing a Gsp1p concentration gradient. Despite pronounced consequences for nuclear transport, mutations in the essential C-terminal domain of Nsp1p had less severe effects on Gsp1p distribution and concentration (Table 2.3). For instance, at room temperature *nsp1-5* displayed a poor nucleocytoplasmic Gsp1p gradient, with elevated cytoplasmic levels of Gsp1p (Fig. 2.4B). Upon exposure to heat, however, Gsp1p gradients improved, and the GTPase became restricted to nuclei (Fig. 2.4D and Table 2.2). One possible interpretation of these results is the preferential degradation of Gsp1p in the cytoplasm in response to heat stress. Indeed, when equal amounts of protein from unstressed and stressed cells were analyzed by Western blotting, the concentration of Gsp1p was found to be slightly decreased at 37°C (Fig. 2.3 and Table 2.2). Improved nucleocytoplasmic Gsp1p gradients

at the non-permissive temperature were also detected in other strains mutated in different coils of the C-terminal Nsp1p domain, *i.e.* mutants *nsp1-ala6*, L640S, ts18, or W644C. By contrast, Gsp1p protein levels did not change noticeably at 37°C in these strains (Table 2.2). Taken together, mutations in different coils of the C-terminal segment, which have strong defects in nuclear trafficking, did not drastically alter the concentration gradient of Gsp1p or levels of the GTPase.

Deletion of the C-terminal *NIC96* Domain Changes the Distribution of Gsp1p

We tested the distribution of Gsp1p in different nic96p mutants (see Introduction), with *nic96ΔN* missing residues 28–63, *nic96-1* carrying the mutations L260P and P332L, and *nic96ΔC* lacking residues 532–839 of the C-terminal domain. All of the mutants mislocalized Gsp1p after prolonged heat treatment, but *nic96ΔC* showed the most severe effect (Fig 2.4L). By contrast, levels of Gsp1p were not drastically altered in any of the *nic96* mutants under restrictive conditions (Fig. 2.3 and Table 2.2). In summary, our results show that the C-terminal domain of Nic96p plays a critical role in generating or maintaining a high concentration of Gsp1p in nuclei.

Effect of Mutant Nucleoporins on Classical Nuclear Protein Import

To determine whether classical nuclear protein import is altered in nucleoporin mutants under the conditions used for our experiments, we have introduced the fluorescent reporter protein SV40-GFP. To concentrate SV40-GFP in nuclei, classical nuclear protein import has to be constitutively active, and the inhibition of import can be monitored by the appearance of SV40-GFP in the cytoplasm [47]. At room temperature, all strains

accumulated SV40-GFP in nuclei, although some cytoplasmic localization was detected for several of the mutant strains (summarized in Table 2.3). Upon exposure to 37°C, wild type cells adapted, and the reporter protein was concentrated in nuclei when cells were inspected after 3 and 6 h of heat treatment (Table 2.3). By contrast, cells carrying the mutations *nup133*Δ, *rat2-1, mtr7-1, nsp1-, nsp1-ala6, nsp1-ts18, nic96*Δ*C, nup84*¯, and *nup85*Δ failed to accumulate SV40-GFP in nuclei after heat exposure. However, this is not a general defect in nucleoporin mutants. For instance, *rat7-1*, a mutant impaired in mRNA export, did not show a defect in classical nuclear import when incubated for 6 h at 37°C. Based on the Gsp1p distribution upon incubation at 37°C (see above), we have assigned the classical transport mutants to two different groups: group A, cells for which the Gsp1p gradient collapsed in response to heat stress (this includes *nup133*Δ, *rat2-1, mtr7-1, nic96*Δ*C*, and *nup85*Δ); group B, several *nsp1* mutants and *nup84*¯ display an intact Gsp1p gradient after 6 h at 37°C. Defects in classical nuclear protein transport for members of group A can be explained by the failure of cells to build a nucleocytoplasmic gradient of Gsp1p. By contrast, different mechanisms of import inhibition operate for mutants in group B. In the following, we have further characterized members of group A.

Import of Gsp1p in Mutant Nucleoporin Strains

With a molecular mass of ~25-KDa, Gsp1p is small enough to diffuse in and out of the nucleus. Once diffused through the NPC, retention could concentrate Gsp1p in nuclei. However, transport of Gsp1p across the nuclear envelope could also result in nuclear accumulation of the GTPase. In the second scenario, elevated levels of Gsp1p in the cytoplasm will indicate a defect in Gsp1p nuclear import. To determine whether Gsp1p

nuclear import plays a role in mutants of group A, we have monitored the nuclear accumulation of GFP-Gsp1p, a protein of ~52 KDa, which is too large to diffuse efficiently across NPCs. To this end, cells synthesizing the reporter protein were incubated at room temperature or 37°C (Fig 2.5 and Table 2.3) and the fusion protein was subsequently localized by fluorescence microscopy. In wild type cells, GFP-Gsp1p accumulated in nuclei of unstressed and stressed cells, and the same was observed for several of the mutant strains such as *nsp1-5* (Fig. 2.5). By contrast, strains *nup133*Δ, *rat2-1, nic96*Δ*C,* and *mtr7-1* showed increased amounts of GFP-Gsp1p in the cytoplasm upon exposure to heat. Interestingly, *nup133*Δ and *mtr7-1* also had elevated levels of GFP-Gsp1p in the cytoplasm at the permissive temperature (Fig. 2.5 and Table 2.3), suggesting defects in GFP-Gsp1p nuclear transport even at room temperature. Likewise, in mutant *nup85* Δ cells GFP-Gsp1p was only slightly accumulated in nuclei when cells were grown at room temperature. Exposure of *nup85*Δ to heat stress did not drastically change the GFP-Gsp1p distribution (Fig. 2.5 and Table 2.3).

Nuclear Envelope Association of Ntf2p-GFP Is Altered in Several Nucleoporin Mutants

A possible explanation for the elevated cytoplasmic levels of GFP-Gsp1p under non-permissive conditions could be a failure of Ntf2p, the nuclear carrier for Gsp1p, to properly interact with NPCs. We have addressed this question with Ntf2p-GFP, a reporter protein that concentrates at the nuclear rim in wild type cells at room temperature. A similar distribution is also observed when wild type cells have been incubated at 37°C for 6 h (Fig. 2.6A) Although mutant strains *nup133Δ* and *rat2-1* show a strong association of

Ntf2p-GFP with NPC clusters under permissive conditions, exposure to heat significantly reduced Ntf2p-GFP localization to clusters (Fig. 2.6 A and B). In addition, mutant *nup85Δ* cells displayed reduced binding of Ntf2p-GFP even at room temperature. This supports the idea that Nup133p, Rat2p, and Nup85p are required to promote binding of Ntf2p to NPCs of stressed cells. In contrast, the association of Ntf2p-GFP with nuclear envelopes was only slightly diminished by heat treatment in *mtr7-1*, several *nsp1*, and *nic96* mutants (Fig 2.6). Even though *nup84*⁻ cells showed reduced binding of Ntf2p-GFP to the nuclear envelope under normal and stress conditions, the difference to wild type cells or between control and heat-treated *nup84*⁻ cells was not statistically significant.

The Guanine Nucleotide Exchange Factor Prp20p Mislocalizes to the Cytoplasm in *nup85Δ*, *mtr7-1*, and *nic96ΔC*

Collapse of the Gsp1p gradient may also be caused by the mislocalization of Gsp1p interacting factors, *i.e.* the guanine nucleotide exchange factor Prp20p or the GTPase-activating protein Rna1p. Under normal growth conditions Prp20p is concentrated in nuclei, where it is essential to generate Gsp1p-GTP. By contrast, most of Rna1p resides in the cytoplasm, although nuclear pools of Rna1p have been detected [88]. When analyzed by immunofluorescence, Prp20p was concentrated in nuclei of wild type and mutant cells at room temperature (Fig. 2.7). However, upon heat treatment a significant redistribution of Prp20p is seen in *nup85*Δ, *mtr7-1*, and *nic96*Δ*C*. Prp20p mislocalization was most prominent in *mtr7-1*, and after 3 h of heat stress less than 35% of the cells had Prp20p concentrated in nuclei. Moreover, elevated cytoplasmic levels of Prp20p were detected even after 1 h of incubation at 37°C. A difference in Prp20p distribution was also

detected for *rat2-1*, but the effect was less significant for *rat2-1* ($p = 0.02$) as compared with *nup85*Δ, *mtr7-1,* and *nic96*Δ*C* ($p < 0.01$).

Discussion

Gsp1p/Ran is an essential protein that plays a pivotal role in nucleocytoplasmic trafficking of macromolecules [48,49,51,89]. As such, conditions that prevent the formation of a Gsp1p/Ran gradient across the nuclear envelope interfere with classical nuclear transport in yeast and in higher eukaryotes [47,90]. Nuclear accumulation of Gsp1p/Ran depends on the Gsp1p/Ran-GDP-binding protein Ntf2p that imports the GTPase into the nucleus, possibly followed by nuclear retention [31,34]. During passage through the NPC, Gsp1p and Ntf2p are believed to interact with nucleoporins. In support of this idea, F*X*F repeats of nucleoporins such as yeast Nsp1p or mammalian p62 bind Ntf2p and Gsp1p *in vitro* [55]. By contrast, we show that mutations in the C-terminal essential domain of Nsp1p do not cause mislocalization of Gsp1p under restrictive conditions.

To identify novel components of the nuclear envelope that are involved in Gsp1p nuclear accumulation, we have analyzed yeast strains that carry a deletion or mutation in various nucleoporin genes or in *MTR7*. So far, components of the nuclear envelope other than FG repeat-containing nucleoporins have not been shown to participate in Gsp1p trafficking. Our results demonstrate for the first time that the non-repeat nucleoporins Nup133p, Nup120p/Rat2p, Nup85p, and Nic96p, as well as acetyl-CoA carboxylase regulate the distribution of the small GTPase.

To define the mechanisms that lead to the collapse of the Gsp1p gradient in mutant strains, we have determined whether nuclear import of the fusion protein GFP-Gsp1p is compromised under non-permissive conditions. GFP-Gsp1p is too large to diffuse efficiently across the NPC, and elevated levels of this reporter protein in the cytoplasm

suggest that nuclear import is impaired in *nup133Δ*, *rat2-1*, *nup85Δ*, *nic96ΔC*, and *mtr7-1*. Interestingly, *nup133Δ*, *rat2-1*, *nup85Δ*, and *mtr7-1* showed elevated cytoplasmic levels of GFP-Gsp1p even at room temperature, suggesting that import is already affected under these conditions.

Nup84p, Nup85p, and Nup120p/Rat2p are present in the same NPC subcomplex, called the Nup84p complex, which also contains Nup145p-C, Seh1p, and Sec13p [80]. Furthermore, Nup133p associates with the Nup84p complex, indicating that the Nup84p-Nup133p unit represents a building block of the NPC [84,85]. To assemble the Nup84p module Nup85p, Nup120p/Rat2p and Nup145p-C are required, whereas Nup84p and Seh1p are dispensable [80]. Deletion of *NUP84*, *NUP85*, or *NUP120/RAT2* changes nuclear membrane and NPC organization [79,80]. In particular, a complete disruption of *NUP84* in *nup84⁻* cells leads to an altered distribution of NPCs [79]. Despite the effects of the *nup84⁻* allele on nuclear envelope and NPC assembly, Gsp1p gradients did not collapse in this mutant at the non-permissive temperature. These results emphasize that the changes in Gsp1p localization observed by us for several nucleoporin mutants cannot simply be ascribed to altered nuclear organization. In contrast to *nup84⁻* cells, Gsp1p gradients collapsed in strains *nup85Δ*, *rat2-1*, and *nup133Δ* under restrictive conditions, which points to a specific role of Nup85p, Nup120p/Rat2p, and Nup133p in nuclear accumulation of the GTPase. We have now demonstrated that the defect in *rat2-1* and *nup133Δ* cells can be attributed to the inefficient association of Ntf2p with NPCs upon exposure to heat stress. However, Nup133p is not essential for binding Ntf2p to the NPC under non-stress conditions, demonstrating the presence of redundant binding sites. Nevertheless, failure of Ntf2p to interact with mutant NPCs in stressed cells will

ultimately dissipate the Gsp1p gradient, as Ntf2p is required for Gsp1p nuclear import. In contrast to *rat2-1* and *nup133*Δ mutants, *nup85*Δ, missing another component of the Nup84p complex, displayed reduced Ntf2p-GFP binding even at room temperature. This is in line with the observation that GFP-Gsp1p in *nup85*Δ cells already mislocalized at room temperature, similar to what was detected at 37°C. Furthermore, Prp20p, the guanine nucleotide exchange factor for Gsp1p, redistributed in *nup85*Δ at elevated temperature (see below). Thus we have identified multiple defects for this mutant that will contribute to the collapse of the Gsp1p gradient. We have also carried out experiments to further analyze the interaction between Ntf2p and Nup133p. In line with the idea that Ntf2p forms complexes with this nucleoporin, we were able to co-purify both components (not shown). However, results for this co-purification were variable, most likely reflecting the transient nature of this interaction.

Even though nuclear export of the ribosomal 60 S subunit depends on the Gsp1p-GTPase cycle, members of the Nup84p complex did not show a major defect in this export reaction [69]. These apparent differences can be explained by the distinct conditions used in our analyses. To study export of the 60 S ribosomal subunit, cells were exposed to 33°C, followed by a 4 h shift to 20°C. It is presently not known whether the Gsp1p concentration gradient dissipates at 33°C. Furthermore, it is possible that a collapsed Gsp1p concentration gradient can be rebuilt when cells are returned to 20°C. Our data clearly demonstrate a deficiency of *rat2-1* cells in the formation of a Gsp1p gradient and of Ntf2p binding to the NPC under non-permissive conditions. Moreover, a weak defect was also detected for the nuclear concentration of Prp20p upon exposure to heat. However, it should be noted that previous studies of *rat2-1* did not reveal a nuclear

protein import defect [66]. In these analyses nuclear import was monitored with a reporter protein containing the N-terminal 33 amino acid residues of yeast histone H2B fused to β-galactosidase [66]. Yeast histones H2A and H2B were recently shown to be imported into nuclei by several members of the β-importin family, including Kap114p, Kap121p, and Kap95p [90]. These importins directly bind to N-terminal histone nuclear localization signals [90]. By contrast, SV40-GFP, the reporter protein used by us, is expected to accumulate in nuclei via the classical Srp1p/Kap95p import pathway. The specific requirements for nuclear import may differ for the two substrates, thereby explaining the distinct effects of the *rat2-1* mutation on their nuclear accumulation. Our results reveal the discrete functions of Rat2p in transport of different cargoes. Although non-classical import mediated by the histone H2B nuclear localization signal is not impaired at the non-permissive temperature, a drastic effect is seen for classical nuclear protein import and Gsp1p nuclear concentration. Our data are in accordance with the idea that individual members of the Nup84p-Nup133p NPC module can selectively affect specific aspects of nucleocytoplasmic trafficking.

Nup133p, Nup85p, Nup120p/Rat2p, and Nic96p are located on both the nuclear and the cytoplasmic side of the NPC [57]. None of these nucleoporins contains F*X*FG or GLFG repeats, and only Nic96p carries heptad repeats implicated in coiled-coil interactions. In line with the latest models for nuclear trafficking [91] several mechanisms, not mutually exclusive, can be proposed for the Gsp1p gradient collapse and failure to import GFP-Gsp1p in mutant nucleoporin strains. (*a*) The organization of NPCs may be changed in a fashion that alters the binding or translocation of Ntf2p-Gsp1p to the NPC. (*b*) Mislocalization of Gsp1p-interacting factors could affect the Gsp1p gradient. (*c*)

Mutations might modulate the nuclear pore channel size, thereby preventing passage of macromolecules across the NPC. (*d*) Nuclear retention of the GTPase could be altered by an unknown mechanism. Because SV40-GFP, a protein of ~45 KDa molecular mass, was still able to exit the nucleus in mutants that collapsed the Gsp1p gradient, a more general obstruction of the channel seems unlikely. Moreover, we have demonstrated that nuclear translocation of GFP-Gsp1p was changed in several of the nucleoporin mutants, even at permissive conditions. Therefore, we favor the first two scenarios, *i.e.* changes in the interaction of Ntf2p-Gsp1p with nucleoporins and relocation of Gsp1p-interacting factors, and we have identified these defects in several nucleoporin mutants. As a result, the initial binding of Ntf2p-Gsp1p to NPCs or its subsequent translocation into the nucleus may be prevented. In addition, the redistribution of Prp20p will affect the Gsp1p-GTPase cycle, generating elevated levels of Gsp1p-GTP in the cytoplasm. In either case, the consequence will be a collapse of the Gsp1p gradient.

Like several nucleoporins, *MTR7* is also required for Gsp1p gradient formation. *MTR7* encodes an acetyl-CoA carboxylase, an enzyme required for *de novo* synthesis of long chain fatty acids that were proposed to stabilize the NPC at the pore membrane interface [87]. Under restrictive conditions, destabilization of NPCs is likely to affect all trafficking reactions, including the nuclear accumulation of Gsp1p, as observed by us. However, even at room temperature nuclear import of GFP-Gsp1p was impaired in *mtr7-1* cells, demonstrating that transport already has been altered by changing the lipid composition of the nuclear envelope.

Factors other than Ntf2p, Prp20p, and Rna1p may also be affected and contribute to the dissipation of the Gsp1p gradient in nucleoporin mutants studied by us. As such,

members of the β-importin family have been proposed to play a role in nuclear retention of Ran/Gsp1p [47]. At this point it is not clear how many individual nuclear carriers are involved in this process. As of yet, it is therefore not possible to determine to whichextent a potential redistribution of β-importins in nucleoporin mutants affects the Gsp1p gradient formation.

In addition to the identification of novel factors involved in Gsp1p nucleocytoplasmic distribution, our results help explain the classical and non-classical transport defects seen for several of the mutants used in this study. Many of the nuclear trafficking reactions require Gsp1p and presumably nucleocytoplasmic gradients of the GTPase. If mutant yeast strains fail to generate a Gsp1p gradient under non-permissive conditions, all nuclear trafficking reactions that rely on such a gradient will be prevented. Thus, the failure to concentrate Gsp1p in the nucleus because of altered binding of Ntf2p to the NPC or relocation of Prp20p may be a primary consequence of some of the nucleoporin mutations and contribute to the observed transport defects. Furthermore, reduced Gsp1p levels as observed for some of the mutations analyzed by us can be expected to alter nuclear transport as the GTPase may become the limiting factor for classical or non-classical pathways.

Acknowledgements

We are grateful to Drs. C. Cole (Hanover, NH), A. Tartakoff (Cleveland, OH), and in particular to E. Hurt (Heidelberg, Germany) for providing us with yeast strains and plasmids. We thank N. Matusiewicz for expert technical assistance.

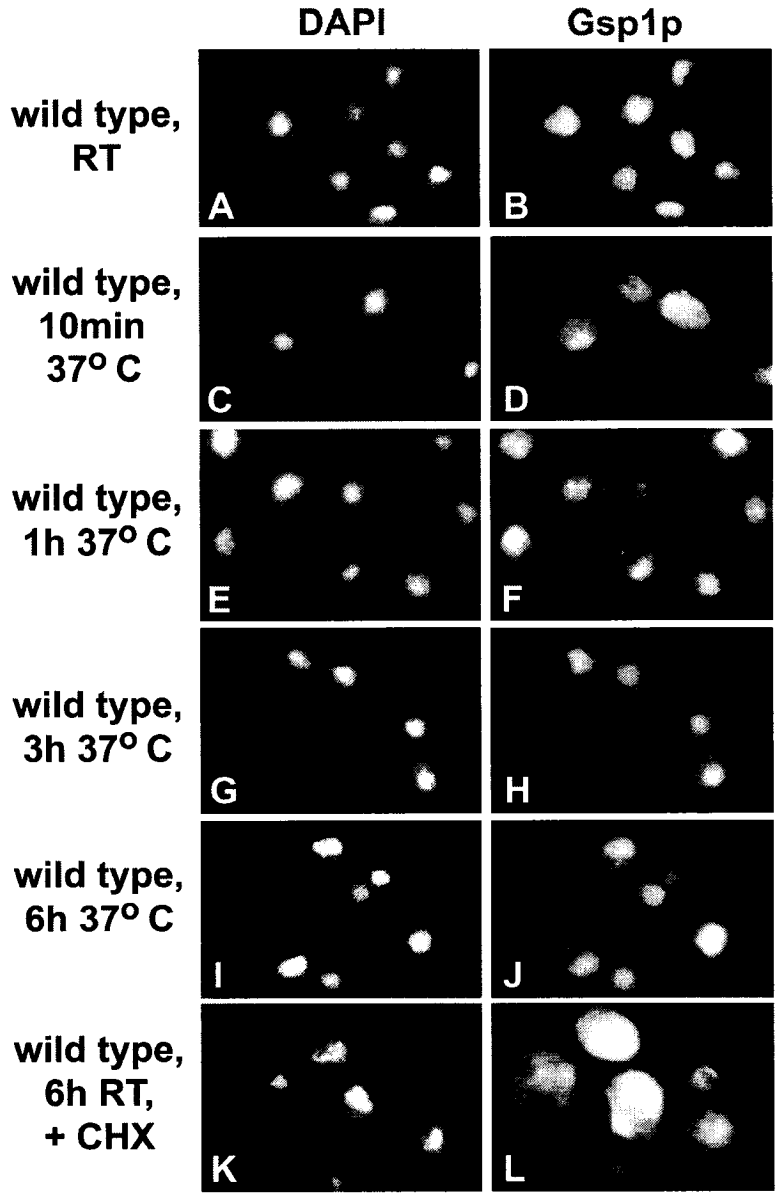


Fig. 2.1
Distribution of Gsp1p in wild type cells exposed to heat shock. Wild type yeast cells were incubated at room temperature (*RT*) (control, *A* and *B*) or at 37°C (*C–J*) for 10 min, 1, 3, and 6 h, as indicated. Cells were also incubated at room temperature in the presence of 50 μg/ml cycloheximide (*CHX*) (*K* and *L*). DAPI staining of nuclei (*A, C, E, G, I,* and *K*) and localization of Gsp1p (*B, D, F, H, J,* and *L*) are shown.

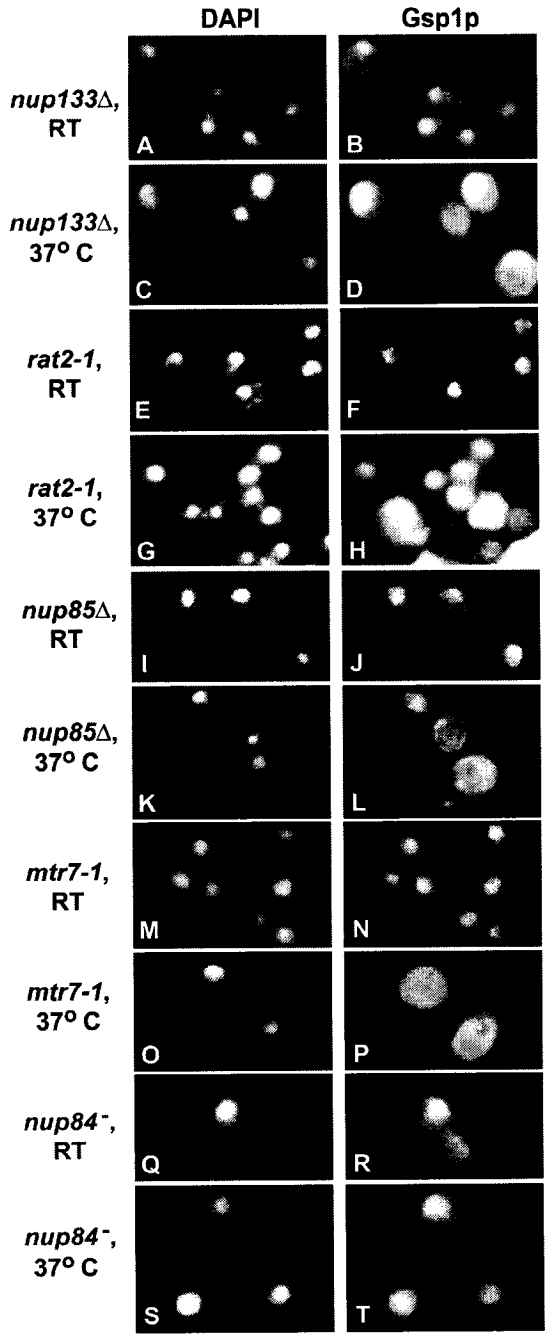


Fig. 2.2

Distribution of Gsp1p in the clustering mutants *nup133 Δ* and *rat2-1, nup85 Δ, mtr7-1,* and *nup84⁻*Yeast cells carrying a deletion of *NUP133* (*A–D*), the mutation *rat2-1* (*E–H*), a partial disruption of *NUP85* (*I–L*), the *mtr7-1* (*M–P*) allele, or *nup84⁻* (*Q–T*) cells were incubated at room temperature (*RT*) or for 6 h at 37°C. Gsp1p was localized by indirect immunofluorescence. Nuclei were visualized with DAPI.

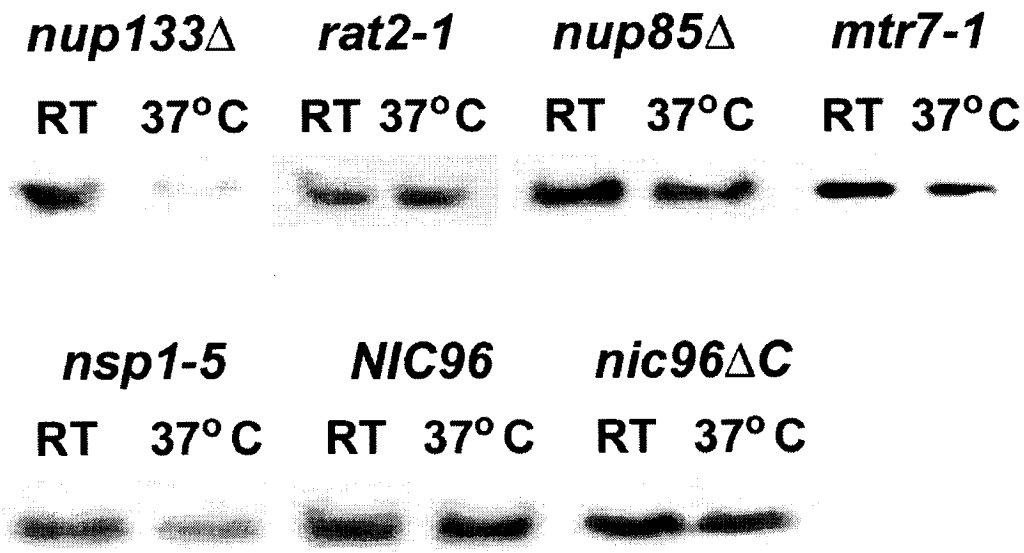


Fig. 2.3
Western blots with anti-Gsp1p for unstressed controls and heat-stressed cells. Strains shown in the figure were kept for 6 h at room temperature (*RT*) or at 37°C. Equal amounts of protein from unstressed and stressed cells were analyzed side-by-side by Western blotting with antibodies against Gsp1p.

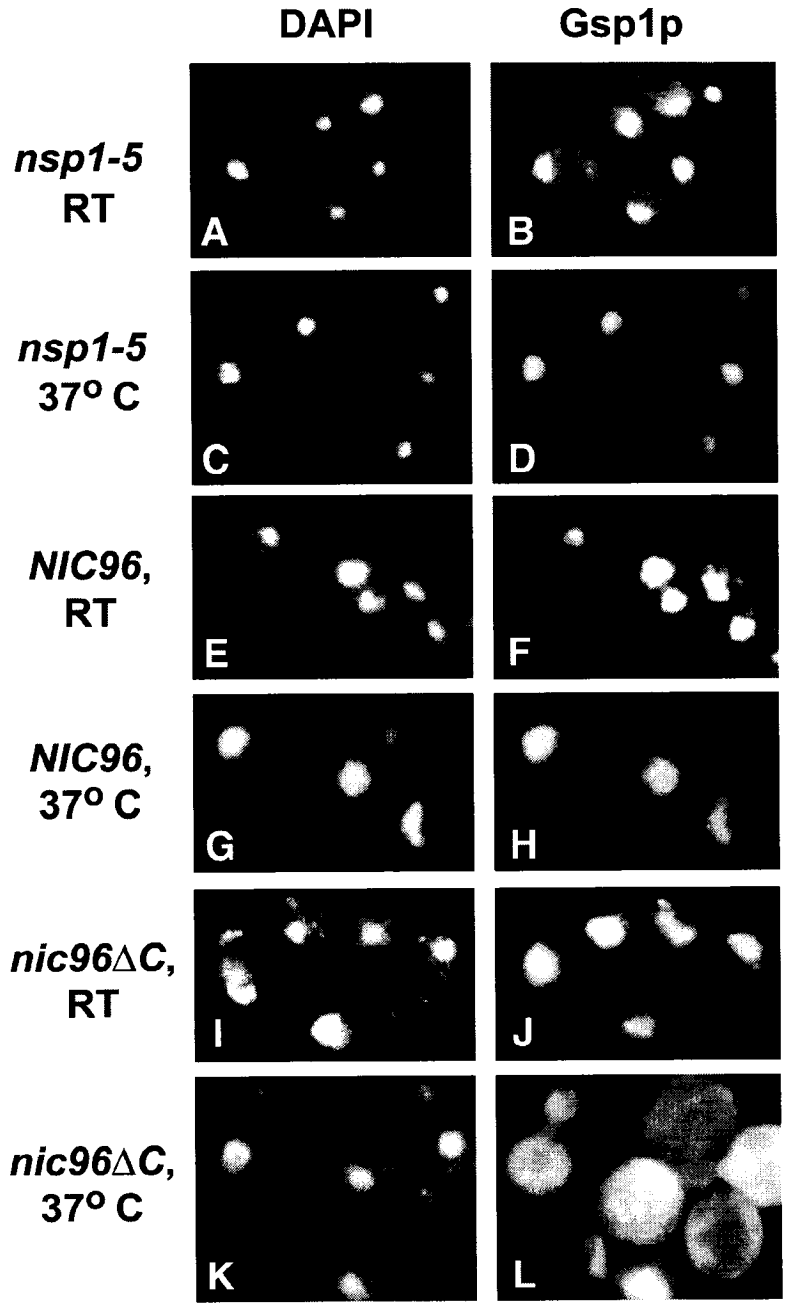


Fig. 2.4
Localization of Gsp1p in cells carrying a mutant allele of *NSP1* or *NIC96*. Yeast strains *nsp1-5* (*A–D*), *NIC96* (*E–H*), and *nic96ΔC* (*I–L*) were grown at room temperature (*RT*) or for 6 h at 37°C as indicated. Gsp1p was located by indirect immunofluorescence and nuclei were stained with DAPI.

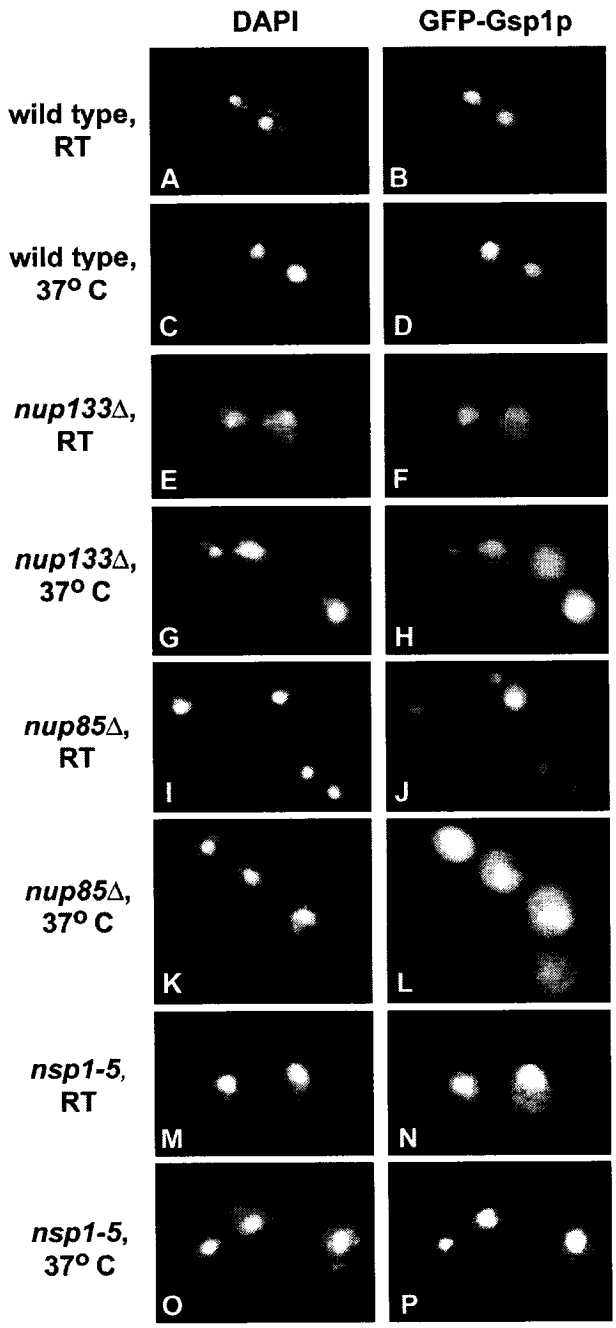


Fig. 2.5
Nuclear accumulation of GFP-Gsp1p in control cells and strains that mislocalize Gsp1p under restrictive conditions. Wild type yeast (*A–D*), mutants *nup133Δ* (*E–H*), *nup85*Δ (*I–L*), and *nsp1-5* (*M–P*) were transformed with plasmids encoding GFP-Gsp1p. Cells kept for 6 h at room temperature (*RT*) or at 37°C were fixed and stained with DAPI as described under "Experimental Procedures."

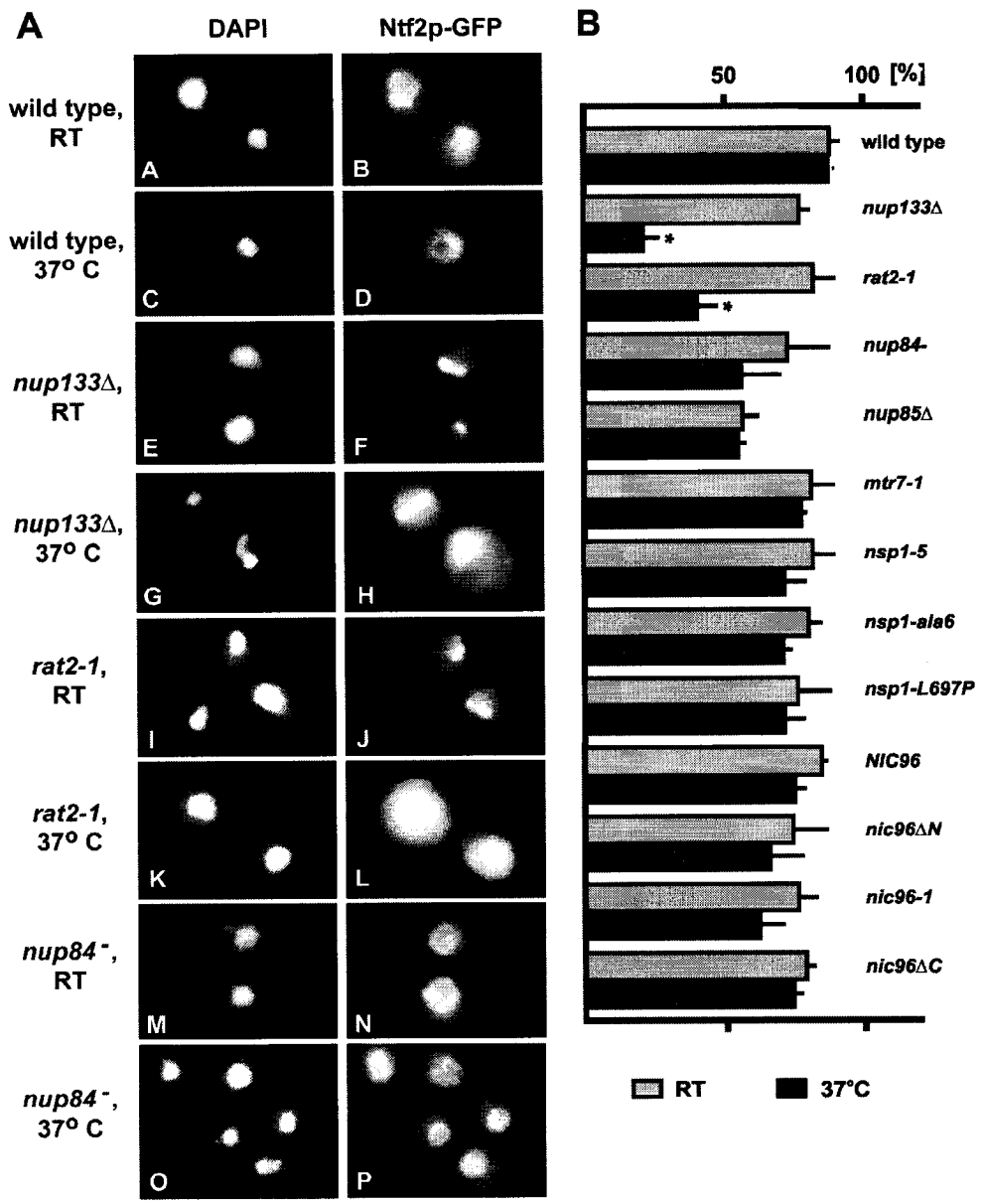


Fig. 2.6
Association of Ntf2p-GFP with the nuclear envelope. *A*, Ntf2p-GFP was localized by fluorescence microscopy upon growth at room temperature or exposure to 37°C for 6 h. Wild type (*A–D*), *nup133Δ* (*E–H*), *rat2-1* (*I–L*), and *nup84⁻* (*M–P*) cells are shown. Ntf2p-GFP accumulates at NPC clusters in *nup133Δ* and *rat2-1* under permissive conditions. *B*, nuclear envelope association of Ntf2p-GFP was monitored by fluorescence microscopy for at least 100 cells that synthesize Ntf2p-GFP. The percentage of these cells that display Ntf2p-GFP nuclear envelope binding is depicted. Mean values and S.D. are shown for at least three independent experiments. *, $p < 0.01$.

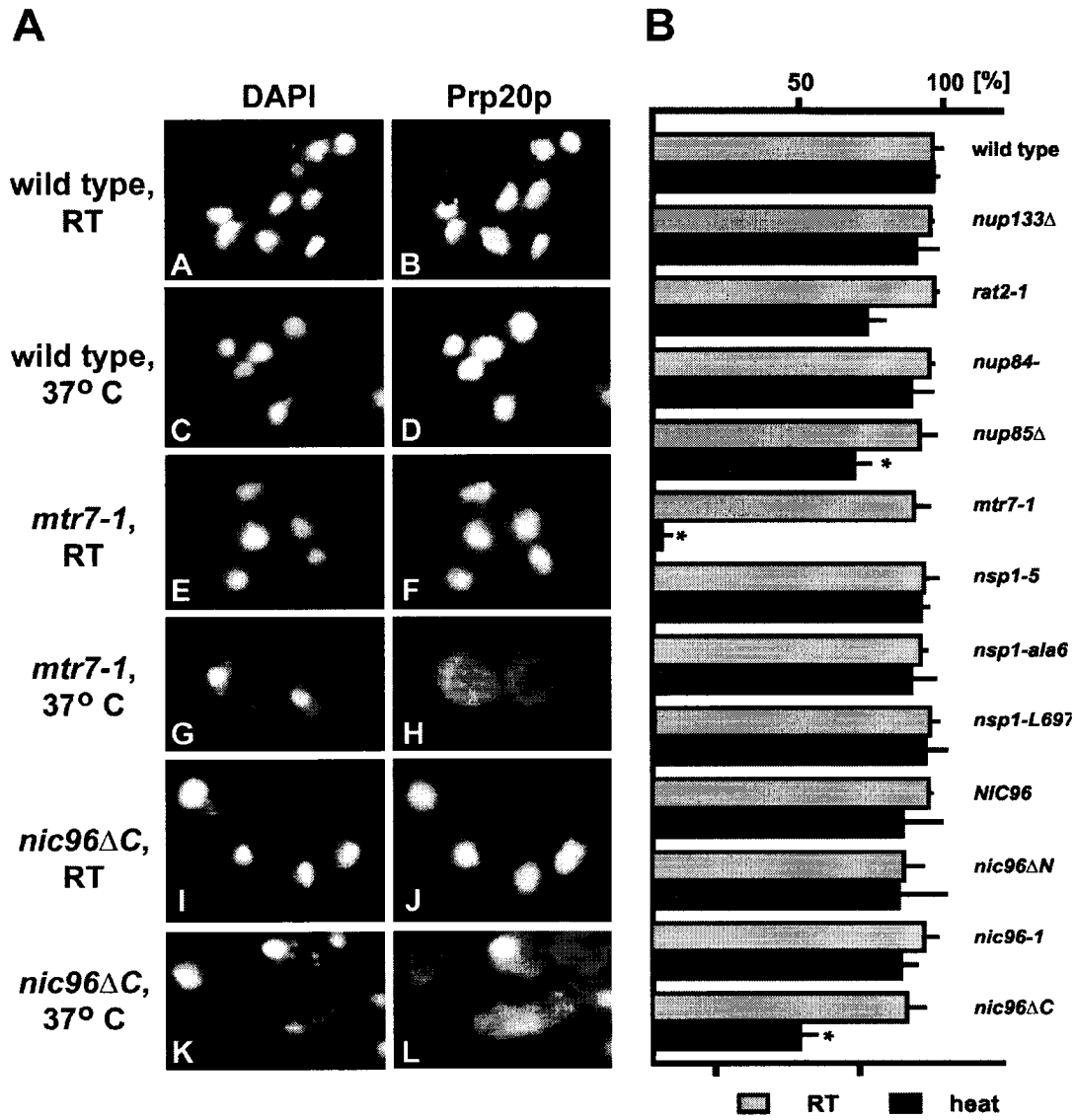


Fig. 2.7
Concentration of the Gsp1p-interacting factor Prp20 in nuclei. *A*, Prp20p was located by immunofluorescence in wild type cells (*A–D*), strain *mtr7-1* (*E–H*), and *nic96ΔC* (*I–L*) at room temperature (*RT*) and upon exposure to 37°C for 6 h. *B*, the distribution of Prp20p was determined by immunofluorescence for at least 100 cells, and the percentage of cells with Prp20p nuclear accumulation is shown. Mean values and S.D. represent at least three independent experiments. *, $p < 0.01$.

Table 2.1: Yeast strains used in this study.

The markers of yeast strains and their previously described phenotypes with respect to nuclear trafficking are listed. NE, nuclear envelope.

Strain	Genotype	Phenotype	Reference
RS453	*Matα ade2 his3 leu2 trp1 ura3*	wild type	61
nup133Δ	*Matα ade2 his3 leu2 trp1 ura3 nup133::HIS3*	NPC clusters, mRNA export	58
CCY282	*Mata leu2 trp1 ura3 rat2-1*	NPC clusters, mRNA export	66
nup84	*ade2 his3 leu2 trp1 ura3 nup84::HIS3*	NE organization	79
nup85Δ	*ade2 his3 leu2 trp1 ura3 nup85::HIS3*	mRNA export, NPC biogenesis	79
T1019	*Mata ade2 leu2 ura3 lys2 mtr7-1*	mRNA export	87
nsp1-C	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pUN100-LEU2-ProtA-TEV-NSP1-(591-823)	-/-	63
nsp1-5	*Mata ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pSB32-LEU2-nsp1-5)	protein import 60S SU export	59, 76
nsp1-ala6	*ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pSB32-LEU2-nsp1-ala6)	protein import, 60S SU export	71
nsp1-L640S	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pSB32-LEU2-nsp1-L640S)	mRNA export	76
nsp1-ts18	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pUN100-LEU2-ProtA-nsp1-ts18)	protein import	63
nsp1-W644C	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pSB32-LEU2-nsp1-W644C)	protein import	[a]
nsp1-L697P	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (pSB32-LEU2-nsp1-L697P)	protein import	[a]
NIC96	*Matα ade2 his3 leu2 trp1 ura3 nic96::HIS3* (pUN100-LEU2-NIC96)	-/-	61

nic96ΔN	*Matα ade2 his3 leu2 trp1 ura3 nic96::HIS3* (pUN100-LEU2-nic96-ΔN)	protein import	61
nic96-1	*Mata ade2 his3 leu2 trp1 ura3 nic96::HIS3* (pUN100-LEU2-nic96-1)	protein import, 60S export, NPC formation	61
nic96ΔC	*Matα ade2 his3 leu2 trp1 ura3 nic96::HIS3* (pUN100-LEU2-nic96-ΔC)		
rat7-1	*Matα his3 leu2 ura3*	mRNA export	65

[a] U. Nehrbass and E.C. Hurt, unpublished data.

Table 2.2: Effect of mutations in nucleoporin genes or in *MTR7* on the localization and cellular concentration of Gsp1p.

Yeast cells were incubated for 3 and 6 h at room temperature or at 37°C. Gsp1p was localized by indirect immunofluorescence, and levels of the GTPase were determined by Western blotting. RT, room temperature. N and C indicate nucleus and cytoplasm, respectively. N>C, the concentration of Gsp1p is higher in the nucleus, and a gradient is clearly detectable. N≥C, Gsp1p concentrations are slightly higher in the nucleus when compared with the cytoplasm. N=C, the Gsp1p gradient is collapsed. In several cases mixed populations of cells were detected, and the distribution for these populations is noted in the table. Data in parentheses indicate that less than 25% of the cells showed this Gsp1p localization. Results depicted in the table have been observed for at least three independent experiments.

Nup	allele	localization upon heat shock			protein levels upon heat shock	
		RT	3h 37°C	6h 37°C	3h 37°C	6h 37°C
---	wild type	N>>C	N>>C	N>>C	no change	no change
NUP133	*nup133Δ*	N>C	N=C, (N>C)	N=C	increased	reduced
RAT2	*rat2-1*	N>C, (N=C)	N=C	N=C, (N>C)	no change	no change
NUP84	*nup84*	N>C, (N=C)	N>C	N>C	no change	no change
NUP85	*nup85Δ*	N>C	N>C, N=C	N=C, (N>C)	no change	no change
MTR7	*mtr7-1*	N>C	N>C, N=C	N=C	reduced	reduced

NSP1	*nsp1-C*	N>C	N>C	N>C	no change	slightly reduced
NSP1	*nsp1-5*	N>C, N≥C	N>C	N>>C	slightly reduced	slightly reduced
NSP1	*nsp1-ala6*	N>C, N≥C	N>C	N>>C	no change	no change
NSP1	*nsp1-L640S*	N>C	N>C, N>>C	N>C, N>>C	no change	no change
NSP1	*nsp1-ts18*	N>C	N>C	N>C, N>>C	no change	no change
NSP1	*nsp1-W644C*	N>C	N>C	N>C, N>>C	no change	no change
NSP1	*nsp1-L697P*	N>C	N>C, N>>C	N>C, N>>C	no change	increased
NIC96	wild type	N>C	N>C	N>C	no change	no change
NIC96	*nic96 ΔN*	N>C	N>C, (N=C)	N>C	no change	no change
NIC96	*nic96-1*	N>C	N>C, (N=C)	N>C	no change	no change
NIC96	*nic96 ΔC*	N>C, (N=C)	N>C, N=C	N=C, (N>C)	slightly reduced	no change

Table 2.3: Localization of the classical import substrate SV40-GFP and of GFP-Gsp1p in control and stressed yeast cells.

Yeast cells synthesizing the classical nuclear import substrate SV40-GFP or GFP-Gsp1p were grown at room temperature or exposed to 37°C for the times indicated. Cells were fixed, and reporter proteins were localized by fluorescence microscopy. The distribution of SV40-GFP and GFP-Gsp1p in nuclei (N) and cytoplasm (C) was monitored as described for Table 2.2; ND, not determined. RT, room temperature.

Strain	SV40-GFP		GFP-Gsp1p	
	RT	6h 37°C	RT	6h 37°C
wild type	N>>C	N>>C	N>C	N>C
nup133Δ	N>C	N=C	N≥C, (N=C)	N=C, N≥C
rat2-1	N>C, N≥C	N=C, N≥C	N≥C, (N=C)	N≥C, (N=C)
nup84	N>C, N≥C	N=C, N≥C	N≥C, N=C	N≥C
nup85Δ	N≥C, (N=C)	N=C, (N≥C)	N≥C, N=C	N≥C, N=C
mtr7-1	N≥C	N=C	N≥C, N=C	N=C
nsp1-C	N>C, (N≥C)	N>C, N≥C	n.d.	n.d.
nsp1-5	N>C	N≥C, N=C	N≥C, N>C	N>C, N≥C

nsp1-ala6	N>C	N=C	N≥C, N>C	N>C, N≥C
nsp1-L640S	N>C, N>>C	N≥C, N=C	n.d.	n.d.
nsp1-ts18	N>C, N≥C	N=C, N≥C	n.d.	n.d.
nsp1-W644C	N>C	N≥C	n.d.	n.d.
nsp1-L697P	N>C, N≥C	N>C	N≥C	N≥C
NIC96	N>C	N>C, N≥C	N>C, N≥C	N>C, N≥C
nic96ΔN	N>C	N≥C, N>C	N>C, N≥C	N≥C, N>C, (N=C)
nic96-1	N>C	N>C, (N=C)	N≥C, N=C	N=C, N≥C
nic96ΔC	N>C, N≥C	N=C, N≥C	N>C, N≥C	N≥C, N=C
rat7-1	N>>C	N>>C	n.d.	n.d.

Table 2.4: Summary of defects identified in nucleoporin mutants that collapse the Gsp1p gradient upon exposure to heat stress.

The nuclear accumulation of GFP-Gsp1p, association of Ntf2p-GFP with NPCs, and nuclear concentration of Prp20p is compared in mutants that collapse the Gsp1p gradient when incubated for 6 h at 37°C. NE, nuclear envelope association. See "Results" and "Discussion" for details. RT, room temperature.

Strain	GFP-Gsp1p		Ntf2p-GFP		Prp20p	
	RT	37°C	RT	37°C	RT	37°C
wild type	N>C	N>C	NE	NE	N>C	N>C
nup133Δ	N≥C, (N=C)	N=C, N≥C	NPC clusters	reduced	N>C	N>C
rat2-1	N≥C, (N=C)	N≥C, N=C	NPC clusters	reduced	N>C	N≥C
nup85Δ	N≥C, N=C	N≥C	NE, reduced	NE, reduced	N>C	N≥C, N=C
mtr7-1	N≥C, N=C	N=C	NE	NE	N>C	N=C
nic96ΔC	N>C, N≥C	N≥C, N=C	NE	NE	N>C	N=C, N≥C

CHAPTER 3:

A non-classical nuclear localization signal accumulates hsc73 in nuclei of heat stressed cells.

Preface

This chapter of my thesis is based on the following manuscript:

Gao, H., Sarmento, C., Stochaj, U. A non-classical nuclear localization signal accumulates hsc73 in nuclei of heat stressed cells.

Contribution to the study:

The study presented in this chapter was designed and supervised by Dr. U. Stochaj. C. Sarmento has generated some of the constructs in Figure 3.1. H. Gao performed all other experiments.

Abstract

I have generated mutant forms of hsc73 and analyzed their nucleocytoplasmic trafficking in control and heat stressed HeLa cells. To this end, the nuclear transport of hsc73 was investigated in growing cells and *in vitro.* With these studies, I have shown that the C-terminal domain of mammalian hsc73 contains a novel targeting signal, which is required and sufficient for stress-induced nuclear accumulation. Upon deletion of this C-terminal segment, nuclear import of hsc73 is compromised even though the cNLS is still present. Fusion of the C-terminal segment to GFP3 generates a protein too large to diffuse across the nuclear envelope. Nevertheless, this fusion protein can shuttle between nucleus and cytoplasm, demonstrating that the C-terminal domain represents a unique shuttling signal in hsc73. Finally, my *in vitro* experiments show that nuclear import of the C-terminal domain needs cytosol and is energy-dependent. Taken together, these results suggest a new signal and a novel mechanism for hsc73 nuclear trafficking upon heat stress.

Introduction

Appropriate responses to stress are essential for survival and repair of stress-induced damage. Exposure to heat results in up-regulation of gene expression for hsp70 and hsc70 proteins. In addition, hsc70/hsp70 proteins accumulate in nuclei of heat-shocked cells. Transport of macromolecules across the nuclear envelope occurs through nuclear pore complexes (NPCs), which are located at the junction of inner and outer nuclear membranes. NPCs mediate diffusion of molecules smaller than 40 KDa. However, components exceeding the diffusion radius of the NPC, such as hsc70s, require specific trafficking signals and a cellular apparatus for translocation [49]. In unstressed cells, proteins of the hsc70/hsp70 family shuttle between nucleus and cytoplasm, and they are located in both compartments [43]. Upon exposure to heat shock, hsc70s/hsp70s concentrate in nuclei where they participate in the repair of heat-induced damage [92]. Heat treatment is the most effective stress to localize hsc70s to nuclei in HeLa cells, whereas exposure to osmotic or oxidative stress is less efficient [93]. Importantly, heat stress inhibits classical nuclear import in growing yeast and mammalian cells, suggesting that hsc70 nuclear accumulation after exposure to heat depends on a specialized non-classical mechanism.

Hsc70s are structurally composed of three domains: a highly conserved 44 KDa N-terminal ATPase domain (amino acid residues 1-400), followed by a polypeptide-binding domain that is less conserved (amino acid residues 401-507) and a variable C-terminal domain (amino acid residues 507-634). Hsc70 proteins contain a single basic amino acid sequence, KRKHKKDAISENKRAVRR, which extends from position 246 to 262 [94, 95]. It was determined, by conjugating multiple peptides to BSA,

that it is capable of promoting nuclear import in unstressed cells. However, inactivation of this signal by deletion of the first six amino acid residues had no effect on hsc70 import [96], suggesting that an alternative signal may also transport hsc70s into nuclei. Proteins of the hsp70/hsc70 family are not only involved in folding of *de novo* synthesized proteins [97] and repair, they also play a role in protein targeting. As such, hsp70s have been proposed to function in cNLS-directed nuclear transport by promoting the formation and stability of NLS-importin α complexes. Shi and Thomas reported that hsc70 is necessary for the import of nucleoplasmin into the nuclei of semi-permeabilized HeLa cells. Specifically, BSA-SV40 large T NLS conjugates were unable to enter the nuclei of cells when coinjected with anti-hsc70 antibodies [42].

The heat shock cognate protein hsc73 is a member of the hsp70 multigene family, which is present in the cytosol. Hsc73 plays an important role in cells by transiently associating with nascent polypeptides to facilitate correct folding [97]. Hsc73 also functions as the uncoating ATPase responsible for the disassembly of clathrin-coated vesicles [98].

In the present study, we have examined the nuclear transport of hsc73 in HeLa cells. We found that the putative cNLS is not required for the nuclear localization, but the C-terminal portion (amino acid residues 588-646) is necessary and sufficient for the nuclear accumulation upon heat stress. Moreover, this part of hsc73 can shuttle between nucleus and cytoplasm. *In vitro* experiments demonstrate that wild type hsc73 and the C-terminal domain depend on energy for heat-induced nuclear accumulation. Moreover, *in vitro* the C-terminal segment shows unique requirements for its nuclear accumulation.

Methods and Materials

Growth and stress exposure of HeLa cells

HeLa cells were grown in multiwell chambers as described [100]. For heat treatment, cells at 50-70% confluency were incubated at 45.5℃ for 30 min and 1 hour as indicated.

Constructs

The gene encoding Flag-tagged mouse hsc73 was cloned into a mammalian expression vector. Gene expression is controlled by five glucocorticoid response elements [100]. Gene truncations were generated by deleting various parts of the 3'- end and insertion of an NheI-linker (12mer; New England, Biolabs) that encodes amber codones in all three frames. To generate EGFP-tagged proteins, the complete hsc73 coding sequence or parts thereof were cloned into pEGFP-C1 (Clontech, Palo Alto). For production of wild type and mutant hsc73 in *E. coli*, His6-tagged versions of the different genes were generated. The correctness of all constructs was verified by DNA-sequencing.

Transfection of HeLa cells

Transient transfection and gene expression in HeLa cells has been described previously [100]. Transfected cells were grown in 6-well plates prior to heat shock to a density of 50-70%.

Treatment with Cytochalasin B

Cytochalasin B (Calbiochem, San Diego, CA) was dissolved in DMSO. Transiently transfected HeLa cells were incubated with 10 μm cytochalasin B at 37℃ for 3 hours.

Controls were incubated with DMSO only.

Purification of His6-tagged proteins

His6-tagged proteins were purified by affinity chromatography under native conditions using Ni-NTA-agarose (Qiagen, Mississauga, ON) as recommended by the supplier. Purified proteins were dialyzed against transport buffer, and concentrated in centrifugal filters (Millipore, Bedford, MA).

In vitro nuclear transport

Untransfected HeLa cells were exposed to 30 min heat stress at 45.5℃. Cells were treated for 5 min with digitonin in buffer B, essentially as described [101]. For energy-depletion, apyrase (Sigma, Oakville, ON) was added to 100 U/ml during treatment with digitonin. Digitonin-treated cells were incubated with or without an energy-regenerating system and 10 mg/ml rabbit reticulocyte lysate (Promega, Madison, WI) as indicated in the figures. For apyrase-treated cells, the energy-regenerating system was omitted during incubation at 30℃. Upon incubation for 1.5 hours at 30℃ samples were washed with ice-cold buffer B, fixed with 3.7% formaldehyde in PBS (20 min at room temperature) and processed for fluorescence microscopy.

Immunofluorescent staining

All steps were carried out at room temperature, essentially as described [93]. Stressed cells and controls were fixed in 3.7% formaldehyde/PBS for 20 min. Cells were permeabilized in 0.1 Triton X-100 in PBS containing 2 mg/ml BSA (5 min), blocked for 1 hour in

PBS/2mg/ml BSA containing 0.05% Tween 20 and incubated overnight with primary antibodies against the Flag-tag (ABR Affinity BioReagents, Golden, CO), diluted 1:500 in PBS/BSA/ Tween 20. Primary antibodies were detected with 1.5 μg/ml FITC-conjugated anti-rabbit secondary antibodies (Jackkson ImmunoResearch, West Grove, PA) diluted 1:200. EGFP-hsc73, EGFP-hsc73(1-480), GFP2-hsc73(588-646) and EGFP-GFP2-hsc73(588-646) were located directly by fluorescence microscopy. DNA was stained with 4', 6-diamidino-2-phenylindole (DAPI) and samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Cells were analyzed with a Nikon Optiphot at 400X magnification and photographed with Kodak T-MAX 400 films. Negatives were scanned and processed with Photoshop 5.5.

Western blot analysis

Transiently transfected HeLa cells were grown on dishes to 50-70% confluency and cells were stressed at 45.5℃ for 1 hour. Plates were washed with PBS and stored at -70℃until use. Samples were solubilized in gel sample buffer and treated as described [102]. Equal amounts of proteins were separated by SDS-PAGE. Proteins were blotted to nitrocellulose and blots further processed as previously described [93,102].

Results

The C-terminal portion of hsc73 is necessary for nuclear localization of hsc73

We have generated mutant versions of mammalian hsc73 to analyze their nuclear trafficking in transiently transfected HeLa cells. To this end, different parts of the coding sequence have been deleted (Fig. 3.1, part A). The various reporter proteins carry a Flag epitope-tag at the N-terminus. The expression of wild type and mutant forms of hsc73 was monitored by immnoblotting with antibodies against the Flag-tag (Fig. 3.1, part B). Upon transfection into HeLa cells, proteins were localized in unstressed cells and cells exposed for 1h to 45.5℃. Upon heat exposure, tagged hsc73 concentrated in nuclei (Fig. 3.2, part A), similar to what is seen for authentic hsc70s [93]. However, deletion of most of the peptide binding domain and the C-terminal domain, Flag-hsc73(1-407), prevented the nuclear accumulation in stressed cells (not shown). As this protein is too large to passively diffuse into nuclei sequences within the C-terminal portion may play a role in the nuclear accumulation. To address this question, additional mutant proteins were analyzed for their distribution in control and heat stressed cells (Fig. 3.1). Truncated proteins lacking the C-terminal portion of the protein, Flag-hsc73(1-480) and Flag-hsc73(1-459) were excluded from nuclei in nonstressed cells (Fig. 3.2, part B). Importantly, these proteins were also excluded from nuclei of heat-shocked cells (Fig. 3.2, part B). Furthermore, Flag-hsc73(1-587), although not excluded from nuclei in unstressed or stressed cells, failed to concentrate in nuclei of heat-stressed cells (Fig. 3.2, part A). This suggests that the C-terminal portion comprising residues 588-646 may function in nucleocytoplasmic trafficking of hsc73.

The C-terminal domain promotes nuclear accumulation of non-nuclear reporter proteins upon heat stress

To further analyze the C-terminal segment of hsc73, fusion proteins containing the C-terminal part of hsc73 and two copies of GFP (GFP2) or three copies of GFP (GFP3) were monitored for their nucleocytoplasmic distribution. GFP2-hsc73(588-646) is 65 KDa and GFP3-hsc73(588-646) is 93 KDa, which are too large to diffuse between the nuclei and cytoplasm. Both proteins were nuclear and cytoplasmic under normal growth conditions and accumulated in nuclei when cells were exposed to heat (Fig. 3.2, part C). It can be seen from these results that the C-terminal part (588-646) of hsc73 is sufficient to accumulate the fusion proteins in nuclei upon heat stress. A quantitation of these results is shown in table 3.1.

The cNLS of hsc73 is not required for the nuclear localization of hsc73 in both stressed and unstressed cells.

Since we have shown that the C-terminal part of hsc73 is sufficient for the stress-induced nuclear accumulation, we wished to define the importance of the potential cNLS in hsc73 nuclear import. Importantly, deletion of the predicted cNLS for hsc73(Δ235-312) did not prevent the accumulation in nuclei (Table 3.1). Since the cNLS is not required for nuclear import of hsc73, we determined whether this signal is sufficient to promote nuclear accumulation after exposure to stress. To this end, GFP-cNLS-β-galactosidase has been generated and transiently synthesized in HeLa cells. The reporter protein located in the cytoplasm of control and heat stressed cells (Table 3.1). Taken together, the putative cNLS of hsc73 is not only unnecessary for nuclear localization of hsc73; it also is not

sufficient to locate the reporter GFP-cNLS-β-galactosidase to nuclei of heat-treated cells.

Hsc73, hsc73(Δ235-312) and GFP3-hsc73(588-646) shuttle in non-stressed cells

Proteins of the hsc70 family have been shown to shuttle between nucleus and cytoplasm. To determine whether the mutant proteins we have generated can shuttle in growing HeLa cells, we have used an approach that was recently developed in our laboratory (Kodiha *et al.*, in preparation). This strategy is based on the fact that cytochalasin B (Cyt B) inhibits nuclear export of proteins and RNA, but does not prevent nuclear import [106]. Proteins that shuttle between nucleus and cytoplasm will therefore accumulate in nuclei in the presence of Cyt B. With this approach, we have shown that treatment of HeLa cells with this drug induces nuclear accumulation of endogenous hsc70s. This supports the idea that in unstressed cells hsc70 nuclear accumulation is prevented because hsc70 is continuously exported from the nucleus. Similarly, Flag-tagged wild type hsc73 concentrated in nuclei under these conditions (Table 3.2). Likewise, Flag-hsc73(1-634), Flag-hsc73(Δ235-312), GFP2-hsc73 (588-646) and GFP3-hsc73(588-646) accumulated in nuclei of cells treated with CytB, suggesting that these proteins shuttle. By contrast, hsc73(1-480), hsc73(1-459) and hsc73(1-407) remained cytoplasmic under these conditions (Table 3.2).

***In vitro* nuclear import of His6-EGFP-hsc73, His6-EGFP-GFP2-hsc73(588-646) and His6-EGFP-hsc73(1-480)**

To further characterize nuclear trafficking of hsc73 we have analyzed nuclear import *in vitro*, using semipermeabilized HeLa cells. To determine the requirements for nuclear import in detail, different combinations of stressed and unstressed components (rabbit

reticulocyte extract, or semi-intact cells) were tested for the capacity to support nuclear accumulation of the reporter proteins. Stressed HeLa cells or other components were exposed to 45.5℃ for 30 min, and different combinations were tested in the *in vitro* import assays as shown in Fig. 3.3, lines 1-12.

His6-EGFP-GFP2-hsc73(588-646) can not accumulate in nuclei under most conditions tested. Only when proteins were pre-stressed together with ATP and cytosol at 45.5℃ for 30 min, and then incubated with stressed or unstressed semi-intact HeLa cells at 30℃ for 1.5 hours, the protein was imported into nuclei. The nuclear import of His6-EGFP-GFP2-hsc73(588-646) was also energy-dependent since depletion of energy with apyrase prevented this accumulation (Fig. 3.3).

For the His6-EGFP-hsc73 wild type protein, under unstressed conditions (Fig. 3.4 lines 1, 2, 3), proteins were both nuclear and cytoplasmic. However, the wild type protein translocated into nuclei (condition 4, 5, 6, 9, 10, 11, and 12), where either semi-intact HeLa cells or cytosol was heat-treated prior to the *in vitro* assay. The reaction was dependent on energy, since energy depletion with apyrase prevented the concentration of EGFP-hsc73 in nuclei. In energy-depleted cells EGFP-hsc73 was restricted to the cytoplasm (Fig 3.4, line 8).

In growing cells, Flag-hsc73(1-480) failed to accumulate in nuclei upon heat stress. To determine its trafficking *in vitro*, we carried out the same experiments as for His6-EGFP-hsc73 and His6-EGFP-GFP2-hsc73(588-646), using His6-EGFP-hsc73(1-480) (Fig. 3.5). To this end, soluble His6-EGFP-hsc73(1-480) was added to the import assays and tested for nuclear import. However, none of the conditions that promoted translocation of His6-EGFP-hsc73 or His6-EGFP-GFP2-hsc73(588-646) support nuclear

import of His6-EGFP-hsc73(1-480).

For all of the *in vitro* experiments, TRITC-HSA has been used as a control. The molecular weight of TRITC-HSA is 90 KDa, which is too large to diffuse between the nuclei and cytoplasm. In the semipermiablized HeLa cells, TRITC-HSA remains in the cytoplasm, but does not enter the nuclei. These control experiments verified that the nuclear envelope remained intact during the 1.5 hours incubation time, since TRITC-HSA was excluded from nuclei under all conditions tested (Fig. 3.6).

Discussion

The aim of our studies was to define the mechanisms that promote nucleocytoplasmic trafficking of hsc73 in control and stressed cells. We found that the C-terminal segment, comprising amino acid residues 588 to 646, are essential for hsc73 nuclear accumulation upon heat exposure. In heat-treated cells nuclear accumulation of EGFP-hsc73 and fusion proteins carrying the C-terminal hsc73 fragment was energy-dependent, indicating active transport across the nuclear envelope. We can exclude that EGFP-hsc73 or fusions containing hsc73(588-646) entered the nucleus by diffusion across the NPC for two reasons. First, diffusion is independent of energy, and second non-nuclear reporter protein without transport signal were excluded from the nucleus. Furthermore, other nuclear transport pathways, such as classical nuclear import, are inhibited in response to heat shock, both *in vivo* and *in vitro* [93; Kodiha *et al.*, unpublished]. Taken together, these results suggest a specialized active transport route for hsc73 and its C-terminal part into the nucleus of heat stressed cells.

With the experiments shown in this chapter, we have identified a novel stress-dependent nuclear import signal located in the C-terminal domain of hsc73. At present, the role of the divergent C-terminal domains found in different members of the hsp70/hsc70 family is not understood. For instance, in *E. coli* the C-terminal segment of DnaK is not required for its function *in vivo*. Likewise, the C-terminal portion of Kar2p, the yeast homology of BiP, is not required for cell growth [57]. Interestingly, the C-terminal part of the cytoplasmic yeast hsp70 Ssb1p is involved in nucleocytoplasmic trafficking of the molecule [103]. This segment contains an NES and prevents the nuclear accumulation of Ssb1p even when cells have been exposed to heat. The C-terminal NES

of Ssb1p, not present in members of the *SSA* family of hsp70s, which do accumulate in nuclei upon heat shock, restricts Ssb1p to the cytoplasm under control and stress conditions [103]. In addition, the C-terminal domain of hsc70s is involved in the interaction with several tetratricopeptide repeat containing proteins, including CHIP and TPR1 [109]. One might therefore speculate that the C-terminal domains of cytoplasmic hsp70s, although not essential to their biological function, can contribute to the nucleocytoplasmic distribution of the chaperone, either directly or by associating with other proteins. In line with this idea, we have shown here that upon heat stress C-terminal truncations of hsc73, such as Flag-hsc73(1-407), Flag-hsc73(1-459) and Flag-hsc73(1-587), fail to accumulate in nuclei, whereas amino acid residues 588 to 646 promote nuclear import (see below).

An alternative interpretation of the data obtained with truncated forms of hsc73 is that the removal of the C-terminal segments generated proteins that are no longer able to fold properly. These misfolded proteins would then remain in the cytoplasm, either because they form aggregates too large to enter the nucleus, or because nuclear targeting signals, such as the potential cNLS in position 246 to 262 of hsc73, are masked and therefore not accessible to the nuclear transport machinery. Future experiments will have to address these questions. It should be noted, however, that truncated forms of hsc73 were soluble when synthesized in *E. coli*. This indicates that, at least in bacteria, the deletion mutants do not have a high tendency to generate large aggregates.

Despite these possible complications with C-terminal truncations, results obtained for the fusion of hsc73 residues 588 to 646 and GFP2 or GFP3 clearly show that this C-terminal segment promote nuclear accumulation of the non-nuclear reporter proteins in

response to heat stress. Therefore, these results suggest that residues 588 to 646 are sufficient to mediate stress-induced nuclear accumulation. Moreover, our experiments with cytochalasin B clearly demonstrate that this segment can function as a shuttling sequence in unstressed cells, similar to what is observed for tagged wild type hsc73 or endogenous hsc70s. Interestingly, the C-terminal hsc73 segment has no clusters of positively charged residues, nor does it contain a leucine rich NES. The lack of classical transport signals in the C-terminal segment of hsc73 is reminiscent to what has been reported for several proteins that are involved in mRNA trafficking [110], whose shuttling sequences are not a simple combination of NLS and NES, but a more complex signal.

Given the fact that the C-terminal portion of hsc73 is sufficient to mediate stress-induced nuclear accumulation as well as shuttling in unstressed cells, it was important to determine the role of the potential cNLS, located between residues 246 and 262 of mouse hsc73. Upon deletion of this signal and flanking sequences, i.e. residues 235 to 315, mutant hsc73 can still concentrate in nuclei of heat-stressed cells. Furthermore, the deletion mutant also shuttles between nucleus and cytoplasm, demonstrating that the basic segment comprising residues 246 to 262 is not essential for stress-induced nuclear accumulation or shuttling. Our results are consistent with data described previously for trafficking of hsc70s in *Xenopus* oocytes; it was shown that the removal of one of the basic clusters present in the predicted cNLS did not abolish nuclear import [96]. However, Dang and Lee [111] reported that a fusion of pyruvate kinase to a basic segment of human hsp70 (residues 250 to 267 of the human protein, homologous to residues 246 to 262 of mouse hsc73) located to nucleoli of unstressed cells. As discussed by the authors, this nucleolar targeting of the fusion protein is unlikely to represent what is seen for

authentic hsp70s. They propose that the potential hsp70 nucleolar targeting signal is cryptic in its normal protein context and may be functional in response to heat [111]. Likewise, conjugation of several copies of the potential hsc70 cNLS to the carrier bovine serum albumin resulted in nuclear accumulation when injected into the cytoplasm of *Xenopus* oocytes [96]. With our studies we attempted to circumvent the complications that may arise by taking a candidate signal out of its normal context or coupling several basic peptides to a carrier protein. To this end, we inserted a larger fragment, residues 235 to 315 of hsc73, into the fluorescent reporter protein GFP-β-galactosidase. Nevertheless, GFP-hsc73(235-315)-β-galactosidase failed to concentrate in nuclei of heat-treated cells. Importantly, this reporter protein was also restricted to the cytoplasm in unstressed cells. Although these results are in line with the interpretation that the basic region in the N-terminal domain of hsc73 is not involved in nuclear import under control or stress conditions, it is still possible that the sequences flanking hsc73(235-315) may interfere with its targeting function. This question will be addressed in future studies by generating reporter proteins for which residues 235 to 315 of hsc73 are present in a different sequence context. In addition, it will be interesting to determine whether the insertion of residues 246 to 262 only into GFP-β-galactosidase supports nuclear transport of the reporter protein.

Nuclear accumulation of shuttling proteins, such as hsc73 or fusion proteins containing amino acid residues 588 to 646, can be achieved by several possible mechanisms: increase of import, decrease of export, enhanced nuclear retention, or a combination of these reactions. Although we cannot exclude that reduced nuclear export or enhanced nuclear retention contribute to the nuclear concentration of GFP-hsc73,

GFP2-hsc73(588-646) or GFP3-hsc73(588-646), our *in vitro* experiments clearly show that nuclear import is increased in response to heat stress. As such, all of the reporter proteins failed to accumulate in nuclei if semi-permeabilized cell assays were carried out with unstressed cells and unstressed cytosol. In the case of GFP-hsc73, nuclear accumulation was observed when either semi-intact cells or cytosol had been heat-treated. By contrast, GFP2-hsc73(588-646) required pre-incubation of the reporter protein with cytosol at elevated temperature. Therefore, conditions that promote the concentration of the C-terminal segment in nuclei are more restrictive than what has been observed for the wild type hsc73 sequence.

At this point, we can only speculate why the C-terminal portion of hsc73 depends on heat treatment together with cytosol for its nuclear accumulation *in vitro*. One possible scenario is that the C-terminal hsc73 fragment requires the association with cytosolic factors in heat-shocked cells for its subsequent transport into nuclei. To address this question, we have isolated protein complexes containing GFP2-hsc73(588-646) after heat stressing the substrate with reticulocyte lysate. Even though we were able to isolate higher molecular mass complexes containing GFP2-hsc73(588-646) under these conditions, their composition did not seem to differ from complexes obtained under non-stress conditions (data not shown). This might suggest that heat stress does not trigger the stable association of a cytoplasmic factor with the C-terminal segment of hsc73.

An alternative hypothesis for the necessity to heat shock the C-terminal hsc73 fragment in the presence of cytosol could be a posttranslational modification of the fragment; this modification may change in response to stress. Indeed, there are several serine and threonine residues between residues 588 and 646, and we have preliminary

evidence that the protein segment is phosphorylated in growing HeLa cells (data not shown). A role of phosphorylation in nuclear trafficking is well established and has been reported for a variety of proteins [112]. Depending on the protein, phosphorylation may increase or reduce nuclear import. Clearly more studies are required to analyze whether and how the modification of the C-terminal hsc73 portion controls its nuclear transport.

Interestingly, EGFP-hsc73 could also accumulate in nuclei, when semi-intact cells were heat-stressed, suggesting an alternative route into the nucleus. This indicates that components of the semi-permeabilized cells have to be "primed" by heat to support nuclear import by this parthway. As nucleoporins are involved in transport of different cargos in and out of the nucleus, they are candidate factors that could be affected by heat stress. In particular, nucleoporins of the FXF family are modified post-translationally by phosphorylation and O-glycosylation, and both of these modifications are dynamic [113]. Moreover, FXF nucleoporins are known to be key components of the nuclear transport apparatus, as they directly interact with transport complexes during NPC binding, translocation across the gated channel and release into the nucleoplasm. Future experiments will have to determine the role of FXF nucleoporins is altered in response to stress.

As discussed above, characteristics of *in vitro* nuclear import are distinct for wild type hsc73 and its C-terminal segment, as wild type hsc73 accumulate in nuclei when cytosol *or* semi-intact cells were heat stressed. The difference could be explained by the presence of multiple import signals in hsc73 that can function in response to heat shock. These signals may not only be diverse in sequence, they might also differ in their dependence on transport factors and possibly post-translational modifications. The

presence of several nuclear import signals has been reported previously for other proteins. For instance, ribosomal protein L5 contains three independent nuclear import signals [107]. These NLSs diverge in their capacity to bind nuclear import factors *in vitro* and their requirements for cytosolic transport factors are distinct. Like protein L5, hsc73 may contain several signals that promote heat-induced nuclear accumulation. It is tempting to speculate that these transport signals have dissimilar properties in nuclear trafficking, which have yet to be defined. However, the presence of several transport signals with unique properties may set the stage to respond to changes in cell metabolism or environment by activating one or more of these signals. Multiple signals with unique import characteristics could then provide a complex regulation of hsc73 nuclear trafficking according to the physiological needs of the cell.

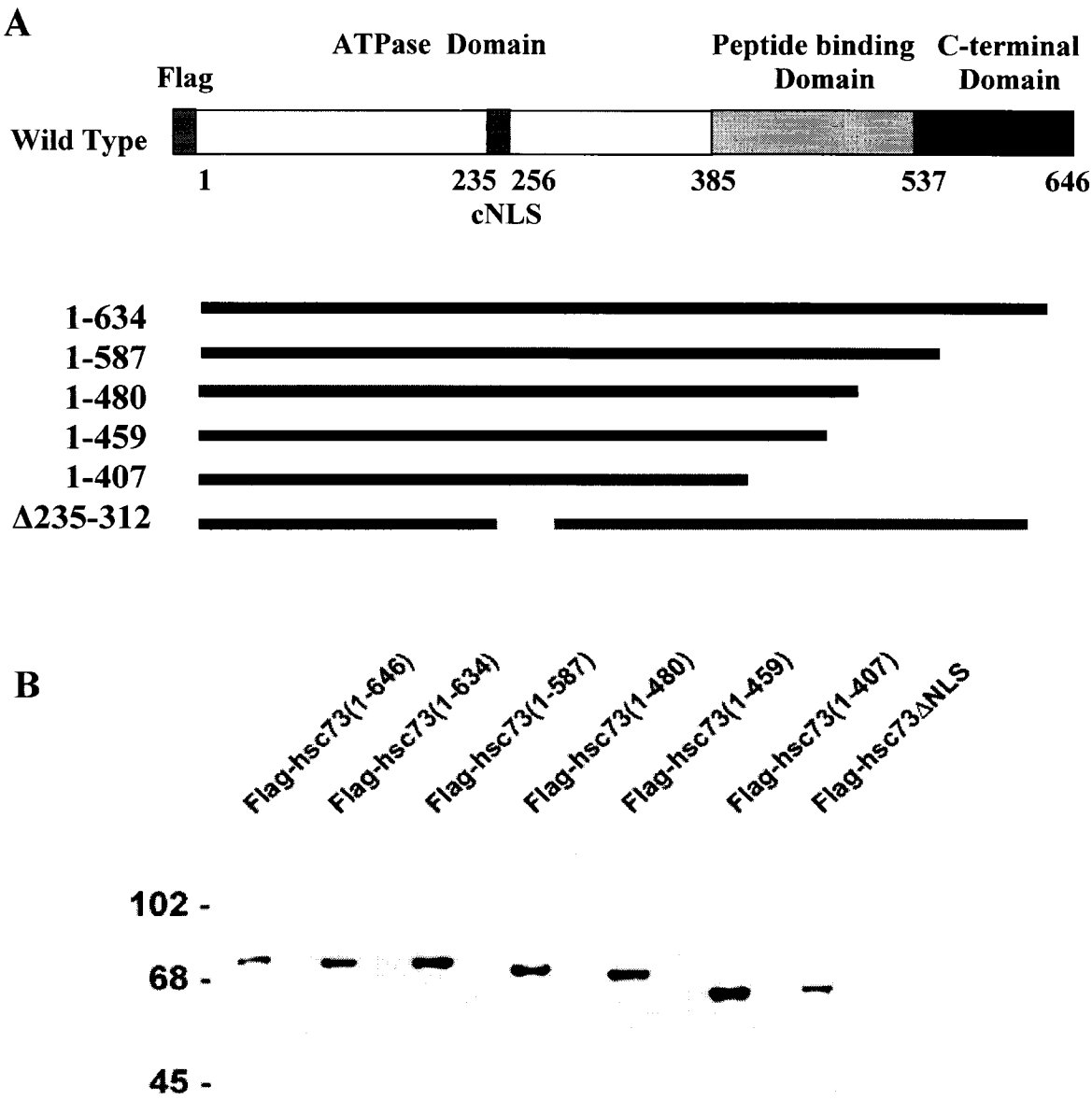
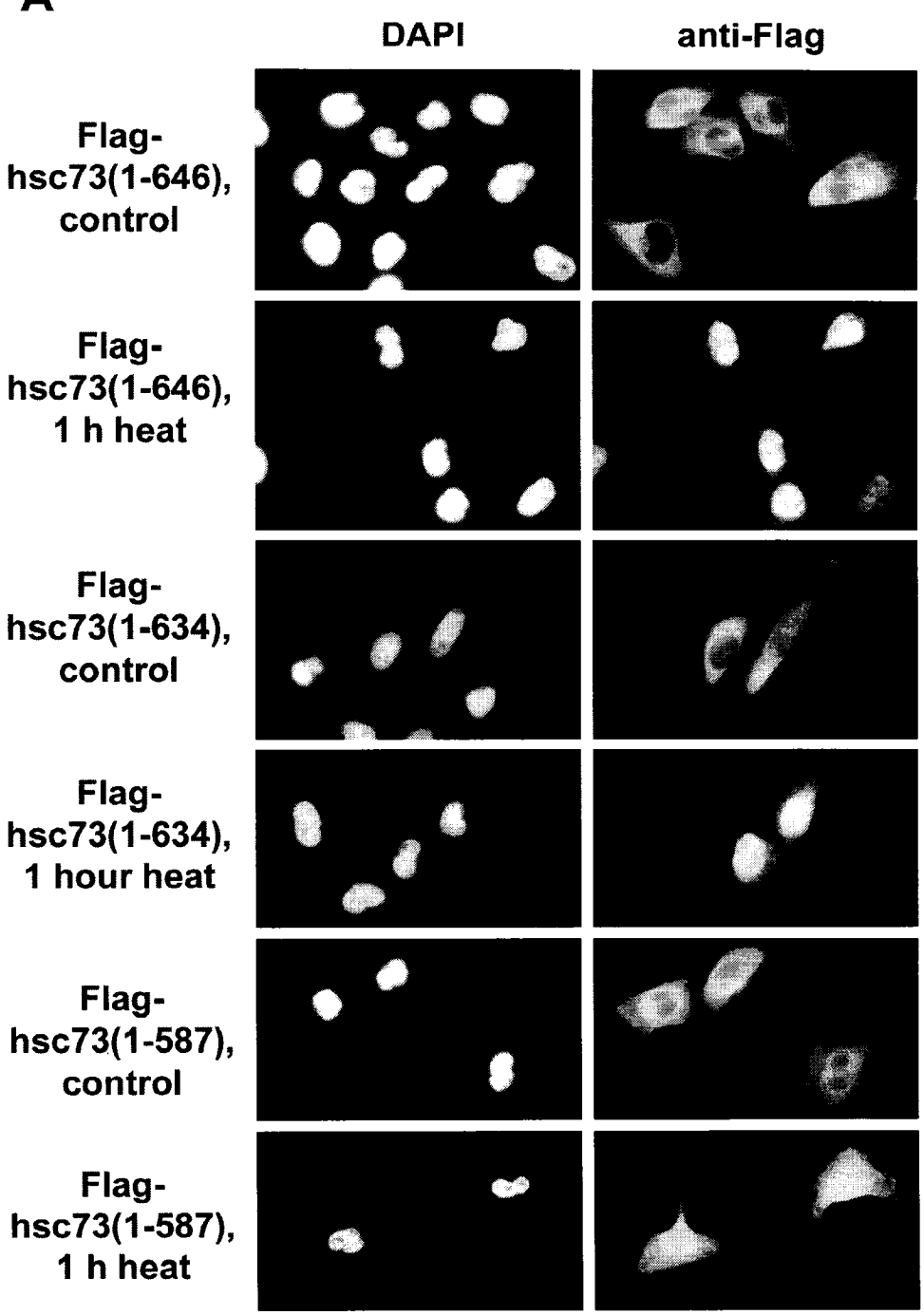


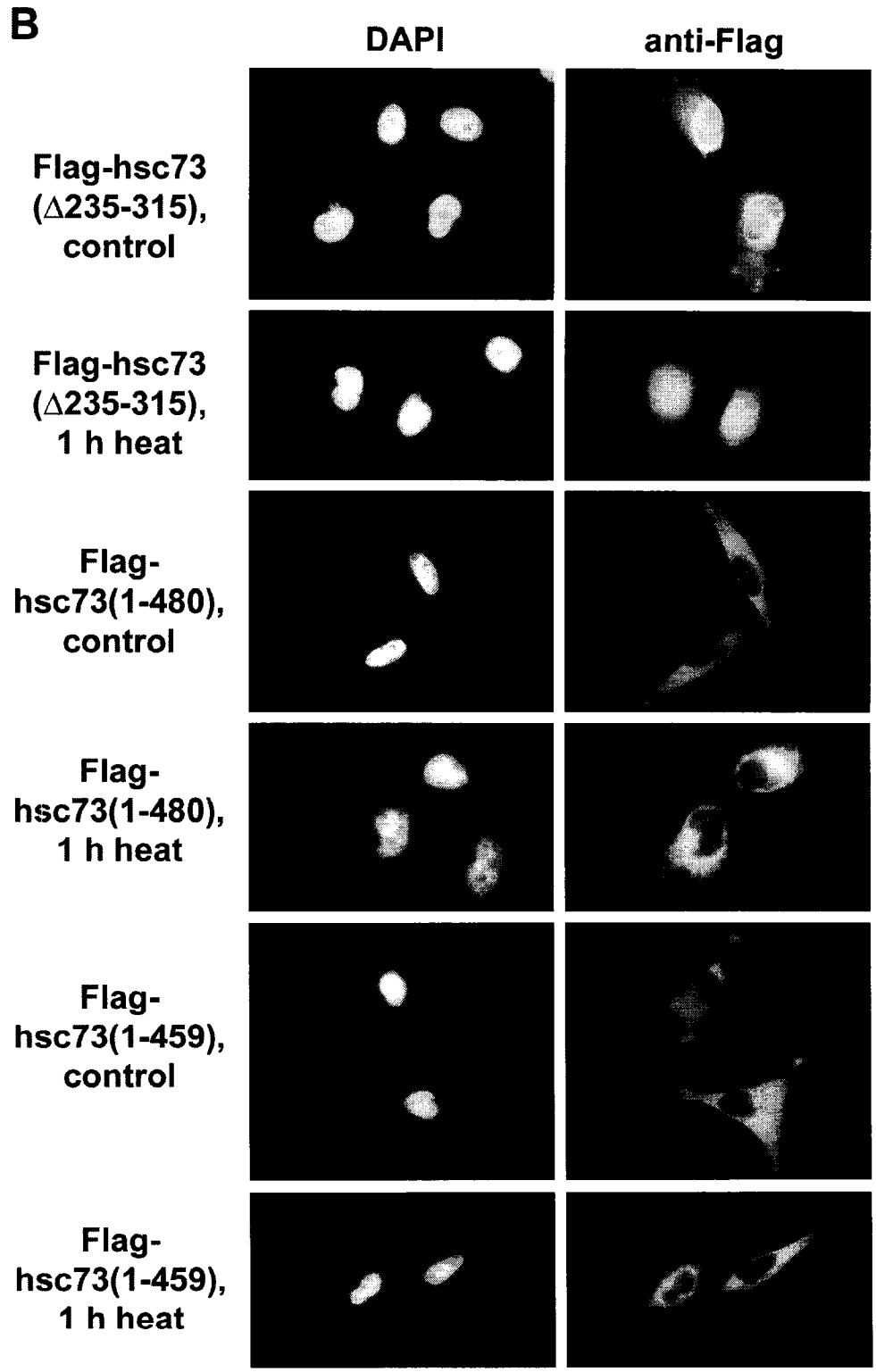
Fig 3.1 Truncated Flag-hsc73 used in the experiments.

A: Schematic representation of different Flag-hsc73 constructs. Wild type Flag-hsc73 contains three domains and the classical predicted NLS is located between residues 235 to 256. All the proteins carry a Flag-tag at the N-terminus. C-terminal truncations used in this study are shown.

B: Upon transfection into HeLa cells, protein synthesis was monitored by Western blotting with antibodies against the Flag-tag.

A


DAPI
anti-Flag
Flag-hsc73(1-646), control
Flag-hsc73(1-646), 1 h heat
Flag-hsc73(1-634), control
Flag-hsc73(1-634), 1 hour heat
Flag-hsc73(1-587), control
Flag-hsc73(1-587), 1 h heat


B
DAPI
anti-Flag
Flag-hsc73 (Δ235-315), control
Flag-hsc73 (Δ235-315), 1 h heat
Flag-hsc73(1-480), control
Flag-hsc73(1-480), 1 h heat
Flag-hsc73(1-459), control
Flag-hsc73(1-459), 1 h heat

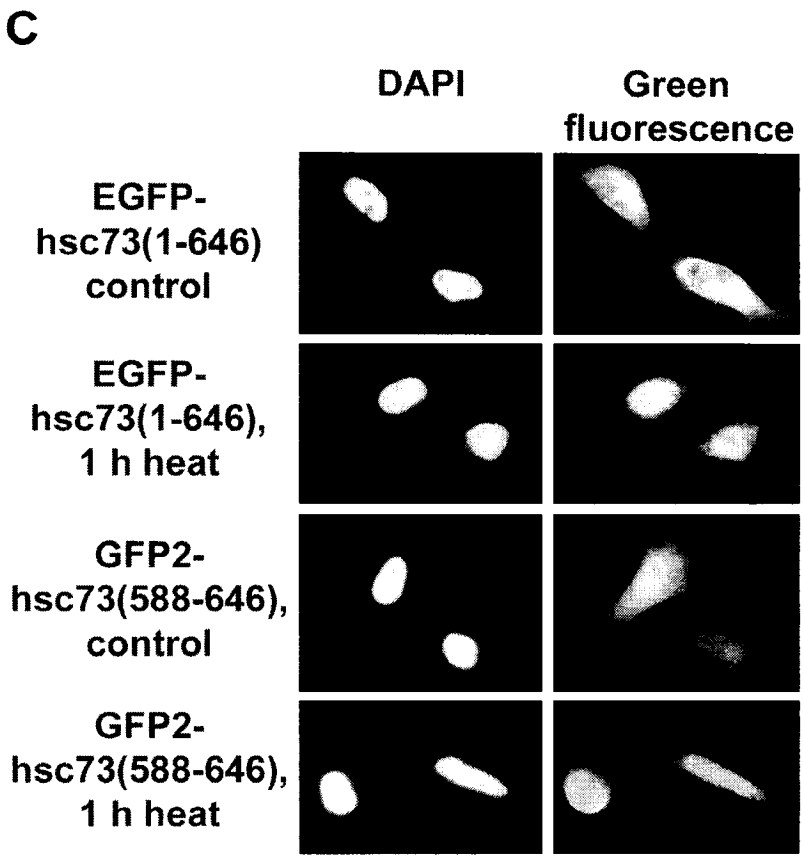


Fig. 3.2 Nuclear import of Flag-hsc73 and truncated Flag-hsc73 in HeLa cells upon heat stress.

Transiently transfected HeLa cells were exposed to 45.5℃ for 1 h. Cells were fixed and reporter proteins localized by fluorescence microscopy. DAPI staining of nuclei is shown for comparison.

	37℃			45.5℃		
	N>C	N+C	C>N	N>C	N+C	C>N
GFP2	0	0	100	0	0	100
GFP2-hsc73(588-646)	34±2.8	65±2.8	0	81±13	18±13	0
GFP3-hsc73(588-646)	17±5.5	83±5.5	0	13±4.5	87±4.5	0
Flag-hsc73(Δ235-312)	15±14	85±14	0	98±2.8	2±2.8	0
GFP- β-Galactosidase	0	0	100	0	0	100
GFP-cNLS-β-Galactosidase	0	5±5.5	95±5.5	0	10±7.9	90±7.9

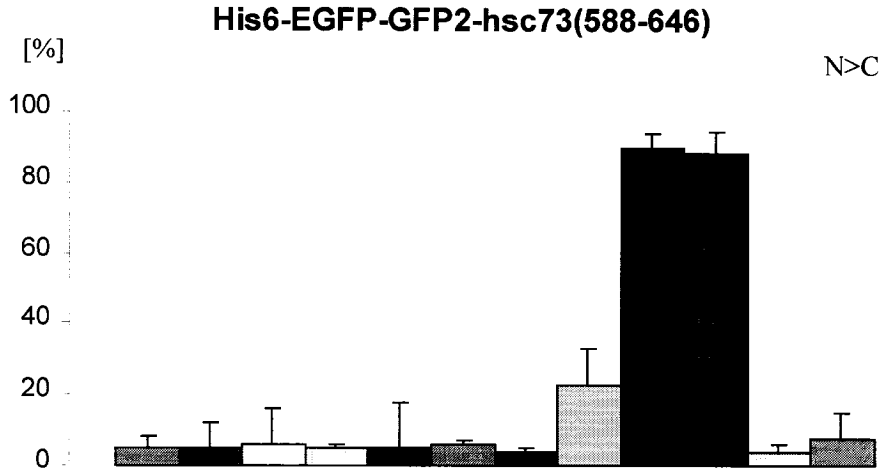
Table 3.1 The C-terminal part of hsc73 is sufficient for the nuclear accumulation upon heat stress. The predicted cNLS is not required for the localization of hsc73 in nuclei.

Transiently transfected HeLa cells were grown at 37℃ or exposed to 45.5℃ for 1 hour. Cells were fixed, and reporter proteins were localized by fluorescence microscopy. N and C indicate nucleus and cytoplasm, respectively. N+C, proteins locate in both nuclei and cytoplasm. N>C, proteins accumulate in nuclei. C>N, proteins are excluded from the nuclei. All of the results represent at least three separate experiments and 100 cells have been scored in each experiment. The numbers shown are means ± S.D.

	3 hours DMSO			3 hours CytB		
	N>C	N+C	C>N	N>C	N+C	C>N
Flag-hsc73	0	30±12	70±12	90±7.9	10±7.9	0
Flag-hsc73(1-634)	0	82±6.8	18±6.8	93±7.6	7±7.6	0
Flag-hsc73(1-587)	12±10	44±35	44±25	90±12	10±12	0
Flag-hsc73(1-480)	0	0	100	0	0	100
Flag-hsc73(1-459)	0	0	100	0	0	100
Flag-hsc73(1-407)	0	8±10.6	92±10.6	0	18±11.3	82±11.3
Flag-hsc73(Δ235-312)	15±14	85±14	0	100	0	0
GFP2-hsc73(588-646)	34±2.8	65±2.8	0	96±6.1	4±6.1	0
GFP3-hsc73(588-646)	17±5.5	83±5.5	0	91±3.3	9±3.3	0

Table 3.2 The effect of Cytochalasin B on the localization of mutant Flag-hsc73; identification of truncated hsc73s that shuttle.

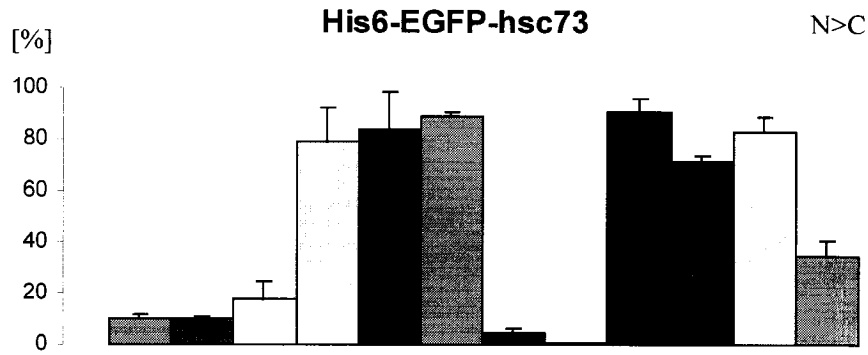
Transiently transfected HeLa cells were exposed to Cyt B dissolved in DMSO or only DMSO for 3 hours at 37°C. Cells were fixed, and reporter proteins were localized by fluorescence microscopy. The distribution of reporter proteins in nuclei (N) and cytoplasm (C) was monitored as described for Table 3.4. Data shown were obtained after performing the experiments at least three times.



	1	2	3	4	5	6	7	8	9	10	11	12
Stressed												
Protein						+	+	+	+	+		
Cytosol								+	+	+	+	+
ATP						+	+	+	+	+		
Semi-intact cells				+	+	+		+	+		+	
Unstressed												
Protein	+	+	+	+	+						+	+
Cytosol			+		+							
ATP		+	+	+	+						+	+
Semi-intact cells	+	+	+				+			+	+	+
Apyrase								+				

Fig. 3.3 Nuclear import of His6-EGFP-GFP2-hsc73(588-646) *in vitro*.

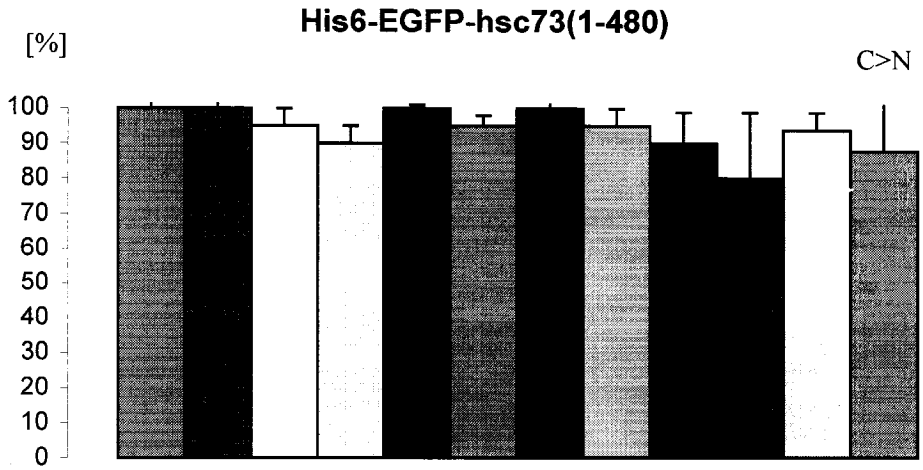
Stressed (exposed to 45.5℃ for 30 min) or unstressed semi-intact HeLa cells were incubated with pre-stressed (exposed to 45.5℃ for 30 min) or unstressed factors (including protein, cytosol or ATP) at 30℃ for 1.5 h. Cells were fixed and reporter EGFP proteins were localized by fluorescence microscopy. The graph shows the percentage of cells that accumulated the substrate in nuclei (N>C).



	1	2	3	4	5	6	7	8	9	10	11	12
Stressed												
Protein						+	+	+	+	+		
Cytosol								+	+	+	+	+
ATP						+	+	+	+	+		
Semi-intact cells				+	+	+		+	+		+	
Unstressed												
Protein	+	+	+	+	+						+	+
Cytosol			+		+							
ATP		+	+	+	+						+	+
Semi-intact cells	+	+	+				+			+	+	+
Apyrase								+				

Fig. 3.4 Nuclear import of His6-EGFP-hsc73 *in vitro*.

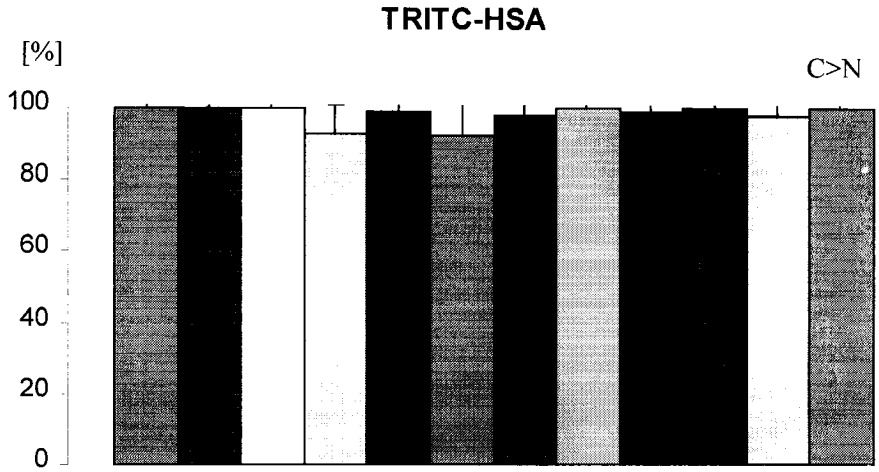
Purified His6-EGFP-hsc73 was used in the *in vitro* experiments, which were carried out as described for Fig. 3.3.



	1	2	3	4	5	6	7	8	9	10	11	12
Stressed												
Protein					+	+	+	+	+			
Cytosol							+	+	+	+	+	
ATP					+	+	+	+	+			
Semi-intact cells				+	+	+		+	+		+	
Unstressed												
Protein	+	+	+	+	+						+	+
Cytosol			+		+							
ATP		+	+	+	+						+	+
Semi-intact cells	+	+	+				+			+	+	+
Apyrase								+				

Fig. 3.5 Nuclear import of His6-EGFP-hsc73(1-480) *in vitro*.

Purified His6-EGFP-hsc73(1-480) was used in the *in vitro* experiments. Transport conditions were as described for Fig. 3.3. The graph shows the percentage of cells for which the substrate remains cytoplasmic (C>N).



	1	2	3	4	5	6	7	8	9	10	11	12
Stressed												
Protein						+	+	+	+	+		
Cytosol								+	+	+	+	+
ATP						+	+	+	+	+		
Semi-intact cells				+	+	+		+	+		+	
Unstressed												
Protein	+	+	+	+	+						+	+
Cytosol			+		+							
ATP		+	+	+	+						+	+
Semi-intact cells	+	+	+				+			+	+	+
Apyrase								+				

Fig. 3.6 Nuclear import of TRITC-HSA *in vitro*.

Purified TRITC-HSA was used in the *in vitro* experiments, which were carried out as described for Fig. 3.3. The localization of TRITC-HSA in the cytoplasm is depicted.

CHAPTER 4:

General Discussion and Conclusions

Proteins destined for nuclei are synthesized in the cytoplasm and, if exceeding the diffusion channel of the NPC, actively transported into the nucleus. In addition, many of the RNAs transcribed in the nucleus or nucleolus need to be exported to the cytoplasm. All of these transport processes are vital under normal physiological conditions; they depend on targeting signals and cellular transport machineries. Moreover, cells exposed to stress have developed specialized mechanisms to target protective molecules to the desired intracellular location, such as the nucleus. In this thesis, trafficking of macromolecules across the nuclear envelope has been studied. Specifically, I have analyzed in detail the generation of the Ran/Gsp1p GTPase gradient and the nucleocytoplasmic transport of heat shock proteins. The results presented in chapter 2 show that the formation of the Ran/Gsp1p nucleocytoplasmic gradient depends on several nucleoporins and the enzyme acetyl-CoA carboxylase, which were previously not known to contribute to the generation of the Ran/Gsp1p gradient. As discussed in detail in chapter 2, our results demonstrate, for the first time, that non-repeat nucleoporins play a role in regulating the nucleocytoplasmic Ran/Gsp1p gradient.

In chapter 3, we show that hsc73 transport upon heat stress has unique properties that clearly differ from the classical nuclear transport route. In particular, we have identified a short segment in the hsc73 C-terminal domain, which can function as a novel non-classical nuclear transport signal that is active in heat treated cells. Furthermore, this portion of hsc73 also promotes shuttling in unstressed cells.

Hsp70/hsc70s are conserved chaperones that participate in a variety of cellular functions, including protein folding and transport as well as the repair of stress-induced damage. Upon heat shock, their synthesis increases and they accumulate in the nucleus.

Nuclear protein transport depends on specific signals, and NLSs promote import across the NPC. The well-characterized cNLSs mediate nuclear import via the classical transport pathway, which has been studied extensively. By contrast, many of the non-classical routes have yet to be defined in detail. The N-terminal domain of hsc70s contains a putative cNLS, however, this signal is not required for nuclear import. This suggests that a non-classical signal promotes hsc73 nuclear import, by an unknown pathway. Indeed, our studies support the hypothesis that hsc73 can enter the nucleus by a non-classical signal and transport route, and we have begun to define this novel pathway. Using HeLa cells as a model, we have demonstrated that the C-terminal segment of hsc73 (amino acid residues 588-646) is necessary and sufficient for the nuclear accumulation upon heat stress. Searching of the protein databases reveals that this fragment does not contain a candidate cNLS, in line with the hypothesis that a non-classical pathway is involved in its nuclear trafficking. Although heat stress inhibits classical nuclear import in growing cells and *in vitro* (Kodiha, Gao, Quan, Lazrak, Varga and Stochaj, in preparation), hsc73 as well as its C-terminal segment can be actively imported into nuclei under these conditions. Our *in vitro* experiments have now defined the unique properties of the heat-induced nuclear accumulation of hsc73 and its C-terminal fragment.

Future studies

Although we are now beginning to understand the molecular mechanisms of nuclear trafficking, many questions remain. As discussed in chapter 2 of this thesis, some members of the importin β family may contribute to the formation of a nucleocytoplasmic Ran/Gsp1p gradient by binding and retaining the GTPase in the nucleus. At present, it is

not clear which of the nuclear carriers are involved in this process and how much of the Ran/Gsp1p gradient formation relies on nuclear retention. To fully comprehend the generation of the Ran/Gsp1p gradient, this question will have to be addressed in the future.

On the basis of the results presented in chapter 3, several aspects of hsc73 nucleocytoplasmic trafficking have to be further analyzed. It is clear from our data that the C-terminal segment of hsc73 promotes nuclear accumulation in an energy-dependent fashion. It is therefore possible that the RanGTPase plays a role in these transport processes. Recent results from our group suggest that Ran is required to accumulate wild type hsc73 in nuclei of heat-stressed cells, and we have identified the nuclear carrier that mediates import (Kodiha, Gao, Quan, Lazrak, Varga and Stochaj, in preparation). The obvious question to ask now is whether the C-terminal fragment relies on Ran and a member of the importin β family for import into the nucleus or whether a distinct route supports its translocation into nuclei.

In chapter 3, we have also discussed the possibility that upon heat exposure several NLSs may target hsc73 to nuclei. To fully understand how hsc73 moves in and out of the nucleus under different physiological conditions, it will now be crucial to identify and characterize these distinct transport signals.

Our preliminary data indicate that residues 588 to 646 of hsc73 are posttranslationally modified by phosphorylation. The NetPhos program predicts several residues in this protein segment to be phosphorylated with high probability. In future experiments, it will be important to determine whether the phosphorylation or dephosphorylation of any of these residues can regulate the nucleocytoplasmic distribution

of hsc73 or its C-terminal fragment.

Although divergent in sequence, C-terminal domains of hsp70s and hsc70s may be involved in targeting to different intracellular locations. It will therefore be interesting to examine whether these C-terminal domains, not required for the folding activity of the chaperone, have a more general role in regulating the nucleocytoplasmic distribution of the chaperones. As such, they could target hsp70/hsc70s to distinct subcompartments within the cytoplasm or nucleus. Within the nucleus, the nucleolus would be of major interest, as hsc70s transiently associate with nucleoli of stressed cells.

Conclusions

The results described in this thesis make new contributions to our understanding of the nucleocytoplasmic trafficking of proteins. We have shown that several non-repeat nucleoporins, including Nup133p, Rat2p/Nup120p, Nup85p and Nic96p, as well as the enzyme acetyl-CoA carboxylase control the distribution and cellular concentration of Gsp1p. In addition, we have identified a novel transport signal in the C-terminal part of hsc73, which mediates nuclear accumulation upon heat stress by a unique transport pathway and promotes shuttling in unstressed cells. These results set the stage to define the molecular mechanisms of hsc73 nuclear transport under normal physiological conditions and the changes in hsc73 trafficking that occur in response to stress.

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