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MOLECULAR AND ULTRASTRUCTURAL ANALYSIS OF TPR, A NUCLEAR PORE COMPLEX-ATTACHED COILED-COIL PROTEIN

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To my family

Summary

Anchored in the nuclear envelope, nuclear pore complexes (NPCs) are large multiprotein complexes which serve as the gateway for nucleocytoplasmic transport. In vertebrates, a 267-kDa protein termed Tpr has been localized to the nucleoplasmic side of the NPC. The function of Tpr has remained largely elusive. The main focus of this thesis has been to gain insight into Tpr's ultrastructural properties and investigate its potential contribution to NPC and intranuclear architecture.

The Tpr protein is divided into a large, coiled-coil forming aminoterminal domain and a shorter, highly acidic carboxyterminal domain. By constructing expression vectors encoding various Tpr deletion mutants, we mapped the position of Tpr's nuclear localization signal within the carboxyterminal domain, whereas a short segment of the aminoterminal domain was shown to be sufficient for NPC-binding. Individual amino acid substitutions introduced into this region were shown to abolish Tpr's ability to bind to the NPC and instead rendered the protein soluble, resulting in its accumulation in the nuclear interior.

To gain insight into Tpr's ultrastructural properties, we studied recombinant Tpr polypeptides by circular dichroism spectroscopy, chemical cross-linking, and rotary shadowing electron microscopy. We showed that Tpr's aminoterminal domain forms coiled-coil homodimers *in vitro*, and has an extended rod-like shape. Using a yeast-two hybrid approach, the arrangement of the coiled-coil was shown to be in parallel and in register. However, Tpr was neither found to self-assemble into extended linear filaments nor stably bound to other intranuclear structures.

At this point it was still uncertain whether or not Tpr acts as a scaffold onto which other NPC proteins (termed as nucleoporins) need to assemble. Moreover, it was also unknown which nucleoporin is the binding partner for Tpr at the NPC. To assess these questions we studied the sequential disassembly and reassembly of NPCs in mitotic cells, paralleled by studies of cells depleted of Tpr by RNA interference. The loss of Tpr was shown to neither have an effect on the assembly of the NPC nor the recruitment of any of various other nucleoporins. Using two-hybrid approaches and affinity chromatography, the binding partner that tethers Tpr to the NPC was found to be a nucleoporin termed Nup153. Whereas RNAi-promoted loss of Tpr had no effect on correct incorporation of Nup153 into the NPC, cellular depletion of Nup153 by RNAi abolished NPC binding of Tpr and caused mislocalization of Tpr to the nuclear interior.

In summary, this study has outlined all basic structural characteristics of Tpr and has provided insight into its architectural properties as a protein peripherally attached to the NPC.

ABBREVIATIONS

aa	amino acid(s)
AD	activation domain
AL	annulate lamellae
BD	binding domain
bp	base pair(s)
cDNA	complementary deoxyribonucleic acid
D	aspartatic acid
DTSSP	3,3'-dithiobis[sulfosuccinimydal propionate]
EM	electron microscopy
EST	expressed sequence tag
FA	formaldehyde
FG	phenylalanine-glycine
GDP	guanosine-diphosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine-triphosphate
hnRNP	heterogenous nuclear RNP
HR	heptad repeat
IF	immunofluorescence
kb	kilo base pair
kD	kilo Dalton
M	methionine
mAb	monoclonal antibody
NBD	nuclear pore complex binding domain
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
NS	nucleocytoplasmic shuttling
nt	nucleotide(s)
NuMA	nuclear mitotic apparatus
ORF	open reading frame
P	proline
PML	promyelocytic leukaemia
Ran	Ras-like nuclear GTPase
RNA	ribonucleic acid
RNAi	RNA interference
RNP	ribonucleoprotein particles
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
snRNP	small nucleolar ribonucleoprotein particle
siRNA	small interfering RNA
Tpr	translocated promotor region
TSS	transcription start site

CONTENTS

PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I N.V. Kuznetsov, L. Sandblad, M.E. Hase, A. Hunziker, M. Hergt, and V.C. Cordes
The evolutionarily conserved single-copy gene for murine Tpr encodes one prevalent isoform in somatic cells and lacks paralogs in higher eukaryotes
Chromosoma 111, 236-255, 2002

II V.C. Cordes, M.E. Hase, and L. Mueller
Molecular segments of protein Tpr confer nuclear targeting and association with the nuclear pore complex
Exp. Cell Res. 245, 43-56, 1998

III M.E. Hase, N.V. Kuznetsov, and V.C. Cordes
Amino acid substitutions of coiled-coil protein Tpr abrogate anchorage to the nuclear pore complex but not parallel, in-register homodimerization
Mol. Biol. Cell 12, 2433-2452, 2001

IV M.E. Hase and V.C. Cordes
Direct Interaction with Nup153 mediates binding of Tpr to the periphery of the nuclear pore complex
Mol. Biol. Cell 14, 000-000, 2003

INTRODUCTION

Research is not only to make new discoveries,
but as well to create order and thereby reduce complexity
(Nikos Panajotopoulos)

Characterization and visualization of proteins steadily contribute to our understanding of how proteins function within the highly organized eukaryotic cell. The subcellular location of a protein is often used as a key characteristic in elucidating the protein's function. However, without undertaking a comprehensive analysis of the protein itself, conclusions based only on the protein's environment might leave us with the wrong impression.

The present thesis describes a molecular and ultrastructural analysis of a protein called Tpr. When this study was begun, Tpr was known to localize at intranuclear fibers that are attached to the nuclear pore complex (Cordes et al., 1997). In amphibian oocytes, these fibers of unknown function appear to be several hundred nanometers long and occasionally connect the NPCs with the nucleoli (Cordes et al., 1993; Ris and Malecki, 1993; Arlucea et al., 1998). Moreover, in different types of *Drosophila* cells, Tpr has also been localized throughout the extrachromosomal nuclear interior where it has been suggested to be part of a filamentous nucleoskeleton (Zimowska et al., 1997). Indeed, predictions of Tpr's secondary structure and some of its sequence characteristics pointed at a protein potentially capable of forming filamentous structures. Consequently, Tpr had been proposed to act as an architectural protein within the nucleus and in association with the nuclear pore complexes. The functions of Tpr remain unknown, but it has been proposed to be involved in the overall organization of nuclear architecture, and in intranuclear and nucleocytoplasmic transport (Gant and Wilson, 1997; Ris, 1997; Singer and Green, 1997; Paddy, 1998; Pemberton et al., 1998).
This thesis provides experimental evidence that will allow us to argue in favour or against these notions. An overview of nuclear architecture, intranuclear and nucleocytoplasmic transport, and the nuclear pore complex will be given in the following section.

1. Architecture of the cell nucleus - Functional organization

The nucleus is a compact and highly organized organelle, host of the genome, and the site of DNA replication, transcription, and RNA processing. A double membrane, the nuclear envelope, separates the contents of the nucleus from the cytoplasm and provides the structural framework of the nucleus. One of the fundamental cell biology questions in nuclear architecture is how nuclear processes are spatially and temporally co-ordinated within the three-dimensional (3D) confines of the nucleus. To date, we know that an internal organization of the nucleus is indicated by the localization of nuclear processes to distinct nuclear compartments (Lamond and Earnshaw, 1998; Lewis and Tollervey, 2000; Dundr and Misteli, 2001).

1.1. Nuclear compartments

In interphase, the chromatin occupies a considerable portion of the nuclear volume. Although chromatin appears to be uniformly distributed, the chromosomes are arranged into discrete functional domains, in which individual chromosomes occupy distinct territories. These chromosome territories are separated by the interchromatin space compartment (Cremer et al., 1993; Zirbel et al., 1993), and within this compartment several discrete nuclear bodies, such as nucleoli, Cajal bodies, speckles, gems, PML bodies, and other small nuclear bodies have been located and their molecular constituents characterized. Consequently, various nuclear functions have been assigned to some of the nuclear bodies. For example, Cajal bodies are believed to function as sites of small nucleolar ribonucleoprotein particles (snRNPs) assembly, components of ribosomal RNA transcription and processing are concentrated within the nucleolus, and components of the splicing machinery are found within speckles (Lamond and Earnshaw, 1998; Matera, 1999; Spector, 2001).

The high degree to which the nucleus is organized has led to the proposal that a nuclear matrix or nucleoskeleton might support nuclear architecture. Such nucleoskeletal framework could help to maintain compartmentalization by providing attachment sites for the chromatin and nuclear proteins, or serve as tracks along which nuclear components could be transported (Pederson, 1998; Nickerson, 2001). However, the existence of a nucleoskeleton has remained a matter of debate. Its appearance was first published in 1974 on the basis of experiments in which nuclei were treated with DNAse and extracted with high salt buffer, and non-ionic detergents to remove histones and other soluble nuclear proteins. Such treatment left a residual network of fibers, consisting of the nuclear lamina, and an internal fibro-granular endoskeleton (Berezney and Coffey, 1974).

Meanwhile, the nuclear lamina - a network of intermediate filaments underlining the nuclear envelope - has been well characterized and its influence on both nuclear structure and function has been revealed to be profound (Hutchison, 2002). In contrast, the molecular composition of the internal endoskeleton has not yet been clearly defined (Pederson, 2000; Nickerson, 2001). Some believe that the observed nucleoskeleton might be the result of an artificial aggregation of proteins and nucleic acids during specimen preparation (Pederson, 1998). Nevertheless, there are proteins deep within the nuclear interior which have been shown to form filamentous structures. These include NuMA (Gueth-Hallonet et al., 1998; Harborth et al., 1999), actin, actin-binding proteins (Pederson and Aebi, 2002), and lamins (Moir et al., 2000). However, none of these proteins have been seen to form any naturally occurring filamentous network extending throughout the nuclear interior. Morover, the majority of proteins that have recently been isolated from the nuclear matrix and localized by immunofluorescence (IF) microscopy were rather found in punctuate structures, suggesting the existence of perhaps smaller local nuclear matrices (Hancock, 2000).

1.2. Nuclear envelope and nuclear organization

The nuclear envelope has a complex structure, consisting of two nuclear membranes, the underlying nuclear lamina, and large proteinaceous channels perforating the nuclear membranes - the nuclear pore complexes (NPCs).

The term lamina includes filamentous proteins - called lamins, plus numerous integral and peripheral proteins of the inner nuclear membrane, some of which bind to chromatin or to the lamins (Gruenbaum et al., 2000). These proteins have been shown to be essential targets in mitotic nuclear assembly (Burke and Ellenberg, 2002). They play a critical role in determining the shape of the nucleus (Liu et al., 2000), and in the maintenance and correct segregation of chromosomes into daughter nuclei during mitosis (Wilson, 2000).

Among additional roles in gene regulation (Hutchison, 2002), the lamina has also been found to be involved in chromatin positioning. In interphase, chromosomes are folded into a 3D-flexible network in which actively transcribed genes appear to be localized at the periphery of chromosome territories, whereas inactive genes face the territory interior or the nuclear periphery (Cremer et al., 2000). Most structurally repressed chromatin, including constitutive heterochromatin (telomeric and centromeric) and silenced chromatin, has been located near the nuclear envelope and has been shown to dynamically associate with the nuclear lamina (Wilson, 2000; Cohen et al., 2001). Also, there is increasing evidence that positioning of genes near peripheral heterochromatin regions promote gene silencing. However, it remains to

be seen whether the association of these regions with the nuclear lamina actively facilitates repression (Cockell and Gasser, 1999).

The relationship between NPC and nuclear organization was proposed many years ago by Günther Blobel (Blobel, 1985). Recently, several studies in yeast have given the first insights into possible molecular links. In these studies, two NPC-attached proteins, Mlp1 and Mlp2 (which are the two homologues of Tpr in yeast), have been suggested to be involved in perinuclear chromatin organization, since their absence leads to a mislocalization of telomeres and a disintegration of telomeric clusters. These effects have been proposed to be mediated by interaction of Mlp2 with the telomere binding protein Yku70 (Galy et al., 2000).

On the other hand, Ishii et al. have reported that physical tethering of chromatin boundaries to the NPC dramatically altered their epigenetic activities (Ishii et al., 2002). Regulatory DNA elements, like boundaries, affect gene regulation by segregating the chromosome into units of independent gene activity (Gerasimova and Corces, 2001). In their study, Ishii et al. demonstrated that various proteins involved in nucleocytoplasmic transport can exhibit boundary activity, when directly interacting with the NPC component Nup2p.

Taken together, both nuclear lamina and the nuclear pore complexes seem to organize nuclear architecture by establishing distinct compartments: a silencing heterochromatin compartment juxtaposed to the nuclear membrane, and a non-repressive euchromatic space adjacent to the nuclear pore (Carmo-Fonseca, 2002).

2. Intranuclear and nucleocytoplasmic transport

2.1. Intranuclar transport

The transport of RNAs and proteins within the nucleus is presumed to take place mainly in the interchromatin space compartment. In vivo experiments using IF techniques have revealed that most proteins are in continuous flux between different nuclear compartments (Phair and Misteli, 2000; Misteli, 2001), and nuclear bodies have been seen to move within the nucleoplasm as well (Swedlow and Lamond, 2001). RNAs have been found in association with a number of proteins forming large ribonucleoprotein particles (RNPs). An early model suggested that newly synthesized RNPs move by an active and directed process along tracks to the closest near pores (Blobel, 1985; Xing et al., 1993; Pemberton et al., 1998). However, to date no active transport mechanism has yet been found inside the nucleus. Instead, recent data have revealed that similar to the movement of proteins, RNP molecules also move by passive diffusion (Politz et al., 1998; Daneholt, 1999; Phair and Misteli, 2000). Nevertheless, RNP molecules have been seen to transiently interact with some structural elements in the

interchromatin space. An electron microscopic tomography study of Chironomus Balbiani ring mRNP particles has shown that some RNPs are in contact with thin fibers in the nucleoplasm (Miralles et al., 2000). These authors have proposed a model in which these fibers mediate binding of pre-mRNPs to other nucleoplasmic structures, and thus might be involved in an active, guided transport mechanism. However, the components of those fibers and their role in intranuclear transport remains to be analysed.

2.2. Nucleocytoplasmic transport

Multiple classes of RNAs, including messenger RNAs (mRNAs), transfer RNAs (tRNAs), and ribosomal RNAs (RNAs), must be transported from their site of transcription to the cytoplasm, where they then function in protein synthesis. Proteins required for nuclear functions, such as transcription factors and protein kinases, need to be continuously imported into the nucleus (Pines, 1999; Komeili and O'Shea, 2000). The molecular traffic across the nuclear envelope solely takes place through the NPCs. Whereas small molecules and ions traverse the NPC by diffusion, proteins and RNAs pass through in a signal-dependent and factor-mediated manner (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999). Most proteins to be transported into or out of the nucleus contain internal signals, nuclear localization signals (NLSs) or nuclear export signals (NESs), which are recognized by soluble transport receptors. These transport receptors bind to their cargoes, either directly or indirectly, with the help of adaptor molecules, and facilitate the passage of the receptor-substrate complexes through the pore. The majority of RNAs passes through the pore in dense complex with specific transport receptors (Cullen, 2003). Some of them are proteins containing nucleocytoplasmic shuttling (NS) signals, and can direct both nuclear import and nuclear export (Michael, 2000).

However, the actual process of translocation through the NPC is still a matter of debate. Many transport factors have been shown to directly interact with components of the NPC (Stoffler et al., 1999a; Vasu and Forbes, 2001). The NPC is a very large protein assembly of ~60 MDa (Cronshaw et al., 2002) that is composed of different proteins, which are referred to as nucleoporins. Many of these nucleoporins directly involved in transport share sequence repeats containing phenylalanine–glycine dipeptides and termed FG nucleoporins (Ryan and Wente, 2000). A common idea is that these FG nucleoporins build a core scaffold along which the transport receptors traffic. However, the exact arrangement of these FG nucleoporins, and their contribution to the translocation process, have been differently discussed (Paschal, 2002).

3. The Vertebrate Nuclear Pore Complex

3.1. 3D architecture of the NPC

The general outline of a nuclear pore has been studied by transmission electron microscopy (Akey, 1989; Hinshaw et al., 1992; Akey and Radermacher, 1993; Yang et al., 1998), scanning electron microscopy (Jarnik and Aebi, 1991; Goldberg and Allen, 1993; Ris and Malecki, 1993; Goldberg and Allen, 1996; Ris, 1997; Worman and Courvalin, 2002), and atomic force microscopy (Rakowska et al., 1998; Stoffler et al., 1999b; Danker and Oberleithner, 2000), and a consensus model of its 3D structure has emerged (Fig. 1). Accordingly, the NPC exhibits an eightfold symmetric basic framework (also called the spoke complex), sandwiched between a cytoplasmic and a nuclear ring. The ring-like spoke complex embraces the central pore channel and is often plugged with a distinct particle, called central plug or transporter, that has a highly variable appearance. In addition to this core structure of the NPC, peripheral fibrils emanate from the cytoplasmic ring as short tufts of ~50 nm in length, and from the nuclear ring as rectilinear fibrils of 5-7 nm in diameter and of variable length. Approximately 60-100 nm from the nuclear ring, the intranuclear fibrils are laterally interconnected by another ringlike structure, called the terminal ring. Together, the terminal ring and the fibrils represent a structural and functional entity, called the nuclear basket (Ris, 1989; Jarnik and Aebi, 1991; Ris, 1991; Goldberg and Allen, 1992).

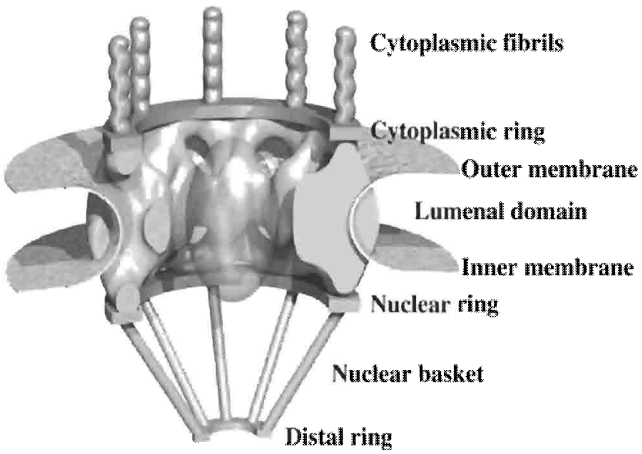

Figure 1: Architecture of the nuclear pore complex

This picture shows a three-dimensional reconstruction of the nuclear pore complex seen from an axis perpendicular to the nuclear envelope, (from Stoffler et al., 1999a).

3.2. Molecular constituents: The Nucleoporins

A recent proteomic analysis has identified all protein components of the vertebrate NPC. On the basis of sequence homology and subcellular localisation, 29 proteins were classified as nucleoporins and a further 18 as NPC-associated proteins (Cronshaw et al., 2002). To build an NPC of eightfold symmetry, each of the nucleoporins is believed to contribute to NPC architecture with 8 copies or multitudes thereof.

3.2.1. Localization of nucleoporins

In order to understand the organization and function of NPCs much effort has been put into the localization of nucleoporins within the 3D architecture of the NPC. However, a complete map of nucleoporins in the vertebrate pore is not yet available. Moreover, since different antibodies and immunolabeling procedures have been used in different investigations, the exact localization of various nucleoporins has remained unclear.

In summary, three nucleoporins have been mapped to the cytoplasmic side of the NPC so far. These comprise **CAN/Nup214** (Kraemer et al., 1994; Pante et al., 1994; Walther et al., 2002), **Nup84** (Pante et al., 1994; Bastos et al., 1997; Fornerod et al., 1997), and **RanBP2/Nup358** (Wu et al., 1995; Yokoyama et al., 1995). Furthermore, two NPC membrane proteins have been identified, namely **POM121**, and **gp210** (Gerace et al., 1982; Wozniak et al., 1989; Hallberg et al., 1993). **p62, p58, p54, p45** have been localized within the translocation channel (Cordes et al., 1995; Grote et al., 1995; Guan et al., 1995), along with **Nup155** (Radu et al., 1993). Nucleoporins located on the nuclear side comprise **Nup153** (Sukegawa and Blobel, 1993; Pante et al., 1994; Walther et al., 2001; Fahrenkrog et al., 2002), **Nup98** (Powers et al., 1995; Radu et al., 1995), **Nup96**, (Fontoura et al., 1999), **Nup 50** (Fan et al., 1997; Guan et al., 2000; Smitherman et al., 2000), **Nup93, Nup188, Nup205** (Grandi et al., 1997; Miller et al., 2000), and a 267-kDa protein termed **Tpr** (Cordes et al., 1997; Zimowska et al., 1997; Frosst et al., 2002). **Nup133** and **Nup107** have been reported to be located at both the nuclear and cytoplasmic sides of the NPC (Radu et al., 1994; Belgareh et al., 2001), and most recently **Nup98** has also been found on both sides (Griffis et al., 2003).

3.2.2. Interaction between nucleoporins

At present, there is only limited information on how nucleoporins interact with each other in order to build the NPCs. However, biochemical co-fractionation experiments have indicated that within the NPC different sets of nucleoporins are organized in subcomplexes, some of which remain stable during disassembly of the NPC during mitosis (Macaulay et al., 1995; Fornerod et al., 1997; Fontoura et al., 1999; Matsuoka et al., 1999; Miller et al., 2000). The

first nucleoporin subcomplex identified was the p62 complex, consisting of p62 and its partners p58, p54 and p52 (Dabauvalle et al., 1990; Finlay et al., 1991; Kita et al., 1993; Guan et al., 1995; Hu et al., 1996). p62 was also found to be a component of a second subcomplex together with the nucleoporins Nup214/CAN and Nup84 (Bastos et al., 1997; Fornerod et al., 1997; Hu and Gerace, 1998). Furthermore, the Nup160-Nup133-Nup96-Nup107 subcomplex (Radu et al., 1994; Fontoura et al., 1999; Belgareh et al., 2001; Vasu et al., 2001), and the Nup93-Nup188-Nup205 complex (Grandi et al., 1997; Miller et al., 2000) have been isolated. By characterizing the nucleoporins Nup84, Nup98, and Nup153, domains have been defined which directly or indirectly mediate nucleoporin-nucleoporin interactions (Bastos et al., 1997; Vasu et al., 2001; Griffis et al., 2003). Furthermore,. the components of the p62 complex have been shown to form homotypic or heterotypic protein-protein interactions via coiled-coil domains (Buss and Stewart, 1995; Guan et al., 1995).

3.3. NPC biogenesis: assembly and disassembly

In all eukaryotes, NPCs assemble during the S-phase of the cell cycle when the nucleus grows and the number of pore complexes doubles (Maul and Deaven, 1977). The newly assembled NPCs are inserted into the double membrane of the nuclear envelope by a so far unknown mechanism (Burke, 2001). However, some details are emerging from NPC assembly studies using scanning electron microscopy (Goldberg et al., 1997; Gant et al., 1998). NPC assembly is initiated by invagination of the inner and outer membranes until they meet and fuse to create a pore. The pore is then stabilized, possibly by the assembly of parts of the spoke ring complex. Central material is inserted simultaneously with a build up of the components of the rings, and is followed by the addition of peripheral filaments.

In higher eukaryotes, both NPC assembly and disassembly also occur as part of the dynamic rearrangements of the nuclear envelope during mitosis. As soon as the cells enter mitosis, the nuclear envelope breaks down. At this time, the disassembly of the nuclear pore is initiated by direct phosphorylation of a subset of nucleoporins (Macaulay et al., 1995; Favreau et al., 1996; Matsuoka et al., 1999; Miller et al., 1999). Subsequently, nuclear membrane proteins merge into the endoplasmic reticulum network (Ellenberg et al., 1997), and NPC subunits are dispersed into the cytoplasm. From a structural point of view, NPC disassembly has been described as a step by step process, starting with sequential loss of peripheral components, and followed by release of the central constituents of the pore (Kiseleva et al., 2001). However, at the end of mitosis, as soon as reformation of the nuclear envelope around the separated chromosomes starts, the nucleoporins are sequentially recruited back to form NPCs, whereby an apparent polarity underlies the order of recruitment. In fact, the

nucleoplasmic Nup153 has been shown to be recruited first, followed by the p62 complex of the central core and later by the cytoplasmic CAN/Nup214 (Bodoor et al., 1999; Haraguchi et al., 2000).

In addition, some knowledge about the role of nucleoporins in pore assembly is derivided from an *in vitro* nuclear reconstitution system in *Xenopus* egg extract. This system takes advantage of large stockpiles of disassembled nuclear components present in the egg, which after addition of DNA assemble into functional nuclei. Biochemical depletion of nucleoporins from these egg extracts has been used to produce nuclei whose NPCs lack specific components (Finlay et al., 1991; Powers et al., 1995; Grandi et al., 1997; Walther et al., 2001). For example, the lack of p62 has been shown to result in loss of NPCs (Dabauvalle et al., 1990). Depletion of Nup93 was found to cause a pronounced decrease in the number of pores formed per nucleus (Grandi et al., 1997). Furthermore, NPCs assembled in the absence of Nup153 lacked the nucleoplasmic nucleoporins Nup96, Nup98, and Tpr, and were mobile within the nuclear envelope (Walther et al., 2001). Lately, it has been shown that the cytoplasmic filaments and the nuclear basket actually can be removed without affecting NPC formation (Walther et al., 2002).

More recently, RNA interference (RNAi) has begun to become a valuable tool for studying NPC assembly in cultured mammalian cells. RNAi is a post-transcriptional gene silencing mechanism initiated by the introduction of short double stranded RNA which are homologous in sequence to the silenced gene (Tuschl, 2001). Using this approach, silencing of the *Nup153* gene has been reported to cause cell growth arrest (Harborth et al., 2001). Most recently, depletion of the nucleoporin Nup107 by RNAi has been shown to prevent the assembly of Nup358 and Nup214 on the cytoplasmic side, and Nup153 and Tpr on the nucleoplasmic side, indicating that Nup107 might act as a central module within the NPC (Boehmer et al., 2003).

In summary, *in vitro* and *in vivo* assembly studies allow us to conlude that NPC assembly is a step by step process, in which nucleoporins assemble into an NPC in a defined order.

3.3.1. Dynamic and turnover of nucleoporins

NPCs have long been considered a homogeneous population. Recent data suggest that some nucleoporins, such as Nup50, Nup98, and Nup153 might not merely represent structural elements of the NPC, but can also exist in highly mobile forms that rapidly bind to and detach from the NPC (Nakielny et al., 1999; Daigle et al., 2001; Griffis et al., 2002; Lindsay et al., 2002). Since all three nucleoporins have been reported to shuttle between nucleus and

cytoplasm, and have also been found to interact with various transport receptors, it has been suggested that these nucleoporins might act as mobile chaperons involved in transporting cargoes through the pore. In the case for Nup98, this nucleoporin has furthermore been found deep within the nucleus, suggesting that it might associate with its cargoes near their site of production and then target them to the NPC (Fontoura et al., 2001; Griffis et al., 2002).
Taken together, the idea has emerged that a subpopulation of nucleoporins with a low turnover constitute to the backbone of the NPC, whereas mobile forms of some nucleoporins with a high turnover are loosely associated to the NPC.

3.4. Peripherial NPC structures

From both morphological and functional perspectives, the effect of the peripheral structures, both the cytoplasmic and nucleoplasmic filaments, has been thought to endow the NPC with an overall asymmetry. Considering that both filaments provide the first NPC structure encountered by imported and exported cargoes, they have been proposed to contribute to unidirectional nucleocytoplasmic transport by an asymmetric arrangement of receptor-binding sites. Moreover, electron microscopy studies have revealed that the overall appearance of both types of filaments changes along with the transport of distinct cargo molecules (Kiseleva et al., 1996; Goldberg et al., 2000; Kiseleva et al., 2000). However, a full understanding of the functions of these filaments attached to the NPC requires knowledge of their composition and structure.

The cytoplasmic filaments

Based on electron microscopical observations and biochemical depletion studies, Nup358/RanBP2 has been identified as the main component of the cytoplasmic filaments (Pante et al., 1994; Walther et al., 2002). The structure of Nup358/RanBP2 itself was investigated by electron microscopy of negatively stained purified protein from rat liver nuclear envelopes, revealing long filamentous structure of ~35 nm (Delphin et al., 1997). From a functional point of view , Nup358/RanBP2 has long been implicated to be the initial docking site for import cargo complexes, especially since this protein contains various binding sites for transport receptors (Wu et al., 1995; Ohno et al., 1998; Ben-Efraim and Gerace, 2001). However, recent studies have revealed that the cytoplasmic filaments are dispensable for selective nuclear protein import (Walther et al., 2002). This finding has opened the field for discussion of more alternative functions. For example, it has been demonstrated that Nup358/RanBP2 also has SUMO1 E3 ligase activity (Pichler et al., 2002). SUMOylation appears to regulate protein-protein interactions and intracellular localization,

and protects some modified targets from ubiquitin-dependent degradation. Deductively, an alternative function of the cytoplasmic filaments could be the specific modification of cargoes, perhaps to direct their localization after nuclear entry or to regulate their activity before their assembly into macromolecular complexes within the nucleus (Pichler and Melchior, 2002).

The nucleoplasmic filaments

In contrast to the cytoplasmic filaments, the composition and function of the nuclear filaments have remained elusive. Among the bulk of proteins which so far have been localized at the nucleoplasmic side of the NPC, several nucleoporins, including Nup50, Nup98, Nup153, and Tpr, have been reported to be involved in nucleocytoplasmic transport (Shah et al., 1998; Ullman et al., 1999; Fontoura et al., 2000). However, how these nucleoporins would form or contribute to the NPC-attached basket structure remains unknown.

Early electron microscopy studies of certain cell types described additional fibers that emanate from the terminal ring region and project further into the nuclear interior (Franke, 1974; Maul, 1977; Kessel, 1983). In amphibian oocytes, this fibrous material appears to form hollow cylinders several hundred nanometers in length that occasionally connect the NPCs with the cortex of amplified nucleoli (Cordes et al., 1993; Ris and Malecki, 1993; Arlucea et al., 1998). However, the function of these filamentous structures is still not known, and so far only Nup153 and protein Tpr have been shown to be part of these filaments in Xenopus oocytes (Cordes et al., 1993; Cordes et al., 1997). Functions proposed for these filaments include roles in nucleocytoplasmic or intranuclear transport, or in structural organization of the nucleus (Franke and Scheer, 1970a,b; Scheer et al., 1988; Gant and Wilson, 1997; Ris, 1997; Singer and Green, 1997; Pemberton et al., 1998). On the other hand, these filamentous structures might be specific for oocytes, and might merely represent a common stockpile of nuclear proteins, including Tpr and Nup153, to be used later during embryogenesis.

Protein Tpr

Originally, the *tpr* gene (tpr: translocated promotor region) had been identified by its rearrangement in certain tumors (Park et al., 1986; Ishikawa et al., 1987; Soman et al., 1991; Greco et al., 1992), resulting in fusions between short Tpr segments and proto-oncogenic kinases.

Tpr and its homologs in yeast, Mlp1 and Mlp2, have been considered likely candidate proteins capable of forming filamentous networks or long filaments within the nucleus and attached to the NPCs that would provide a nucleoskeleton or filamentous tracks for

intranuclear transport (Zimowska et al., 1997; Paddy, 1998; Strambio-de-Castillia et al., 1999; Kosova et al., 2000; Fontoura et al., 2001).

In fact, Tpr is a protein of 2363 amino acids (aa) of which the first 1640 aa are dominated by consecutive copies of so-called heptad repeats (HRs) of seven aa residues (a-b-c-d-e-f-g). These HRs are a characteristic for α-helical proteins organized in coiled-coils (Lupas, 1996). Coiled-coil domains have been shown to be involved in homo- or hetero-oligomerization of proteins. The two-stranded coiled-coil is the simplest form. Here, the hydrophobic residues a and d form the interface between two α-helical proteins, which can run either in parallel or antiparallel. However, coiled-coils in which up to five α-helices twisted around one another to form a supercoil exist as well, but are less frequent than the two-stranded coiled-coil (Burkhard et al., 2001).

Within the nucleus, coiled-coil domains serve as dimerization domains of several types of DNA-binding proteins (Lupas, 1996), and as structural motifs within putative nucleoskeletal proteins like lamins and NuMA. In contrast to the transcription factors, NuMA and lamins dimers form extended coiled-coils and filamentous networks. NuMA has been shown to assemble into 5 nm filaments (Saredi et al., 1996) and its overexpression results in three-dimensional, ordered lattices within the nucleus of cultured cells (Gueth-Hallonet et al., 1998; Harborth et al., 1999). Lamins assemble into homo-and heteropolymeric filaments of 8-12 nm in diameter, building up the nuclear lamina network (Herrmann and Aebi, 1998).

With respect to Tpr, in the beginning of this study, little was known about its actual molecular architecture and ultrastructural properties. A short aminoterminal segment (aa 1-142) of Tpr, as part of the oncogenic Tpr-Met fusion proteins (Park et al., 1986), was known to be capable of homodimerization and thus mediate dimerization of this chimeric oncogene product (Rodrigues and Park, 1993).

However, whether the native Tpr protein forms homodimers or homopolymers, or whether it might rather engage in heteropolymeric interactions with other proteins, remained to be investigated. Moreover, proposed contributions to the NPC structure or to a nuclear skeleton were matters of debate, but in fact unclear.

RESULTS AND DISCUSSION

3. Protein Tpr: From Structure to Function

3.1. Gene structure and homologes

Sequencing of human Tpr cDNAs revealed ORFs of different lengths; the longest encoding a protein of ~267 kD (Mitchell and Cooper, 1992a,b; Byrd et al., 1994; Cordes et al., 1997; Gorlich and Kutay, 1999). The homologue in the fruit fly *Drosophila melanogaster* is of similar size ~262 kD; (Zimowska et al., 1997), whereas the two homologs in the budding yeast *Saccharomyces cerevisiae*, termed Mlp1 and Mlp2, are smaller ~218 kD and ~195 kD (Strambio-de-Castillia et al., 1999; Kosova et al., 2000).

In **paper I**, we address the question whether and to what extent isoforms, splice variants, or paralogs of Tpr may exist in mammals or in other metazoa. One prerequisite to answer this question was the isolation and thorough characterization of the murine *tpr* gene. We screened a mouse cosmid library with human Tpr cDNAs as hybridization probes, and sequenced the largest clone. This clone contained an insert of 32260 bp comprising *tpr*'s promoter region and the first 27 of a total of 52 exons. The sequence for the second half of the mouse *tpr* gene was assembled from various contigs that have been deposited in public mouse EST databases. Finally, the *tpr* gene sequence located between the transcription start site (TSS) and the polyadenylation signal comprised nearly 57 kb. The promoter region was found to be embedded in a CpG island, and lacks a TATA-box. The TSS is flanked by numerous potential binding sites for ubiquitous and tissue-specific transcription factors. Such promoter characteristics are known to be typical for many house-keeping genes (Novina and Roy, 1996) and support earlier reports suggesting *tpr* to be a gene widely expressed in mammalian cells (Park et al., 1986; Cordes et al., 1997). Completing the *tpr* gene characterization, we performed genomic Southern hybridization and radiation hybrid mapping and were able to show that the murine *tpr* exists as a single-copy gene on chromosome 1.

In the course of searching for Tpr splice variants, we first characterized *tpr* transcripts by Northern blot analyses. Although the expression levels strikingly differ in embryonic and most adult tissues, *tpr* expression was found to result in only one major transcript of ~8 kb in size. To confirm the presence of the Tpr translation product *in situ*, IF microscopy was performed on cryostat sections from mouse embryos and adult tissues. In all somatic tissues investigated, protein Tpr was detected near-ubiquitously in the cell nuclei, with a predominant localization at the nuclear periphery. A different Tpr staining pattern was only seen in certain stages of the spermatogenic process.

Additionally, in order to find very small transcripts that can not be distinguished by Northern blot analysis, we searched the mouse and human EST databases for Tpr sequences. Since all known intron-exon boundaries of *tpr* have been found to be evolutionarily conserved in mammals, we inferred that any regularly occurring splice variant of physiological significance would be evolutionarily conserved too, and thus would be present in both EST databases. Indeed, we found a few rare splice variants of both mouse and human tpr transcripts, but none of these splice variants found in either species was detected in the other. Consequently, alternative splicing of *tpr* transcript does not appear to be evolutionarily conserved.

However, at this point we could still not exclude that post-translational processing could result in Tpr variants. To address this issue, we raised novel monoclonal and peptide-specific antibodies against different domains of Tpr and used them for immunoblotting of total cell extracts. As a common result, the antibodies exclusively recognized a major band at ~270 kDa, indicating that only one major isoform of Tpr exists in mammalian cells.

Having established that no isoforms and splice variants of Tpr exist, we started to search for paralogs of Tpr in metazoans. To this end, we defined several sequence features that characterize all known Tpr homologs and used these as guidelines to search whole-genome sequence databases. Similar to *Saccharomyces cerevisiae*, two putative Tpr homologs were identified in the fission yeast *Saccharomyces pombe*. Furthermore, we found one probable Tpr ortholog each in *Arabidopsis thaliana* and *Caenorhabditis elegans*. However, no further paralogs of Tpr neither in different metazoan model organisms nor in humans were found.

3.2. Localization of Tpr in mammalian cells

Previous studies on the localization of Tpr and its homologs have reported partly inconsistent data. In somatic mammalian cells and amphibian oocytes, Tpr has been shown to be attached to the inner side of the NPCs as thin fibrils (Cordes et al., 1997). In addition, Tpr has also been described as an abundant fiber-forming protein present throughout the nuclear interior (Fontoura et al., 2001) or concentrated in discrete intranuclear foci instead (Frosst et al., 2002). Similarly, the homolog of Tpr in the fruit fly has been located both at the intranuclear side of the NPC and distributed throughout the extrachromosomal and extranucleolar spaces of the nuclear interior (Zimowska et al., 1997). In the budding yeast, the Tpr homolog Mlp2 has been found in proximity of the NPC, whereas Mlp1 has been located also deeper in the nuclear interior and at innerporous regions along the inner side of the nuclear envelope (Strambio-de-Castillia et al., 1999; Kosova et al., 2000).

In contrast to the two different yeast homologs, no paralogs or isoforms of Tpr can explain the controversial reports on Tpr's subcellular localization in mammalian cells. Since Tpr has been localized by different groups, using different monoclonal and polyclonal antibodies in different labeling procedures, we saw a need in reinvestigating Tpr's localization by using different Tpr antibodies under identical conditions for confocal IF microscopy.

As described in **paper I**, most of these Tpr antibodies revealed an intense and predominant labeling of the nuclear periphery. However, some antibodies also showed additional intranuclear staining. In order to distinguish between specific Tpr staining and possible cross-reactions with other proteins, we decided to make use of the RNA interference (RNAi) approach. By transfecting cells with small interfering RNAs, homologous in sequence to the mouse and human Tpr mRNA, we were able to suppress the expression of *tpr* in different mouse and human cell lines. In fact, three days after the initial transfection we observed near-complete disappearance of Tpr staining at the nuclear periphery, independently of which antibody was used. However, the strong intranuclear staining seen with some antibodies was not significantly affected by *tpr* RNAi, indicating that some Tpr antibodies not only recognized Tpr itself but also cross-reacted with other proteins *in situ*. Based on this observation we concluded, that in somatic mammalian cells Tpr is located predominantly at the nuclear periphery.

3.3. Secondary and quaternary structural properties

Predictions of a secondary structure suggested that protein Tpr is divided into two major domains; a large aminoterminal domain dominated by consecutive copies of heptad repeats (HR), and a short acidic carboxyterminal domain. In **paper III**, we provide experimental evidence for Tpr's ultrastructural properties, that allowed us to predict certain structural functions of the protein.

In order to gain insight into Tpr's basic secondary and quaternary structural properties, we first studied recombinant Tpr segments by circular dichroism spectroscopy and rotary shadowing electron microscopy (EM). We could show that polypeptides of the aminoterminal domain are dominated by α-helices which form extended rod-like shapes, whereas polypeptides of the carboxyterminal domain are of nonhelical structure. The rod-shape appearance of aminoterminal domain segments suggested that Tpr might form homomeric coiled-coils. Indeed, chemical cross-linking studies confirmed that homodimeric protein-protein interactions take place between recombinant polypeptides of Tpr's aminoterminal, but not carboxyterminal domain. Furthermore, by studying *in vivo* protein-protein interactions in the yeast two-hybrid system we found that only identical segments of the aminoterminal

domain homodimerize, indicating that the arrangement of the two strands within the coiled-coil is in parallel and in register.

In order to clarify whether Tpr's homodimers can self-assemble into homopolymeric filaments, we overexpressed the full-length molecule and deletion mutants thereof in human cells and analysed these by confocal IF microscopy and immuno-EM. In general, the full-length recombinant Tpr was found mainly bound to the NPC. However, in transfected cells with high amounts of the recombinant protein, surplus Tpr was also found dispersed throughout the extranucleolar nuclear interior or occasionally also within aggregate-like cytoplasmic structures. However, none of these Tpr polypeptides exhibited structures of marked filamentous appearance. In this sense, Tpr clearly differs from other coiled-coil proteins which have been seen to form rectilinear filament bundles and paracrystalline structures upon overexpression in transfected cells (Bader et al., 1991; Saredi et al., 1996; Klapper et al., 1997; Bridger et al., 1998; Yuan et al., 1998).

In addition, immuno-EM labeling of the recombinant Tpr revealed a clear spatial separation of the intranuclear pool of surplus Tpr from the NPC-attached pool. In contrast to those Tpr polypeptides stably associated to the NPC, the surplus Tpr was not structure-associated and could be quantitatively released by brief detergent permeabilization of cells. Gel filtration chromatography and sucrose gradient centrifugation revealed the soluble pool to be in a monodisperse form of 7,5 S, with a Stokes radius of ~161 Å. These values allowed us to calculate a corresponding molecular weight of ~509,000, which is close to the sequence-deduced value of 534,670 predicted for a homodimer of full-length Tpr.

In summary, since human Tpr did not self-assemble into homopolymeric filaments of marked length within the nuclear interior and also did not stably interact with any other intranuclear structures, an architectural role of Tpr deep within the nuclear interior semms unlikely. However, providing that Tpr's homodimers of extended rod-like shape are usually tightly associated with the NPCs, we believe any architectural function of Tpr only to be performed when bound to the NPC.

3.4. Tpr domains required for nuclear targeting and NPC-association

In order to identify which of the Tpr domains determines its localization at the NPC, we constructed expression vectors encoding different myc-tagged Tpr deletion mutants, expressed them in cultured cells, and studied their cellular localization by confocal IF microscopy.

In **Paper II**, we show that Tpr's carboxyterminal domain contains a bipartite nuclear localization signal (NLS) that is necessary and sufficient to mediate nuclear import of the

protein. Transfection of cells with expression vectors encoding only the myc-tagged aminoterminal domain resulted in nuclear exclusion of the truncated protein. However, as soon as the aminoterminal domain was fused to the protein's NLS or to an artificial NLS (e.g. SV40-NLS), the imported protein was targeted to the NPC. By further shorting the aminoterminal domain, we could define a segment (aa 1-513) that was sufficient to confer association of protein Tpr with the NPCs.

Figure 2: Schematic illustration of Tpr's domain structure

The rectangle represents the Tpr protein comprising 2363 aa. Black boxes indicate HR clusters within the aminoterminal domain; the short hatched rectangle represents the carboxyterminal domain; NBD: NPC binding domain; NLS: Nuclear localization signal.

In **paper III**, we then aimed to characterize the sequence elements essential for NPC binding. Knowing that Tpr's aminoterminal domain forms a double stranded coiled-coil, it remained to be clarified whether the heptad repeats (HRs) or other sequence elements contribute to NBC binding. Within the aminoterminal domain 14 clusters of heptad repeats, called HR clusters, exist (see Fig. 2). By constructing expression vectors encoding smaller Tpr polypeptides we could narrow down Tpr's NPC binding domain (NBD) to only a single HR cluster, no. 5, located between aa 437 and 513. This HR cluster contains 11 consecutive copies of the heptad aa consensus sequence "abcdefg", which are arranged in tandem, yielding at least five HRs.

To determine whether the sequence integrity of individual HRs is essential for NPC binding, we introduced aa substitution mutations. Expecting more dramatic effects with proline than with other aa substitution mutations on α-helical structure and coiled-coil formation, these were preferably studied. Indeed, we could completely abolish NPC binding of Tpr recombinant protein by double proline substitutions at L458 and M489 (both in d positions of the tandem repeats 2 and 4). The same effect was observed when aa L458 and M489 were exchanged for aspartic acid residues. Remarkably, in contrast to NPC binding, none of these substitutions abolished Tpr's general ability to homodimerize, as monitored by the yeast two hybrid system. This allowed us to conclude, that Tpr binds to the NPC as a homodimer.

However, at this point we could still not rule out that binding of recombinant Tpr to the NPC might be the result of homo-oligomeric interactions with the wild-type Tpr homodimers, rather than of direct binding to other NPC proteins. To clarify this issue, we first depleted cultured cells of wild-type Tpr by RNAi and later post-transfected Tpr deficient cells with expression vectors encoding the intact ot mutated NBD. However, in order to allow synthesis of recombinant Tpr variants in the presence of siRNAs that target the wild-type Tpr mRNA, concomitant degradation of the recombinant mRNA had to be prevented. To this end, we introduced silent point mutations into the nucleotide sequence encompassing the start codon. These sequence alterations allowed the expression of recombinant Tpr despite on-going silencing of the wildtype *tpr* gene. Eventually, we could show that recombinant polypeptides possessing an intact NBD were stably bound to the NPC even though the wild-type protein was no longer present. In contrast, Tpr polypeptides with aa substitution mutations L458P and M489P, bound neither to the NPCs of control cells nor to those of wild-type Tpr-depleted cells. Instead, these mutants remained in a soluble, detergent-extractable form within the nuclear interior.

In summary, we demonstrated that Tpr binds to the NPC as a homodimer in which a single HR cluster, no. 5, represents the direct binding site for perhaps one or several NPC proteins. Since this binding has been shown to depend on the sequence integrity of the HR cluster, NPC binding may occur via heteromeric coiled-coil interactions between Tpr's HR and that of the binding partner. On the other hand, in case that a homodimeric cluster 5 coiled-coil would be recognized by the binding partner as a structural unit, the binding partner would not have to be a coiled-coil protein itself.

3.5. Binding partner at the NPC

Although in previous reports several nucleoporins have been proposed to interact with Tpr, the binding partner responsible for positioning Tpr at the NPC remained elusive. Amphibian NPCs reassembled from *Xenopus* egg extracts, that had been immuno-depleted of Nup153, have been reported to lack Nup93, Nup98, and Tpr (Walther et al., 2001). However, whether Nup153 might serve as a direct binding partner for any of these proteins was not determined. Independently, the nucleoporin Nup98 has been described as an interaction partner of Tpr (Fontoura et al., 2001), but whether Nup98 serves as an NPC-attachment site for Tpr, or whether Tpr itself provides an anchor site for Nup98 remained unanswered. In yeast, several nucleoporins have been found to be associated with either Mlp1 or Mlp2, including Nup145p-C and Nic96p, the probable yeast homologs of Nup96 and Nup93, respectively (Galy et al.,

2000; Kosova et al., 2000), but also here, whether these proteins interact directly with Mlps, or whether binding is mediated by additional linker proteins, remained uncertain.

In **paper IV**, we present different experimental approaches aiming at identifying Tpr's direct binding partner at the NPC. *In vivo* crosslinking of cultured cells followed by immunoprecipitation of Tpr revealed a close spatial relationship of Tpr with various nucleoplasmic nucleoporins, including Nup93, Nup96, Nup98, Nup107, Nup153, and Nup205. In contrast, p62 (a component of the central pore channel) could not be co-immunoprecipitated. However, despite variations in DTSSP concentration and length of incubation, these and other cross-link approaches did not allow us to distinguish unequivocally between nucleoporins that were bound directly to Tpr, and those located only in close proximity. Consequently, all of those basket proteins could be potential binding partners for Tpr.

In order to find the nucleoporin(s) that would directly interact with Tpr's NPC binding domain, we designed bacterial expression vectors encoding GST-fusion proteins, comprising Tpr aa 172-651 with and without aa substitutions. The purified recombinant proteins were immobilized on glutathione Sepharose beads and used for affinity chromatography of human cell extracts. In this way, Nup153 was found to bind specifically to the intact NBD of Tpr. Moreover, since only traces of Nup153 were found associated with the NBD mutant, we concluded that this interaction clearly depends on sequence integrity of Tpr's NBD. In contrast, the nucleoporins Nup50, Nup93, Nup96, Nup98, Nup107, and Nup205, did not interact with Tpr's NBD.

As an independent approach to study interactions between Tpr and other nucleoporins, we made use of the yeast two-hybrid methodology. We constructed two-hybrid expression vectors encoding the full-length sequences and/or segments of Nup50, Nup93, Nup96, Nup98, Nup107, Nup153, Nup160, and Nup205, and studied their abilities to interact with the intact and mutated form of Tpr's NBD. Again, the yeast two-hybrid study confirmed the specific interaction between Tpr and Nup153. The intact NBD did not interact with any of the other nucleoporins, and aa substitutions within the NBD near-completely abolished binding to Nup153. Furthermore, Tpr's NBD was found to interact only with a short segment of Nup153 aminoterminal domain, comprising aa 228-439. Interestingly, this Nup153 region has been shown to be essential for anchoring Nup153 to the NPC itself (Enarson et al., 1998), and also to contain the binding sites for the NPC subcomplex containing Nup96, Nup107, Nup133, and Nup 160 (Vasu et al., 2001). In fact, except for Nup96 and Nup107, our findings so far did not rule out the possibility that another protein acts as a linker between Tpr and Nup153. For clarification, we investigated whether or not purified recombinant Tpr and Nup153

polypeptides were capable of direct interaction *in vitro*. Indeed, immobilized GST-Tpr 172-651 was found to bind recombinant Nup153 polypeptide 228-611, whereas binding between mutant GST-Tpr and Nup153 was impaired. Consequently, we concluded that the interaction between Tpr and Nup153 is direct and specific. Moreover, semiquantitative analysis of the amount of Nup153 bound to Tpr revealed an average Nup153:Tpr ratio of 1:2,1, consistent with our conception that Tpr homodimers are bound to Nup153 and thus to the NPC.

In summary, we demonstrated that the nucleoporin Nup153 is the direct binding partner of Tpr at the NPC. However, we could not rule out the possibility that other nucleoporins might interact with domains downstream or upstream of Tpr's NBD, and that such additional interactions might help to stabilize Tpr's association with the NPC.

3.6. Role in NPC-assembly

NPC biogenesis in post-mitotic cells is understood to be a hierarchically organized succession of events, in which the different nucleoporins are sequentially recruited to the reassembling NPC. Previous studies have shown that re-association of Tpr is preceeded by incorporation of several nucleoporins of the cytoplasmic side (RanBP2/Nup358, Can/Nup214) and the central NPC channel (p62, Pom121), but also by the nucleoplasmic Nup153 (Bodoor et al., 1999; Haraguchi et al., 2000). However, the sequence by which other nucleoplasmic nucleoporins are recruited back to the NPC was not known. In fact, it was possible that Tpr itself might act as a scaffold for other nucleoporins, and thus, Tpr's presence would be essential for their recruitment to the NPC.

In order to understand the role of Tpr in NPC assembly, in **paper IV**, we analyzed the composition of NPCs during different stages of mitotic HeLa cells by double-labeling IF microscopy. Tpr was found to be recruited back to the nucleus only late in telophase, when chromosomes were mostly enclosed by a continuous nuclear envelope. In striking contrast, other nucleoplasmic nucleoporins, including Nup50, Nup93, Nup96, Nup98, Nup107, and Nup153, were already present at the periphery of the newly segregated chromatids in anaphase. At this time point, Tpr was still found dispersed throughout the cytoplasm, indicating that recruitment of nucleoplasmic nucleoporins to the NPC clearly preceeded recruitment of Tpr.

Similar observations were made in HeLa cells in which Tpr synthesis had been suppressed by RNAi. Such post-transcriptional *tpr* gene silencing did not prevent the other nucleoporins from binding to the NPC. On the other hand, cellular depletion of Nup153 by RNAi clearly caused a mislocalization of Tpr, and also Nup50, to the nuclear interior.

However, loss of Nup153 did not prevent other nucleoporins from binding to the NPC, suggesting that Nup153 might provide a link between the NPC core and Tpr.

In summary, these observations indicated that Tpr does not act as an anchoring site for other nucleoplasmic nucleoporins at the NPC. Furthermore, since NPC distribution within the nuclear envelope remained unaffected by the lack of Tpr, Tpr is also not an essential anchoring element for the NPC as had been proposed earlier (Walther et al., 2001).

However, the finding that Tpr binds only to properly assembled NPCs, renders Tpr a protein only peripherally attached to the NPC. Assuming that the other nucleoplasmic nucleoporins formerly envisioned to be basket components might instead be located closer to the NPC proper, the coiled-coil homodimer of Tpr now emerges as the most likely candidate for the central architectural element of the nuclear basket.

CONCLUSIONS AND PERSPECTIVES

The aim of this thesis has been to provide insight into Tpr's ultrastructural properties and its proposed architectural functions within the nucleus and at the NPC. Tpr is shown to exhibit a bipartite domain organization: the long aminoterminal domain is dominated by α-helices arranged in double-stranded coiled-coils of extended rod-like shape, whereas the short acidic carboxyterminal domain is of non-helical structure. Each domain exhibits different targeting properties: the carboxyterminal domain targets the protein into the nucleus, and the aminoterminal domain mediates binding to the NPC. NPC binding involves a single HR cluster that directly interacts with the nucleoporin Nup153. Aa substitution mutations introduced into this HR cluster abolish Tpr's ability to bind to Nup153, rendering the protein soluble, resulting in its accumulation in the nuclear interior. Since Tpr has neither the potential to self-assemble into extended filaments nor stably bind to other intranuclear structures, an architectural function within the nucleus seems unlikely. Instead, Tpr's homodimers stably associated with the NPC alone suggest a role in NPC architecture and function. Moreover, since Tpr is dispensable for NPC binding of other nucleoporins, we conclude that this protein is peripherally attached to the NPC core. Based on its localization and ultrastructural properties we postulate that Tpr is the central architectural element of the NPC-attached basket fibers.

Meanwhile, in our laboratory, specific antibodies against various nucleoporins and different segments of the Tpr protein have been used to study Tpr's location at the NPC in spatial relationship to other nucleoporins by post-embedding immuno-EM. Additionally, posttranscriptionally gene silencing of other nucleoporins, gave insight into their role in NPC architecture. Based on these data, a new detailed 3D map of the NPC has been drawn, in which the bulk of nucleoporins is located within NPC core structures, whereas Tpr indeed emerged as the central component of the nucleoplasmic basket fibers (*Manuscript* in preparation).

Nuclear basket assembly

Provided that Tpr is the main constituent of the nuclear basket, the question arises how the Tpr homodimers assemble into this structure of eight-fold rotational symmetry.

Some insight has emerged from recent studies in our laboratory, in which antibodies recognizing different domains of Tpr have been used for immuno-EM. The resulting data allow us to propose a model in which the basket fibers consist of folded Tpr homodimers or homotetramers. These homomers are anchored to the nucleoplasmic ring of the NPC via Tpr's NBD, whereas both amino- and carboxy-termini are oriented towards the nuclear interior, and position near the basket's distal end. In fact, assuming that Tpr's rod-shaped coiled-coil domain can reach more than 100 nm when fully extended, the distance of ~50 nm between nucleoplasmic ring and distal ring could easily be bridged by the Tpr protein, even when folded back onto itself. Since the basket fibers are of ~3-6 nm in diameter (Cordes et al., 1993; Ris, 1997), in principal, each fiber could harbor several Tpr homodimers. Guided by fiber models for other coiled-coil proteins (Herrmann and Aebi, 1999), we can estimate that a 6 nm thick basket fiber could accommodate either up to 16 rectilinear Tpr homodimer or eight dimers folded back onto themselves. A 3 nm fiber could instead only accommodate a tetramer or a single folded-up dimer. One approach to distinguish between these possibilities would be to purify the full-length Tpr protein and study its self-assembly characteristics. So far, attempts to purify full-length Tpr from mammalian tissues, and from bacterial and insect expression systems, did not yet yield sufficiently high amounts of undegraded protein in pure form. However, once such purified Tpr will be available, we could study Tpr's overall appearance, and the manner in which it might assembles *in vitro* into a higher-order structure such as the nuclear basket. Furthermore, the addition of other purified nucleoporins, for example Nup153, might gain insight into whether such proteins act as nucleation points for Tpr fiber assembly or be essential for nuclear basket formation in any other way.

Additional functions of Tpr at the NPC

Apart from elucidating Tpr's structural properties, research in our laboratory has focussed on finding other proteins that interact with Tpr, and might give insight into additional functions of Tpr at the NPC.

Using the carboxyterminal Tpr domain as a bait in yeast two hybrid screens of human cDNA expression libraries, several proteins with known or potential roles in DNA-binding and repair, in cell cycle checkpoint control, and tumor suppression have been identified. Interaction with these proteins are suggestive of an involvment of Tpr in chromatin

organization and mRNA metabolism. However, the physiological relevance of Tpr's interaction with these proteins still remains to be investigated.

When immobilizing recombinant polypeptides of Tpr's carboxyterminal domain on Sepharose beads, and using those for affinity chromatography of mammalian cell extracts, we have identified several heterogeneous nuclear ribonucleoproteins (hnRNPs) and splicing factors that interact with different domain segments of Tpr. hnRNPs have been shown to be involved in RNA-related biological processes such as transcription, pre-mRNA processing, nuclear retention and nucleocytoplasmic transport of mRNAs (Nakielny and Dreyfuss, 1999). In fact, earlier functional studies of Tpr in mammalian cells have pointed at potential roles of Tpr in mRNA and nuclear protein export (Bangs et al., 1998; Frosst et al., 2002), as well as in the processing and intranuclear transport of mRNAs (Shibata et al., 2002). In order to gain insight into Tpr's putative role in nucleocytoplasmic transport, HeLa cells were depleted of Tpr by RNAi and analysed for their ability to import and export both recombinant and natural transport cargoes. To this end, Tpr depleted HeLa cells were also post-transfected with mammalian expression vectors that encode nuclear transport factors and other karyophilic proteins. In summary, Tpr has been found to be dispensable for NLS-mediated nuclear protein import, leptomycin B-sensitive protein export, and mRNA export. However, using splicing reporters that allowed us to study expression of different reporter genes with exon- and intron-embedded coding sequences, we could demonstrate that Tpr-deficiency leads to facilitated nuclear export of both correctly, and incompletely spliced mRNAs, the latter normally are retained within the nuclear interior in the presence of Tpr (*Manuscript* in preparation).

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