

# Therapeutic Targeting of Nuclear Protein Import in Pathological Cell Conditions

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**Abstract**—Proteins enter the nucleus through the nuclear pore complex. Once in the nucleus, some proteins, such as transcriptional regulators, can turn genes on or off, and change the composition of the cell and its function to meet the demands of its environment. This process of protein import into the nucleus is highly controlled and regulated by the expression or function of single cargoes, transport receptors, or the transport channels themselves. Thus, these components of the import process have

an impact on transport capacity, which subsequently affects gene expression, signal transduction, and cell growth and development. With such a key position in the process of cell growth, it is reasonable to hypothesize that alterations in nuclear protein transport may play an important role in pathological cell conditions that have abnormal cell growth as a central feature. Indeed, there are now sufficient data to demonstrate that alterations in nuclear protein transport participate in alterations in cell proliferation and hypertrophy. Further study is needed to provide definitive proof that changes in nuclear protein import directly participate in the pathogenesis of diseases such as hypertension, atherosclerosis, cancer, viral infection, and diabetes. However, the data to date have, on select occasions, led to a clear association of alterations in nuclear transport with disease states. Furthermore,

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this research has led to the important identification of new targets within the process of nuclear protein import that hold therapeutic promise to inhibit viral rep-

lication, to improve drug delivery during cancer therapy, and, in general, to modify cell growth and viability during disease conditions.

## I. Introduction

The movement of proteins from the cell cytoplasm into the nucleus can induce gene expression and alter cell function, composition, and morphology. Nuclear protein import is essential, therefore, for cell growth. Many factors are important for nuclear transport. These include targeting signals responsible for protein import [nuclear localization signal (NLS<sup>1</sup>)] and protein export (nuclear export signal), transport receptors called karyopherins (or the importin and exportin superfamilies), other proteins constituting the nuclear pore complex (NPC) including the nucleoporins (Nups) and Ran GTPase-activating protein (RanGAP) (Poon and Jans, 2005). The transport of molecules (>40 kDa) in or out of the nucleus consists of three phases: recognition by a receptor, docking, and transport through the NPC (Kau et al., 2004) (Fig. 1). Each loading and unloading of cargoes and the rate of transport through the NPC are highly controlled by the Ran GTPase (Kau et al., 2004). Thus, the process that participates in the nuclear import of key proteins, such as transcription factors, DNA-binding proteins, polymerases, and kinases, is responsible for regulating not only gene expression but also larger processes, such as cell proliferation and cell differentiation, phenomena occurring during cell mitosis, and embryonic development. Alterations in this highly regimented process have been observed in a wide spectrum of pathological cell conditions (e.g., cell hypertrophy, angiogenesis, and a wide range of diseases including atherosclerosis, hypertension, cancer, viral infection, and diabetes). This review discusses the dysregulation of the nuclear protein import machinery that is highly associated with the pathological states cited in section III and therapeutic interventions recently developed to address these prob-

lems. Targeting the nuclear protein machinery now constitutes an approach used to avoid viral replication in the nucleus (used as antiviral therapies), improve drug delivery (used in anticancer therapies such as chemotherapy or for drugs lacking cellular selectivity), and aid in nonviral gene transfer.

## II. Nucleocytoplasmic Trafficking

Access to the nucleus is limited by the nuclear envelope, a double-membrane structure in which large pore structures named nuclear pore complexes are embedded (Poon and Jans, 2005). Mammalian NPC is composed of 30 to 50 Nups (Kau and Silver, 2004) that play precise roles in NPC assembly and function (Terry et al., 2007). Proteins smaller than 40 kDa can diffuse through the NPC, whereas larger proteins are actively transported

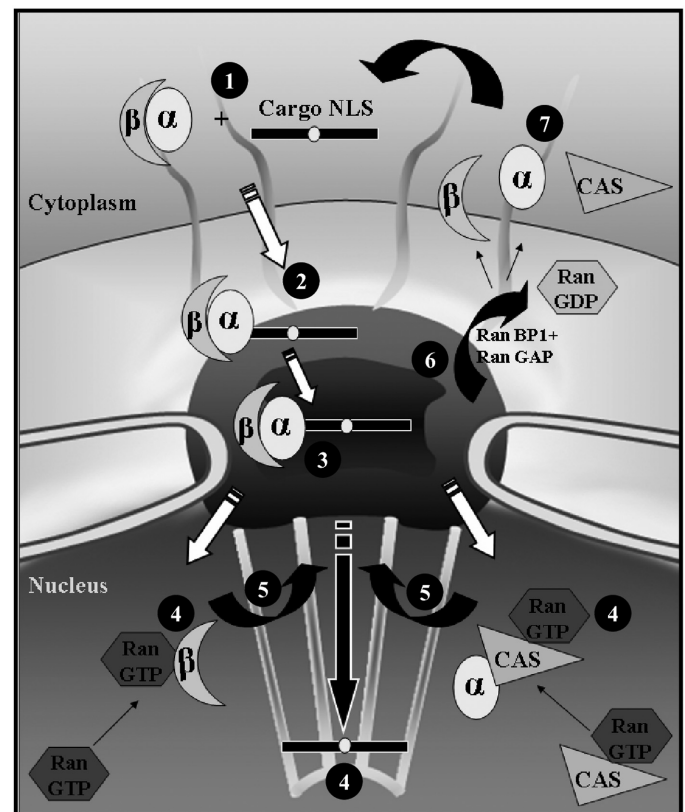


FIG. 1. The nuclear protein import cycle. An NLS-containing cargo (molecules >40 kDa) is recognized by importin- $\alpha$  that forms a heterodimer with importin- $\beta$ . The trimeric complex translocates into the nucleus through the NPC by docking of Nups. The release of the NLS cargo in the nucleus requires the binding of RanGTP to importin- $\beta$  dissociating the complex. Importin- $\alpha$  is exported by its own export receptor known as CAS. The complex RanGTP-importin- $\beta$  is recycled to the cytoplasm and the RanGTPase-activating protein RanGAP stimulated hydrolysis of Ran GTP to RanGDP, thus releasing the importins for another transport cycle.

<sup>1</sup> Abbreviations: CAS, cellular apoptosis susceptibility; CNI-H1194, 2-amino-4-(3,5-diacetylphenyl)amino-6-methylpyrimidine; cNLS, classical NLS; DTPA, diethylenetriamine pentaacetic acid; EBOV, Ebola virus; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HSP70, 70-kDa heat shock protein; IFN, interferon; I $\kappa$ B, inhibitor of nuclear factor- $\kappa$ B; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NFAT, nuclear factor of activated T cells; NLS, nuclear localization signal; NPC, nuclear pore complex; NPI, nuclear protein import; Nups, nucleoporins; oxLDL, oxidized low-density lipoprotein; PARP, poly(ADP-ribose polymerase); PBC, primary biliary cirrhosis; PCNA, proliferating cell nuclear antigen; PD98059, 2'-amino-3'-methoxyflavone; PIC, preintegration complex; PY-STAT1, tyrosine-phosphorylated signal transducer and activator of transcription 1; RanGAP, Ran GTPase-activating protein; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; LN-50, NF- $\kappa$ B cell-permeable inhibitory peptide (AAVALLPAVLLALLAPVQRRKQKLMF); VSMC, vascular smooth muscle cell; WGA, wheat germ agglutinin.

through the nuclear import machinery (Gasiorowski and Dean, 2003).

The import of cargo proteins into the nucleus is mediated by different types of NLSs. These NLSs are recognized by members of the importin superfamily, of which there are multiple  $\alpha$  and  $\beta$  forms. Nuclear protein import is mediated either by one of a number of importin- $\beta$  or by a heterodimer of importins  $\alpha/\beta$  (Fig. 1) if the proteins to be imported contain classic NLSs (cNLSs) (Goldfarb et al., 2004; Poon and Jans, 2005; Lange et al., 2007; Ratan et al., 2008).

#### A. Classic Nuclear Import Cycle

During the classic nuclear protein import cycle, importin- $\alpha$  plays the role of an adapter to bond cNLS cargoes. The heterodimer of importins  $\alpha/\beta$  is translocated into the nucleus through the NPC by docking to Nups (Köhler et al., 1999; Ratan et al., 2008). In the nucleus, the transport receptor importin- $\beta$  binds to the small nuclear GTPase Ran-GTP, which induces a conformational change thus causing the dissociation of importin- $\alpha$  and the release of the cNLS cargo (Stewart, 2007; Ratan et al., 2008). Although importin- $\beta$  returns rapidly to the cytoplasm, importin  $\alpha$  is exported to the cytoplasm via its nuclear export factor cellular apoptosis susceptibility (CAS), which binds to importin  $\alpha$  in the presence of RanGTP (Köhler et al., 1999). Hydrolysis of the GTP in the cytoplasm releases importin- $\alpha$  for a new import cycle (Ratan et al., 2008).

Note that the RanGTPase, a small GTPase, provides the required energy to control transport in each direction through two forms of Ran: RanGDP at high levels in the cytoplasm and RanGTP at high levels in the nucleus (Gasiorowski and Dean, 2003; Lange et al., 2007). As described above, the gradient of RanGTP/RanGDP across the nuclear envelope constitutes the driving force for protein trafficking through the NPC (Gasiorowski and Dean, 2003) (Fig. 2).

#### B. Alternative Nuclear Import Pathways

It is also important to note that there are alternative nuclear protein import pathways using other carriers and NLSs from those observed in the classic nuclear import pathway. This allows the import of ribosomal proteins, histones, and heterogeneous nuclear ribonucleoproteins (Stewart, 2007). Importin- and Ran-independent nuclear import pathways have also been reported for the import of molecules such as  $\beta$ -catenin, importin- $\beta$ 1, calmodulin, ERK, importin- $\alpha$ , and STAT family proteins (Poon and Jans, 2005; Sorokin et al., 2007).

### III. Dysregulation of Nuclear Protein Import: Evidence for Its Involvement in Pathological Cell Conditions

Channels found in the plasma membrane of a cell have been associated with organ function and dysfunction for

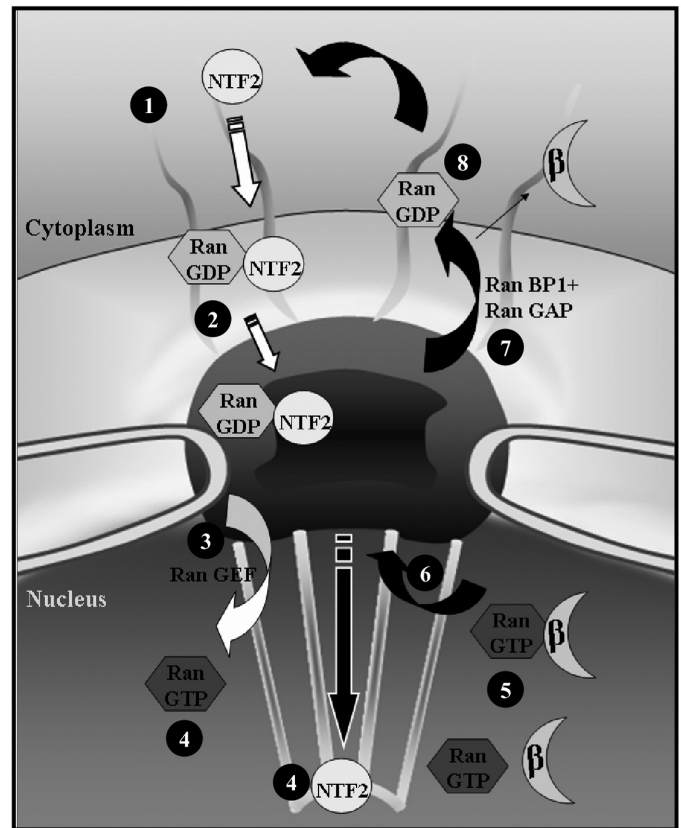


FIG. 2. The Ran cycle. RanGTP is hydrolyzed by the cytoplasmic RanGAP into RanGDP, which is then transported into the nucleus by NTF-2. In the nucleus, RanGEF catalyzes recharging of RanGDP with GTP, which is then free to bind importins and mediate their translocation into the cytoplasm through the NPC.

decades. For example, changes in the expression, structure, and/or function of ion channels will produce significant effects on tissue function and viability (Mukherjee and Spinale, 1998; Mukherjee et al., 1998). Modification of the function of molecules that interact with ion channels can also modulate tissue function (Pitt, 2007). Thus, these channels represent an important therapeutic target to minimize the clinical effects of a disease. The concept that changes in nucleocytoplasmic trafficking through the nuclear pore may be involved in disease processes is generally the same. Changes in the expression, structure, and/or function of the pore represent a critical juncture to modify cell morphology, function, growth, proliferation, and even cell death. These effects may be achieved in three basic ways:

1. The transport of a specific cargo is affected when changes occur at the level of that cargo.
2. The transport of all cargoes recognized by a specific receptor is affected when modifications occur at the level of that transport receptor.
3. The transport process as a whole can be affected when alterations occur at the level of the NPC itself. This may have the most dramatic effects on protein transport into the nucleus (Terry et al., 2007).

Although this field is relatively young, evidence is emerging that all of the processes involved in the movement of signaling proteins into the nucleus may be altered during disease and may participate in the pathological characteristics of that disease. The nuclear accumulation of proteins ultimately represents an imbalance of both the import and export processes. Even though this review will focus on the effects occurring during nuclear import only, it is important to remember that signaling proteins are normally in constant movement in and out of the nucleus in a balance between the rates of import and export (Gama-Carvalho and Carmo-Fonseca, 2001). This review article focuses on the potential for this balance to be disturbed during a disease process (Table 1).

### A. Dysfunctional Cargo Trafficking

Alterations in the regulatory mechanisms for nucleocytoplasmic trafficking of cargo proteins (essentially the movement of transcriptional factors from the cytoplasm into the nucleus) could result in the localization of these proteins in the wrong subcellular compartments and, subsequently, pathological cell states. Several proteins have been associated with a variety of diseases through an abnormal nuclear localization. For example, the transcriptional activator nuclear factor  $\kappa$ B (NF- $\kappa$ B) has a predominant nuclear localization in breast, ovary, colon, pancreas, and thyroid tumor cells (Brand et al., 1996; Rayet and G elinas, 1999; Huang et al., 2000; Lee et al., 2007), and in vascular smooth muscle cells, endothelial

TABLE 1  
*Dysregulation of nuclear protein import and associated pathological cell conditions*

	Protein	Pathological States
Target cargo protein	Activation and nuclear mislocalization of NF- $\kappa$ B	Tumors of breast, ovary, colon, pancreas, thyroid Atherosclerotic plaques
	Activation and nuclear mislocalization of HIF-1 $\alpha$	Breast and prostate cancers, hepatocarcinogenesis
	Activation and nuclear mislocalization of NFAT	Malignant transformation, cancer development, tumor invasion, and metastasis
	Nuclear mislocalization of cdk2, cd4, cyclin B1, cyclin D1 in cells exposed to oxLDL	Diabetes Cell proliferation associated with atherosclerosis
	Nuclear mislocalization of PCNA and phospho- ERK in cells exposed to oxLDL (short exposure)	Cell proliferation associated with atherosclerosis
	Nuclear mislocalization of PCNA in cells exposed to stretch	Cell proliferation associated with hypertension
	Nuclear mislocalization of phospho-p38 in cells exposed to oxLDL (long exposure) and to ceramide	Cell apoptosis associated with atherosclerosis
Transport receptors and the transport-driving force	Aberrant expression in importins $\alpha$ 1 $\alpha$ 2	Defects in fertility and embryogenesis
	Truncated form of importin- $\alpha$ lacking NLS-binding domain	Breast cancer cell lines
	Importin- $\alpha$ 1 overexpression	Breast cancer, melanoma patients
	Karyopherin $\alpha$ 1 sequestered in the cytoplasm by EBOV protein	Ebola virus infection
	Nuclear mis-localization of importin- $\beta$ in cells exposed to hydrogen peroxide	Cell stress
	Nuclear mislocalization of importin- $\alpha$ in cells exposed to oxidative	Cell stress
	Cytoplasmic mislocalization of CAS in cells exposed to oxidative	Cell stress
	Cytoplasmic mislocalization of CAS in cells exposed to ceramide	Cell stress
	CAS overexpression	Tumors and cancer cells (colon cancer, breast cancer, and liver neoplasms)
	Cytoplasmic mislocalization of Ran/TC4 in cells exposed to hydrogen peroxide	Cell stress
Nuclear pore complex	Overactivation of RanGAP in cells exposed to LPC	Atherosclerosis and cancer
	mNup98 down-regulation	Influenza virus
	Nup 153 and p62 degradation	Poliovirus and rhinovirus
	Nup214/Nup88 complex up-regulation	Acute myelogenous leukemia
	Nup88 overexpression	Tumor cell lines, primary human solid tumors
	Autoantibodies against gp210 protein and Nup p62	Primary biliary cirrhosis
	Nup 153 degradation induced by hydrogen peroxide	Cell stress
	p62 and Nup 153 upregulation	Cell stretch associated with hypertension
	p62 up-regulation induced by oxLDL (short exposure)	Cell proliferation associated with atherosclerosis
	p62 down-regulation induced by oxLDL (long exposure)	Cell apoptosis associated with atherosclerosis
Nup155 mutation	Atrial fibrillation and early sudden cardiac death	
p62 mutation	Infantile bilateral striatal necrosis Neuroendocrinological disease (triple A syndrome)	



cells, and macrophages of atherosclerotic plaques (Brand et al., 1996). The molecular mechanism leading to NF- $\kappa$ B activation and its subsequent import into the nucleus is very well described. Stimuli, such as inflammatory cytokines, reactive oxygen intermediates, and microorganisms, induce the phosphorylation of NF- $\kappa$ B inhibitor (I $\kappa$ B) by the I $\kappa$ B kinase complex. This allows the unmasking of the NF- $\kappa$ B NLS and the activation of NF- $\kappa$ B followed by its nuclear import (Kau and Silver, 2003; Kau et al., 2004). Abnormal localization of NF- $\kappa$ B in the nucleus could be due to an alteration in I $\kappa$ B activity, a hyperactivation of the upstream kinases resulting in I $\kappa$ B phosphorylation and degradation, or an improper acetylation by p300 (Rayet and G elinas, 1999; Chen and Greene, 2003; Kau et al., 2004).

Cell cycle protein regulators (cdk2, cd4, cyclin B1, and cyclin D1) and proliferating cell nuclear antigen (PCNA) translocate into the nucleus to modulate the cell cycle. Oxidized low-density lipoprotein (oxLDL) is thought to be involved in the accelerated cell proliferation and cell death associated with atherosclerosis. oxLDL can induce cell proliferation through, in part, affecting the nuclear import of cell cycle proteins (Zettler et al., 2003). Low concentrations of oxLDL or short exposure times of aortic vascular smooth muscle cells (VSMCs) to oxLDL can induce cell proliferation (Zettler et al., 2003; Chahine et al., 2009). On the other hand, the nuclear translocation of cell cycle proteins is inhibited by higher oxLDL concentrations or longer exposure times (Zettler et al., 2004; Chahine et al., 2009). VSMCs exposed to ceramide respond with a decrease in PCNA and cyclin A translocation into the nucleus (Faustino et al., 2008). Ceramide blocks cell proliferation and induces cell apoptosis. Nuclear transport, therefore, may represent the mechanism through which oxLDL and ceramide regulate the degree of cell proliferation (Chahine et al., 2009) or cell apoptosis (Faustino et al., 2008; Chahine et al., 2009) that are relevant to the atherosclerotic process.

Regulatory molecules are not the only factors that have the capacity to alter cell growth and nuclear protein import. When cells were exposed to mechanical factors such as cell stretch, cell growth (hyperplasia and hypertrophy) was stimulated (Richard et al., 2007). At the same time, nuclear protein import was increased in these cells compared with control cells. Continuous, intermittent cell stretch is an important component of vascular hypertension. The changes in nuclear protein import, therefore, represent a mechanism to explain the accelerated growth of the arterial wall in hypertension (Richard et al., 2007) and a checkpoint that could be targeted to block this response.

### *B. Transport Receptors and the Transport Driving Force*

Many pathological cell conditions or diseases are characterized by a mutation or an altered expression of the nuclear protein import receptors (such as importin- $\alpha$  or

- $\beta$ ) or a disruption of the RanGTP/GDP gradient (called the transport-driving force) (Terry et al., 2007).

*1. Transport Receptors.* Importin- $\alpha$  binds to cNLS-containing proteins and links them to importin- $\beta$ , the karyopherin that ferries the ternary complex through the NPC. Importin- $\alpha$  is exported from the nucleus back to the cytoplasm by CAS protein (Goldfarb et al., 2004; Kodiha et al., 2008). Because importins play a major role in the regulation of gametogenesis and early embryogenesis (Terry et al., 2007; Ratan et al., 2008), it is understandable that aberrant expression patterns for the importins  $\alpha$ 1 and  $\alpha$ 2 would result in an altered import of cNLS cargoes and, consequently, defects in fertility and embryogenesis as demonstrated in *Drosophila melanogaster* (Goldfarb et al., 2004; Terry et al., 2007).

Alterations in importin- $\alpha$  have been reported during cancer. A truncated form of importin- $\alpha$  lacking the NLS binding domain has been discovered in breast cancer cell lines (Kim et al., 2000; Kau et al., 2004). Overexpression of importin- $\alpha$ 1 has been also found in breast cancer (Dahl et al., 2006). BRCA1 is a nuclear protein transported into the nucleus by the karyopherin pathway (Thakur et al., 1997) possibly linking importin- $\alpha$ 1 expression with breast carcinogenesis (Dahl et al., 2006). Importin- $\alpha$ 1 overexpression has also been closely associated with poor survival and prognosis in patients with melanoma (Winnepeninckx et al., 2006).

During viral pathogenesis, abnormalities in transporter receptor trafficking have been observed (Reid et al., 2006). During Ebola virus (EBOV) infection, for example, the virus is able to resist antiviral drugs such as interferon (IFN)- $\alpha/\beta$  or IFN- $\gamma$  by inhibition of the IFN- $\alpha/\beta$  or IFN- $\gamma$  signaling (Harcourt et al., 1999; Reid et al., 2006). One of the major steps in this signaling pathway is the nuclear import of protein tyrosine-phosphorylated signal transducer and activator of transcription 1 (PY-STAT1). Reid and coworkers (2006) showed that infection of cells with EBOV blocks the IFN-induced accumulation of PY-STAT1. They reported that, to inhibit this signaling pathway, the protein EBOV VP 24 sequesters karyopherin- $\alpha$ 1, the previously identified NLS receptor for STAT1 (Sekimoto et al., 1997; McBride et al., 2002; Melen et al., 2003), in the cytoplasm. This, in turn, alters the interaction of karyopherin- $\alpha$ 1 with the NLS of PY-STAT1 leading to inhibition of the nuclear import of this protein, a key step in both IFN- $\alpha/\beta$  or IFN- $\gamma$  signaling (Reid et al., 2006).

Cell stress is an essential component of the pathophysiology of ischemia, heart failure, hypertension, diabetes, and cancer. It has been reported that in response to cell stress (such as UV irradiation, oxidative stress, and heat shock stress), transport receptors such as importins are mislocalized (Kodiha et al., 2004; Miyamoto et al., 2004). For example, importin- $\beta$  has been localized in the nucleus of cells exposed to hydrogen peroxide (Kodiha et al., 2004). Importin- $\alpha$  has been localized in

the nucleus of cells exposed to oxidative stress (Kodiha et al., 2008) and to all cellular stresses cited above (Miyamoto et al., 2004).

Nuclear accumulation of importin- $\alpha$  may be due to an increase in nuclear import of CAS or a decrease in the nuclear export of CAS (Kodiha et al., 2008). Because CAS recycles importin- $\alpha$  to the cytoplasm, any change in its localization will effectively alter nuclear trafficking in both directions. Exposure of VSMCs to ceramide induced a decrease in the nuclear localization of CAS through p38 mitogen-activated protein kinase (MAPK) activation (Faustino et al., 2008). This is thought to decrease the recycling of importin- $\alpha$ , thereby depressing the nuclear import of proliferative genes such as PCNA and cyclin A (Faustino et al., 2008). As discussed in section III.A, ceramide is an important second messenger that stimulates apoptosis and also induces a significant antiproliferative effect on VSMCs (Faustino et al., 2008). On the other hand, CAS overexpression has been reported in several different tumors and cancer cells (colon cancer, breast cancer, and liver neoplasms) (Brinkmann et al., 1996; Behrens et al., 2001; Wellmann et al., 2001; Kau et al., 2004). CAS overexpression was suggested to accelerate the recycling of importin- $\alpha$  to the cytoplasm which enhanced the nuclear import of cargo proteins such as p53, BRCA1, and RB (Behrens et al., 2003; Kau et al., 2004) that can induce cell proliferation or prevent cell apoptosis (Kau et al., 2004; Poon and Jans, 2005; Terry et al., 2007).

*2. Transport-Driving Force.* In several pathological cell conditions, particularly during oxidative stress, abnormalities in the enzymes controlling the Ran gradient, such as RanGTPase, have been observed (Czubryt et al., 2000; Kodiha et al., 2004; Miyamoto et al., 2004). Exposure of VSMCs to hydrogen peroxide has been found to increase the RanGTPase (Ran/TC4, a small Ras-related GTPase) in the cytosol, thereby decreasing nuclear import (Czubryt et al., 2000). These results have been confirmed in a subsequent work by use of the same oxidative stress (Miyamoto et al., 2004) and HeLa cells (Kodiha et al., 2004).

During atherosclerosis and in cancer, an important mitogenic second messenger is lysophosphatidylcholine (LPC) (Chai et al., 1996; Fang et al., 2000; Faustino et al., 2007b). Exposure of VSMCs to LPC stimulates ERK1/2 activity, which in turn increases RanGAP activity (ERK1/2 shares docking sequences with RanGAP). This induces the hydrolysis of Ran-GTP leading to higher levels of RanGDP in the cytosol, which causes an increase in RanGTP in the nucleus, enabling more importins ( $\alpha$  and  $\beta$ ) to be recycled to the cytoplasm, which increases nuclear import (Faustino et al., 2007b). In conclusion, it is clear that oxidants are potential triggers for changing the ratio of RanGTP/RanGDP, thereby altering nuclear protein import.

### C. Nuclear Pore Complex

Changing NPC composition or expression levels could markedly alter nuclear transport and subsequently cellular function. Increasing the number of channels may increase transport characteristics. Furthermore, the NPC is made up of a number of nuclear pore proteins called nucleoporins. It is possible that any alteration in the Nups could indirectly or directly lead to a large spectrum of diseases.

Viral infection through DNA and some RNA viruses, including poliovirus, rhinovirus, and influenza, can each target a subset of Nups for degradation or selective inhibition. For example, in 293T and Madin-Darby canine kidney cells, influenza virus down-regulates mNup98, a nucleoporin that is a docking site for mRNA export factors. This disrupts the mRNA nuclear export machinery rendering the cells highly permissive to influenza virus replication (Satterly et al., 2007). This viral effect is in direct competition with interferon- $\gamma$ -mediated up-regulation of mNup98 (Enninga et al., 2002; Satterly et al., 2007; Terry et al., 2007) and constitutes a viral strategy to promote viral replication (Satterly et al., 2007). Poliovirus (Gustin and Sarnow, 2001) and rhinovirus (Gustin and Sarnow, 2002) induce both Nup 153 and p62 degradation. This degradation has functional consequences. Nup153 interacts with Ran GDP, and the protein p62 is found in the central channel of the NPC (Guan et al., 1995). The protein p62 has been shown to interact with importin- $\beta$  family members (Hu et al., 1996; Moroianu et al., 1996; Bonifaci et al., 1997; Yaseen and Blobel, 1997; Kehlenbach et al., 1999; Petersen et al., 2001; Gustin and Sarnow, 2002) and NTF2 (Paschal and Gerace, 1995), which is a critical protein for the proper recycling of Ran between the nucleus and cytoplasm (Ribbeck et al., 1998; Smith et al., 1998; Gustin and Sarnow, 2002). This might explain why an inhibition of nuclear import correlated well with the degradation of Nup153 and p62 in cells infected with poliovirus or rhinovirus (Gustin and Sarnow, 2002). The inhibition of nuclear import by the virus may represent a novel strategy to evade host immune defenses by preventing signal transduction into the nucleus (Gustin and Sarnow, 2001, 2002).

Several cancer-associated fusion proteins have been identified involving Nups. In patients with acute myelogenous leukemia, chromosomal rearrangements can result in the fusion of Nups, such as Nup98 or Nup214 (or CAN) with HOXA9 (a homeobox transcription factor) or DEK (a DNA-binding protein), respectively (Kau et al., 2004; Poon and Jans, 2005). Up-regulation of the Nup214/Nup88 complex has been observed during the development of leukemia (Shu et al., 2008). Nup88 was overexpressed in a series of tumor cell lines and primary human solid tumors (Gould et al., 2002; Agudo et al., 2004; Zhang et al., 2007; Shu et al., 2008). The fusion of Nups prevents them from being incorporated into the NPC, thus retaining them in the

cytoplasm, and impairing their binding to transport receptors (Kau et al., 2004; Faustino et al., 2007a).

Liver diseases have been associated with changes in Nups (Wesierska-Gadek et al., 2008). Primary biliary cirrhosis (PBC) is a chronic, progressive, cholestatic autoimmune liver disease characterized by destruction of intrahepatic bile ducts, portal inflammation, and development of cirrhosis and hepatic failure (Nakamura et al., 2007). PBC targets two major Nups: gp210 and p62 (Wesierska-Gadek et al., 1995, 2007, 2008). Furthermore, autoantibodies against Nups have also been detected in ~30% of patients with PBC (Invernizzi et al., 2005; Nakamura et al., 2007; Wesierska-Gadek et al., 2008). Because the levels of PBC-specific anti-NPC antibodies correlate with the severity of disease, this may be of important prognostic value (Invernizzi et al., 2001, 2005; Wesierska-Gadek et al., 2006, 2008). Indeed, the presence of anti-gp210 antibodies has been identified as a risk factor for the progression to end-stage hepatic failure (Nakamura et al., 2007).

Oxidative cell stress may be a common factor inducing an alteration in NPC composition or NPC permeability. Oxidative stressors such as hydrogen peroxide induce a degradation of Nup153 resulting in a decrease in nuclear import through a mechanism independent of the ERK1/2 MAPK pathway (Kodiha et al., 2004). Oxidant treatment also induced the import of Nup 153 and 88 into the nucleus where they become components of high molecular complexes. Importin- $\alpha$  is sequestered within these high molecular complexes in nuclei of stressed cells, thus inhibiting nuclear import (Kodiha et al., 2008). Oxidized compounds can also affect the NPC. Short-term exposure of VSMCs to oxLDL induced an increase in p62 expression (Chahine et al., 2009). ERK1/2 MAPK activity was directly involved in this up-regulation. Nuclear protein import and cell proliferation were similarly stimulated by this short exposure to oxLDL (Chahine et al., 2009). However, exposure of VSMCs to oxLDL for longer durations induced an increase in p38 MAPK activity resulting in the down-regulation of p62 expression, a depressed rate of nuclear import and an induction of cell apoptosis (Chahine et al., 2009) (Fig. 3).

A mechanical stimulus is also a mechanism to induce cell stress. Cell stretching will stimulate cell growth via cell hyperplasia and hypertrophy (Richard et al., 2007). Mechanical stretch induced an up-regulation of NPC expression (two nucleoporins, Nup153 and p62). This was associated with an activation of the MAPK pathway (Markovics et al., 1974; Maul et al., 1980; Richard et al., 2007). It also correlated with an increase in nuclear protein import (Richard et al., 2007) (Fig. 3).

Changes in the NPC have been directly linked to the development of atrial fibrillation and early sudden cardiac death by Zhang et al. (2008). They used human genetic and mouse gene-targeting approaches to detect a mutation in the gene encoding nucleoporin NUP155 that leads to atrial

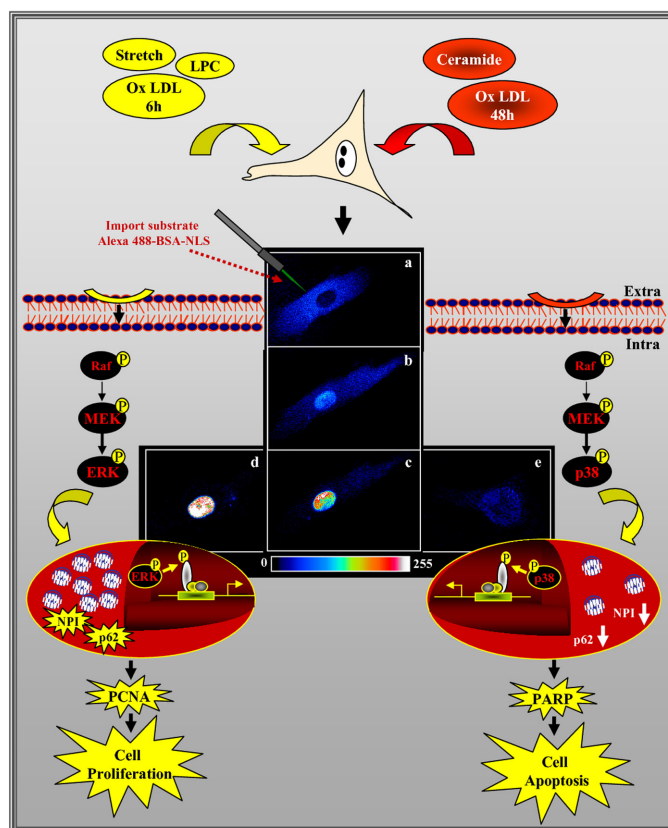


FIG. 3. Effects of different cellular stressors on nuclear protein import (NPI), NPC density, and cell growth through MAPK activation. LPC (Faustino et al., 2007b), mechanical stretch (Richard et al., 2007) and short exposure of VSMC to oxLDL (6 h) (Chahine et al., 2009) induces a high level of activation of the ERK MAPK pathway, an up-regulation of NPI and NPC expression (up-regulation of Nup p62), and a stimulation of the cell cycle (PCNA). The increased import of specific proteins into the nucleus (i.e., transcription factors) could lead directly to the activation of genes that cause cell proliferation (left side). Conversely, ceramide (Faustino et al., 2008) and longer exposure of VSMC to oxLDL (48 h) (Chahine et al., 2009) induces increased levels of activation of the p38 MAPK pathway, and an inhibition of NPI and NPC expression (down-regulation of Nup p62). The decreased import of specific proteins into the nucleus (i.e., transcription factors) could directly induce the activation of genes that cause cell apoptosis (cleavage of PARP) (right side). Nuclear protein import and the technique of microinjection: a to e represent confocal images of VSMC microinjected with Alexa488-BSA-NLS substrate contained in a micropipette into a single cell. Images were taken during (a) and after injecting (b–e) cells at different time points. a to c are images of the same cell. c to e represent images at 30 min after injection; c, image of a control cell (untreated); d, image of a cell exposed to oxLDL for 6 h; e, image of a cell exposed to oxLDL for 48 h. The signal strength in the nucleus is proportional to the amount of substrate present in the nucleus. The pseudocolor scale represents the intensity of fluorescence levels from 0 (black) to 255 nm (white). BSA, bovine serum albumin.

fibrillation and early sudden cardiac death (Zhang et al., 2008). They showed that the NUP155 mutation inhibited HSP70 mRNA export and HSP70 protein nuclear import (Zhang et al., 2008). The authors suggested that “NUP155 may act as a key upstream gene that causes atrial fibrillation by regulating the expression of downstream genes such as HSP70 by controlling mRNA nucleocytoplasmic export.” Thus, this study has provided a novel, ion channel-independent mechanism for the pathogenesis of an electrophysiological disturbance in the heart through an alteration in the NPC (Zhang et al., 2008).



Two other examples directly involving nuclear-trafficking diseases of the central nervous system are both of a nuclear pore complex protein causing a Mendelian disease in humans. A mutation of the nucleoporin p62 protein has been suggested to cause autosomal recessive, infantile bilateral striatal necrosis, which is a neurodegenerative disorder (Basel-Vanagaite et al., 2006). Their findings suggested that p62 has a cell-type-specific role and is important in the degeneration of the basal ganglia in humans (Basel-Vanagaite et al., 2006). The triple A syndrome is an autosomal recessive neuroendocrinological disease in humans that exhibits defective NPC targeting (Cronshaw and Matunis, 2003; Basel-Vanagaite et al., 2006; Kiriya et al., 2008). The causative mutations for this syndrome have been identified in a gene that encodes ALADIN, a component of the NPC. The primary defect caused by the mutant ALADIN is the selective failure to import the DNA repair proteins aprataxin and DNA ligase I into the nucleus, resulting in increased DNA damage and subsequent cell death under oxidant stress (Kiriya et al., 2008).

#### IV. Targeting Nuclear Import as a Strategy to Alter Disease Progression

The preceding discussion has provided evidence from many independent studies identifying the association and the involvement of nuclear trafficking with different pathological states. Because this has occurred at three different general sites within the process of nuclear protein import, it offers multiple therapeutic targets to attempt to normalize nuclear trafficking as a strategy for the treatment of diseases.

##### A. Pharmacological Modulation of Nuclear Import

1. *Targeting the Nuclear Pore Complex.* Several approaches can be used to modulate nuclear import. One of them consists of targeting the nuclear import at the level of the NPC (Gasiorowski and Dean, 2003) specifically by targeting conserved FG or FXFG repeats of the NPC that bind to the importin family members. Monoclonal antibodies (including mAb414 and RL2) against the FG and FXFG epitopes of the nucleoporins have been used successfully in rat liver nuclear envelopes to block the translocation of proteins through the NPC (Snow et al., 1987). Monoclonal antibodies prevent cargo from associating around the rim of an NPC, which ultimately inhibits its movement into the nucleoplasm (Gasiorowski and Dean, 2003). Alternatively, several Nups in higher organisms are characterized by the covalent addition of a single *O*-linked *N*-acetylglucosamine to serine and threonine residues (Gasiorowski and Dean, 2003). This sugar moiety can mediate some of the interactions with importins. For example, wheat germ agglutinin (WGA) is a lectin that binds to *N*-acetylglucosamine. When added to isolated rat liver nuclei, WGA can inhibit nuclear import by associating with the sugar-modified nucleoporins and blocking the channel (Finlay et al., 1987; Adam

and Adam, 1994; Gasiorowski and Dean, 2003). An example of the utility of this approach is provided during adenoviral delivery of DNA. In this process, the NPC serves as a docking site for incoming virus particles, contains a particle dissociation activity, and provides a gateway for nuclear import of the uncoated viral DNA (Horwitz and Loeb, 1990; Greber et al., 1996, 1997). Inhibitors of *O*-linked NPC glycoproteins, WGA and the RL1 antibody, prevent stable virus docking at the nuclear envelope and also prevent the disassembly of the capsid and ultimately inhibit the nuclear import of the DNA-associated protein VII (Greber et al., 1997).

It is important to note that the experiments cited above were not performed on intact cells. The use of monoclonal antibodies is problematic when it is necessary to target intracellular proteins because of a lack of accessibility. However, a new method for the intracellular delivery of antibodies has been developed recently that uses a novel hemagglutinating virus of Japan envelope vector system (Kondo et al., 2008). The hemagglutinating virus of Japan envelope is an inactivated Sendai virus particle that can fuse to the external membrane of a cell and thereby deliver a variety of molecules into living mammalian cells (Kondo et al., 2008). This new method could hold great promise for the future to deliver monoclonal antibodies that selectively block the NPC.

In conclusion, there is still a very limited number of compounds but no drugs to date that can selectively target the NPC. WGA is limited in its use because it nonspecifically blocks all proteins that are normally imported through the NPC (Gasiorowski and Dean, 2003). Thus, this inhibitor can only be useful clinically as a diagnostic test for a protein potentially targeted to the nucleus (Adam and Adam, 1994; Gasiorowski and Dean, 2003), or more generally to detect the presence of autoantibodies to correlate with the severity of disease. Despite its limitations, it may have important prognostic value in autoimmune diseases, such as primary biliary cirrhosis, in particular (Wesierska-Gadek et al., 2008).

2. *Targeting Nuclear Import via Transport Receptors.* Two peptide inhibitors, bimax1 and bimax2, have been identified recently as specific blockers of nuclear import activity mediated by the classic nuclear import pathway (importin- $\alpha/\beta$  pathway) (Kosugi et al., 2008). Both peptides bind tightly to importin- $\alpha$  independently of importin- $\beta$ , which prevents the release of the cargo into the nucleus. These peptides were designed without use of protein structural information. Instead, an activity-based profile was generated for a linear peptide, which represented the functional contribution of various amino acids substituted at each position within a template peptide sequence. This approach was successful in obtaining peptides that bind with high specificity and high affinity (Kosugi et al., 2008).

3. *Targeting Nuclear Localization of Key Transcription Factors in Disease.* Drugs are normally used to directly stimulate or, more frequently, inhibit the activ-



ity of a target protein. However, a drug could be just as effective if it could induce an alteration in the function of a target simply through a change in its localization. Cargo proteins such as transcription factors are down-regulated when localized to the cytoplasm, then once activated they translocate into the nucleus. Thus, the activity of these transcription factors can be regulated by their subcellular localization. Small molecules that alter the localization of transcription factors, for example, can be used to control nuclear transport (Kau and Silver, 2003; Kau et al., 2004).

*a. Targeting Nuclear Factor- $\kappa$ B.* Inhibition of NF- $\kappa$ B suppresses carcinogenesis (Holmes-McNary and Baldwin, 2000; Takada et al., 2004; Piva et al., 2005; Lee et al., 2007). However, most of the inhibitors lack specificity and inhibit other signaling pathways (Lee et al., 2007). Different strategies have been developed for cancer therapy, including the use of cell-permeable peptides. The cell-permeable peptide strategy specifically targets the NF- $\kappa$ B complex by blocking its nuclear localization. For example, peptides such as SN-50 and *o,o'*-bismyristoyl thiamine disulfide have been designed to mimic the sequence of p50, which is responsible for transporting the NF- $\kappa$ B complex from the cytoplasm to the nucleus. The peptides compete with NF- $\kappa$ B complexes, thus blocking NF- $\kappa$ B nuclear import (Pieper and Riaz-ul-Haq, 1997; Shoji et al., 1998; Lee et al., 2007).

*b. Targeting nuclear factor of activated T cells.* Involvement of the calcineurin/nuclear factor of activated T cells (NFAT) pathway is critical in many diseases. The conventional inhibitors for NFAT pathway are immunosuppressive drugs such as CsA and FK506 (Zhu and McKeon, 1999). After entry into cells, CsA and FK506 form complexes with their cellular partners and block the phosphatase activity of calcineurin (Zhu and McKeon, 1999; Hallhuber et al., 2006). However, because CsA and FK506 block the enzymatic activity of calcineurin, they do not specifically target NFAT. Therefore, these immunosuppressive agents have severe side effects (Kiani et al., 2000; Lu and Huan, 2007). The ultimate goal is a highly selective inhibitor for NFAT signaling. More specific targeting has been developed in myocardial hypertrophy in which the calcineurin/NFAT signaling cascade is crucial. Calcineurin is responsible for dephosphorylating NFAT in the cytosol, thus enabling its nuclear import (Zhu and McKeon, 1999). Its presence in the nucleus is also significant in ensuring the full transcriptional activity of NFAT (Zhu and McKeon, 1999). In angiotensin II-stimulated hypertrophy, inhibition of the calcineurin/importin- $\beta$ 1 interaction by a synthetic competitive peptide (KQECKIKYSERV), which mimics the calcineurin NLS, subsequently prevented calcineurin nuclear import (Fig. 4). The inhibition of calcineurin nuclear import by peptide competition for the binding of the nuclear import protein importin- $\beta$ 1 represents a sophisticated approach to abolishing the deleterious effects of exaggerated NFAT transcriptional activity (Hallhuber et al., 2006). This results in a suppressed tran-

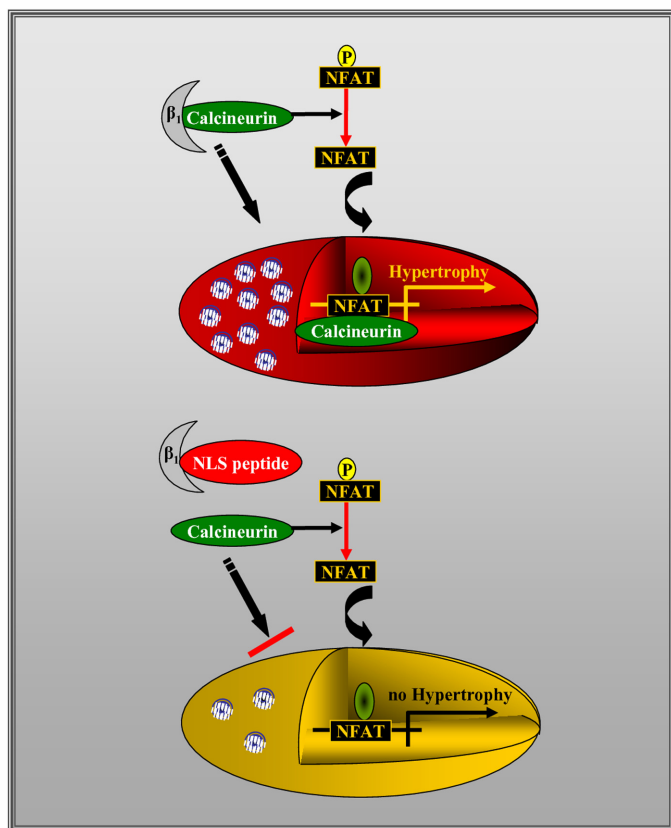


FIG. 4. Calcineurin/NFAT shuttling and an approach to abolish the deleterious effects of exaggerated NFAT transcriptional activity. Calcineurin, translocated into the nucleus via importin- $\beta$ 1, plays a crucial role in the transcriptional activity of the calcineurin/NFAT signaling cascade leading to myocardial hypertrophy. To inhibit the interaction between calcineurin and importin- $\beta$ 1 and, consequently, the harmful effects of calcineurin/NFAT signaling, a synthetic NLS peptide has been designed to mimic the NLS sequence of calcineurin that is recognized by importin- $\beta$ 1. This creates a competition, thus inhibiting the import of calcineurin into the nucleus and abolishing the transcription of genes important in myocardial hypertrophy (model proposed by Hallhuber et al., 2006).  $\beta$ 1, importin- $\beta$ 1.

scription of genes important in myocardial hypertrophy and identifies a potentially novel therapeutic strategy to inhibit myocardial hypertrophy (Hallhuber et al., 2006).

The same approach may be used in other diseases. For example, in diabetes, a slight increase in endogenous calcineurin/NFAT signaling may increase  $\beta$ -cell mass and improve function in patients with type 2 diabetes mellitus (Heit et al., 2006; Heit, 2007). Future studies might test the use of small-molecule activators of NFAT proteins as novel therapeutics in the treatment of type 2 diabetes mellitus (Crabtree and Olson, 2002; Heit, 2007).

*4. Targeting Nuclear Import with Kinase Inhibitors.* Alterations in MAPK activation have important consequences on the movement of proteins into the nucleus. Therefore, MAPK inhibitors have been used extensively in vitro and have a regulatory effect on uncontrolled cell growth and cell apoptosis through an action on nuclear protein import (Czubryt et al., 2000; Faustino et al., 2007b; Richard et al., 2007; Chahine et al., 2009). For

example, the ERK1/2 MAPK inhibitor PD98059 normalized the increases in p62 expression levels, nuclear import rate, and cell proliferation induced by a short exposure of VSMCs to oxLDL (Chahine et al., 2009), mechanical stretch (Richard et al., 2007), or LPC (Faustino et al., 2007b). In addition, the p38 MAPK inhibitor SB203580 normalized the decreases in p62 expression levels, nuclear import rate, and cell number induced by longer exposure of VSMCs to oxLDL (Chahine et al., 2009) or to ceramide (Faustino et al., 2008). The mechanism responsible for the effects of the p38 inhibitor involved CAS localization (Faustino et al., 2008). CAS was delocalized from the nucleus after exposing cells to ceramide and treatment with the p38 MAPK inhibitor redistributed CAS to the nucleus, which allowed for the progression of the transport cycle. This suggests that CAS could be a target of p38 MAPK. CAS contains target motifs to MAP/ERK kinase, a kinase upstream to p38 (Faustino et al., 2008). Taken together, these results have shown that even when nuclear import is depressed, it is possible to restore it by targeting the upstream kinases instead of directly targeting the different components of the transport machinery. Thus, these very encouraging *in vitro* results have led to the use of kinase inhibitors in clinical therapy. However, when applied as a clinical therapy, kinase inhibitors lack cellular selectivity. Because they are mostly small lipophilic molecules, their distribution throughout the body would be widespread, exposing both diseased tissues and healthy tissues to the drug. Thus, they can be toxic, particularly with more chronic treatments (Kerkelä et al., 2006; Force et al., 2007; Temming et al., 2008). A drug delivery approach for cell-type-specific kinase inhibitors might be a better strategy (Temming et al., 2008). The first step consisted of the recognition of the receptor present on diseased cells by the kinase inhibitor-carrier conjugate, followed by their binding, thus leading to receptor internalization (Temming et al., 2008). This intracellular delivery approach ensures that the coupled drug acts specifically in diseased cells, whereas normal cells are not affected by the drug. These approaches may be very useful tools for improving drug delivery specifically into tumor cells, angiogenic endothelial cells, or fibrotic hepatic and renal cells (Temming et al., 2008). By using this strategy, a kinase inhibitor can specifically interact with the cytoplasmic kinases of diseased cells that subsequently can regulate nuclear import.

### *B. Improving Therapeutic Delivery of DNA to the Nucleus*

Peptides resembling NLS sequences can be used to facilitate nuclear uptake of exogenous DNA (van der Aa et al., 2006). Another new approach has used histone to mediate DNA delivery. Histones contain protein transduction domains that enable them to enter an intact cell in a receptor- and energy-independent manner (Wagstaff et al., 2007). Very recently, it was demonstrated

that histone H2B proteins that were optimized for nuclear targeting could be used to reconstitute chromatin. The histone/DNA cargo is condensed and protected from degradation by nucleases that, together with the NLS, ensure an efficient delivery of the DNA to the nucleus of intact living mammalian cells (Pouton et al., 2007; Wagstaff et al., 2008). In their study, they achieved an efficient delivery of DNA to the nucleus of intact living mammalian cells with a 6-fold higher level of transgene expression than commercial liposomal delivery approaches (Wagstaff et al., 2008). This method, called “chromofection,” resulted in the successful expression in 35% of cells to which DNA had been delivered, which is 40-fold more efficient than that observed for traditional nonviral mediated techniques and 4- to 5-fold better than previously engineered histone H2B monomers or dimers (Pouton et al., 2007; Wagstaff et al., 2008). Chromofection may represent an efficient means to deliver large pieces of DNA (plasmid DNA of 6000 base pairs) encoding multiple genes, with the potential to treat complex genetic disorders (Wagstaff et al., 2008). In addition to treating fatal monogenic diseases, the use of gene therapy as an adjuvant treatment with radiotherapy and chemotherapy has shown promising results. The transfer of gene therapy from rare genetic conditions to more common disorders, such as cancer or cardiovascular diseases is likely to benefit patients who will have a wider choice of treatments available (Räty et al., 2008).

## **V. Applications for Therapy**

### *A. Viral Therapy*

Viruses have evolved to exploit the host's cellular machinery to replicate their own genomes. For example, adenoviruses target their genome to the cell nucleus by a multistep process involving endocytosis, membrane penetration, and cytoplasmic transport, and finally import their DNA into the nucleus (Greber et al., 1996; Leopold and Crystal, 2007).

Most current anti-human immunodeficiency virus (HIV) therapies have focused on developing drugs that inhibit viral entry, viral genome replication, and virus-specific proteolysis. However, many viruses exploit cellular kinases to facilitate subcellular targeting during infection and to achieve specific phosphorylations of their gene products (Alvisi et al., 2008). The kinase CK2 phosphorylates most viral proteins (Meggio and Pinna, 2003) by inducing an increase in the affinity between importin and an NLS, thereby increasing nuclear protein import (Krosky et al., 2003; Marschall et al., 2003; Alvisi et al., 2008). For example, the DNA replication of the human cytomegalovirus (HCMV), a Herpesviridae family member, depends on the DNA polymerase holoenzyme, which is composed of a catalytic subunit (pUL54) and a polymerase accessory protein or processivity factor (ppUL44) (Ertl and Powell, 1992; Mocarski and Kemble, 1996; Alvisi et al., 2008). It has been re-

ported that, although the ppUL44-NLS is directly recognized by importin- $\alpha/\beta$  for nuclear translocation, the affinity of this interaction is strongly increased by the presence of upstream sequences containing a site (Ser413) for CK2 phosphorylation (Alvisi et al., 2008). Thus, critical viral protein products such as ppUL44 exploit CK2 activity for proper intracellular localization. This, in turn, implies the development of new antiviral drugs directed against CK2 and may be a valuable direction. Indeed, inhibition of the activity of these kinases with cdk inhibitors, for example, blocks the replication of many viruses including herpes simplex virus, HCMV, varicella-zoster virus, Epstein-Barr virus, and HIV. Thus, inhibiting kinases and specifically CK2 phosphorylation may be effective, at least in part, through the inhibition of protein transport into the nucleus (Alvisi et al., 2008).

To prevent viral integration and transcription from occurring, other compounds have been developed to inhibit the nuclear import of HIV-1 (Gasiorowski and Dean, 2003). Nuclear translocation of HIV-1 preintegration complex (PIC) (a nucleic acid/protein complex) is essential for viral replication, because it allows the PIC to get into contact with the cellular chromatin (Haffar et al., 2005). Arylene bis (methylketone) small-molecular-weight compounds, such as the agent CNI-H1194, show therapeutic promise by disrupting the formation of the PIC, which could lead to inefficient nuclear import (Dubrovsky et al., 1995; Popov et al., 1996; Gasiorowski and Dean, 2003). However, there is still no consensus about the mechanisms that govern nuclear import of the HIV-1 PIC (Nisole and Saib, 2004; Haffar et al., 2005). Three HIV-1 proteins, matrix protein, integrase, and viral protein R, have been proposed as karyophilic agents that recruit the cellular nuclear import machinery to the PIC (Haffar et al., 2005). However, the ability of HIV-1 to transport its cDNA through an intact nuclear envelope depend on the karyophilic potential of HIV-1 integrase via an importin- $\alpha/\beta$ -dependent mechanism (Hearps and Jans, 2006). Integrase harbors multiple NLSs that interact with importin- $\alpha$  and the importin- $\alpha/\beta$  heterodimer (Hearps and Jans, 2006; Faustino et al., 2007a). This classic nuclear import mechanism of integrase highlights important potential therapeutic targets for impeding the progression of HIV/AIDS. Indeed, targeting matrix protein NLS-dependent nuclear import by use of excess NLS mimetics has been shown to inhibit HIV replication (Bukrinsky et al., 1993; Friedler et al., 1998; Glushakova et al., 1999; Haffar et al., 2005). For example, backbone cyclic peptides that have amino acid sequences corresponding to the NLS of the HIV matrix were able to inhibit nuclear import in *in vitro* assay systems and HIV-1 replication in infected cultured cells (Friedler et al., 1998). The NLS mimetics presumably work by competing with the matrix protein NLS for binding to importin- $\alpha$  (Haffar et al., 2005).

Arylene bis(methylketone) compounds have also been described as a class of small-molecule inhibitors of HIV nuclear import that directly target the matrix NLS (Dubrovsky et al., 1995). For example, one of the compounds of this group, the ITI-002, inactivates the NLS by forming Schiff bases with lysine residues (through the ketone groups) (Al-Abed et al., 2002), which can neutralize the positive charges critical for NLS activity (Haffar et al., 2005).

### B. Cancer Therapy

The major obstacle that must be surmounted to develop therapeutics that modify the general nuclear transport machinery of cancer cells is the problem of specificity for tumor cells versus normal cells (Kau et al., 2004). To address this problem, researchers have used the subcellular distribution of a mislocalized protein in cancer cells to identify small molecules or peptide aptamers that correctly redirect the proteins to the correct compartments or alter the cellular localization of a protein (Kau et al., 2004). Analogous to monoclonal antibodies, peptide aptamers are small proteins that contain a structurally constrained variable region of ~20 amino acids, expressed as part of an inert scaffold such as thioredoxin or green fluorescent protein (Colas et al., 1996; Kau and Silver, 2003). The advantage of their small size allows their structures to be solved and to function inside cells (Colas et al., 1996). Some aptamers have been designed to incorporate a NLS to translocate their binding partner into the nucleus by use of the nuclear transport machinery of the cell (Colas et al., 2000; Kau and Silver, 2003). For example, peptide aptamers, such as anti-Cdk2 and anti-Ste5 aptamers, have been fused to an NLS as "transporters," which caused their targets to accumulate in the nucleus (Colas et al., 2000). Such screening approaches could lead to the discovery of novel compounds that have anticancer activity, and compounds that provide new insights into the role of nucleocytoplasmic trafficking and regulatory pathways in cancer (Kau et al., 2004).

One of the major problems during cancer chemotherapy is the development of resistance to cancer drugs. Novel approaches have been developed by conjugating an anticancer agent, such as a carboplatin analog, to a poly(ethylene glycol) carrier and an NLS resulting in a rapid internalization of the drug into M109 murine lung carcinoma cells and an efficient accumulation in the nucleus (Aronov et al., 2004). Although underused today, use of an NLS to direct drugs to the nucleus may be a novel and efficient way to improve drug efficacy in the future.

A targeted radiotherapy of malignancies has been successfully achieved through radiolabeled biomolecules, such as peptides that recognize tumor-associated antigens or growth-factor receptors (Goldenberg, 2002; Reilly, 2005). However, these biomolecules have been restricted almost exclusively to the cell surface. Novel



strategies have recently emerged to overcome the delivery challenges through the conjugation of biomolecules with NLSs to promote their internalization and routing to the cell nucleus (Costantini et al., 2008). Most importantly, exciting new opportunities also have emerged by use of NLS motifs to direct auger-electron-emitting radiopharmaceuticals to the nucleus of cancer cells where these electrons are highly potent in causing DNA strand breaks, thereby killing the cells (Kassis, 2003). For example, receptor tyrosine kinases and their cognate ligands are able to translocate to the nucleus through NLS-mediated processes (Jans, 1994). In many types of human malignancies, epidermal growth factor receptor (EGFR) is overexpressed in nuclei from tumors in patients with breast and oropharyngeal carcinomas (Lo et al., 2006). The internalization and nuclear translocation of EGFR has been exploited by introducing auger-electron-emitting  $^{111}\text{In}$ -labeled EGF ( $^{111}\text{In}$ -DTPA-hEGF) to the nucleus of breast cancer cells (Chen et al., 2003; Costantini et al., 2008).  $^{111}\text{In}$  and other auger-electron emitters are highly cytotoxic and damaging to DNA when they decay in close proximity to the cell nucleus. This makes them highly selective for killing targeted single-cancer cells. Indeed, Chen's group found that  $^{111}\text{In}$ -DTPA-hEGF possessed strong antitumor effects (3-fold reduction in tumor growth) when administered to athymic mice bearing EGFR-positive MDA-MB-468 human breast cancer xenografts (Chen et al., 2003; Costantini et al., 2008).

Finally, an increasing number of oncogenes and tumor suppressors have now been identified as important intervention points for next-generation anticancer drugs. In general, cellular p53 exists in low concentrations and is relatively inactive, but during cellular stress, the amount of p53 is increased and the protein is activated. Depending on the cellular environment, activation of the protein leads to either cell cycle arrest or apoptosis. The network microtubules and the dynein motor proteins are involved in facilitating p53 nuclear import (Pouton et al., 2007). For example, treatment with the microtubule-depolymerizing agent nocodazole reduces the extent of nuclear p53 accumulation in vivo (Giannakakou et al., 2000; Lam et al., 2002; Kau and Silver, 2003; Pouton et al., 2007; Roth et al., 2007). This approach may also improve the delivery of therapeutic DNA or drugs to the nucleus in gene therapy or cancer therapy, because viruses and some proteins reach the perinuclear region by following the microtubular network. However, because of safety concerns associated with the use of viral vectors, the development of safe, nonviral gene delivery vectors is preferable (Pouton et al., 2007). For example, Moffatt and colleagues (2006) designed a single vector having multiple factors for both tumor-targeted and nuclear-specific delivery, such as CNGRC/poly(ethylene glycol)/polyethylenimine/DNA-p53/NLS/DNA nuclear targeting signal. The CNGRC peptide was chosen as the targeting ligand because of its high specificity for CD13,

which is expressed at extremely high levels in tumor cells but not in the normal vasculature. This vector not only targeted the nucleus of tumor cells but also the nucleus of tumor-associated endothelial cells (Moffatt et al., 2006). In conclusion, targeting proteins that are involved in nuclear transport, in addition to the nuclear transport of factors that have been associated with cancer, could prove to be a powerful approach for controlling cancer cell growth.

## Conclusions and Perspectives

Transport of macromolecules between the nucleus and cytoplasm is a critical cellular process for eukaryotes, and the machinery that mediates nucleocytoplasmic exchange is subject to multiple levels of control. This regulation is achieved by modulating the expression or function of single cargoes, transport receptors, or the transport channel itself. Each of these targets has an increasingly broad impact on transport patterns and capacity, and this hierarchy of control directly affects gene expression, signal transduction, cell growth, and disease. Naturally, if the import process is involved in the pathogenesis of a disease, then it logically follows that it may represent a potential therapeutic target. Drug action and delivery can try to relocalize a protein to alter its activity instead of simply blocking active enzymatic sites (Gasiorowski and Dean, 2003). Introducing NLS peptides to compete with existing transporters and inhibit protein movement into the nucleus can effectively deter growth processes. Alternatively, peptides resembling NLS sequences can be used to target the DNA toward the nucleus to facilitate nuclear uptake of exogenous DNA. We are just beginning to realize the potential of treating disease through targeting the nuclear import pathway. Increasing our understanding of the factors that modulate nuclear protein import may allow us to fully capitalize on the potential of using this pathway to treat a great number of chronic diseases that have augmented cell growth as a central feature.

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