The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein

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Protein import into the nucleus and export from the nucleus are signal-mediated processes that require energy. The nuclear transport process about which the most information is currently available is classical nuclear localization signal (NLS)-mediated nuclear import. However, details concerning the signal-mediated export of proteins and RNAs as well as alternative nuclear import pathways are beginning to emerge. An example of this is the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein which, by virtue of its M9 domain, is actively exported from the nucleus and imported into the nucleus via a novel pathway mediated by the recently characterized transportin protein. Here we report that the shuttling hnRNP K protein contains a novel shuttling domain (termed KNS) which has many of the characteristics of M9, in that it confers bi-directional transport across the nuclear envelope. KNS-mediated nuclear import is dependent on RNA polymerase II transcription, and we show that a classical NLS can override this effect. Furthermore, KNS accesses a separate import pathway from either classical NLSs or M9. This demonstrates the existence of a third protein import pathway into the nucleus and thereby defines a new type of nuclear import/export signal.

Keywords: hnRNP K protein/nuclear export/nuclear import/shuttling domain/signal

Introduction

Pre-mRNAs are synthesized in the nucleus of eukaryotic cells by RNA polymerase II (pol II) and then processed extensively prior to transport to their site of translation in the cytoplasm. It is known that mRNA export is an active, signal-mediated event, yet the mechanism by which mRNAs are exported to the cytoplasm is a poorly defined process (reviewed in Izaurralde and Mattaj, 1995; Nakielnny *et al.*, 1997). Throughout their nuclear residency, pre-mRNAs/mRNAs are associated with a group of abundant RNA-binding proteins collectively termed heterogeneous nuclear ribonucleoproteins (hnRNPs) (Dreyfuss *et al.*, 1993). Considerable evidence now suggests a tight linkage between the transport of some hnRNPs and that of mRNA export from the nucleus to the cytoplasm. First, it was found that a subset of hnRNPs, including hnRNPs A1,

A2, D, E, I and K, shuttle continuously between the nucleus and cytoplasm in a manner consistent with factors involved in mRNA export (Piñol-Roma and Dreyfuss, 1992, 1993; Michael *et al.*, 1995b). Second, it has been demonstrated recently using immunoelectron microscopy that mRNAs in transit through the nuclear pore complex (NPC) to the cytoplasm are associated directly with shuttling hnRNPs (Visa *et al.*, 1996). Third, an active, transferable nuclear export signal (NES) within the shuttling hnRNP A1 protein has been identified (Michael *et al.*, 1995a). Fourth, a direct role for the A1 NES in mRNA export has been demonstrated recently. In these experiments, it was shown, using *Xenopus* oocytes, that injection of saturating amounts of A1 into the nucleus competitively inhibits export of mRNA while a deletion mutant of A1 which lacks a functional NES does not (Izaurralde *et al.*, 1997), indicating that the A1 NES plays a central role in the export of mRNP particles.

A1 is the most extensively characterized of the shuttling hnRNPs. It is composed of two RNP motif RNA-binding domains (RBDs) as well as a third RNA-binding domain, the RGG box (Burd and Dreyfuss, 1994). A1 typifies a class of shuttling hnRNPs which require continuous pol II transcription for complete nuclear localization (Piñol-Roma and Dreyfuss, 1991; Michael *et al.*, 1995b). Transport of A1 is determined by the M9 domain, a 38 amino acid sequence located at the carboxy-terminus. M9 was identified initially as the A1 nuclear localization signal (NLS) as placement of M9 on normally cytoplasmic reporter proteins results in nuclear localization (Siomi and Dreyfuss, 1995; Weighardt *et al.*, 1995). Interestingly, M9 also supplies the A1 nuclear export activity. When fused to a protein which is otherwise retained within the nucleus, such as the pentameric core domain of nucleoplasmin, M9 can activate nuclear export (Michael *et al.*, 1995a). Mutagenesis experiments have so far been unable to separate the NLS from the NES activities of M9, suggesting that at least some of the factors required for A1 nuclear import may also function in A1 nuclear export (Michael *et al.*, 1995a). M9 is a novel transport signal by several criteria. It is the only sequence identified thus far which specifies both nuclear import and nuclear export and, consistent with this activity, M9 bears no sequence similarity to 'classical' NLSs of the SV40 large T or nucleoplasmin bipartite-basic types (for a review of classical NLSs, see Dingwall and Laskey, 1991).

Classical NLSs represent the NLS for many nuclear proteins and, over the past several years, considerable progress has been made in ordering the events and factors required for nuclear import of classical NLS-containing proteins (reviewed in Görlich and Mattaj, 1996). Classical NLSs are recognized in the cytoplasm by a soluble NLS receptor protein (Adam and Gerace, 1991) termed importin α (Görlich *et al.*, 1994; Imamoto *et al.*, 1995b; Moroianu

Fig. 1. Deletion of the bipartite-basic NLS within hnRNP K results in a nuclear protein with transcription-dependent nuclear localization. HeLa cells were transfected with expression vectors encoding myc-tagged C1 (A and E), A1 (B and F), K (C and G) and K lacking the bipartite-basic NLS (D and H). At 40 h post-transfection the medium was replaced with either fresh medium (control) or medium containing 5 μ g/ml actinomycin D (Actino. D) and incubation was continued for another 4 h prior to fixation and immunostaining with mAb 9E10 which recognizes the myc tag.

et al., 1995). The NLS protein–importin α complex then connects to the other component of the NLS receptor complex, importin β (Chi *et al.*, 1995; Görlich *et al.*, 1995; Imamoto *et al.*, 1995a; Radu *et al.*, 1995), by virtue of the importin β -binding (IBB) domain contained within the N-terminus of importin α (Görlich *et al.*, 1996; Weis *et al.*, 1996). This trimeric complex then docks at the NPC where additional factors, such as the GTPase Ran/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993) and the protein p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995), participate in the energy-dependent translocation of the nuclear protein–NLS receptor complex through the NPC and into the nucleus (reviewed in Görlich and Mattaj, 1996). Consistent with its lack of sequence similarity to classical NLSs, we have demonstrated recently that M9-mediated nuclear import does not utilize this pathway and instead relies on a novel factor, transportin, for import into the nucleus (Pollard *et al.*, 1996). Transportin, which is distantly related to importin β , binds directly to M9 and is the only exogenous factor required for nuclear import of M9-bearing proteins *in vitro* (Pollard *et al.*, 1996). These findings demonstrated that protein import into the nucleus occurs along at least two distinct pathways and raised the possibility that other pathways may also be operational in the cell.

Here we extend our studies on the subcellular trafficking of shuttling pre-mRNA-binding proteins by examining the signals which control transport of the hnRNP K protein (Matunis *et al.*, 1992). We have shown previously that K contains a classic, bipartite-basic NLS (Michael *et al.*, 1995a,b), and here we delineate a second, unique NLS within K and show that it, like M9, specifies bi-directional transport across the NPC and thus functions also as a NES. Because of this ability to promote nucleo-cytoplasmic shuttling, we have named this signal KNS, for hnRNP K nuclear shuttling domain. The NES activity of KNS can be partially separated from NLS activity and corresponds to a 25 amino acid subdomain of KNS. When KNS-

mediated nuclear import is examined *in vitro* using the permeabilized cell assay, we find that import occurs via a pathway which is distinct from those utilized by either classical NLSs or M9. These results broaden our understanding of nucleo-cytoplasmic protein transport through the identification of a new type of shuttling signal and transport pathway.

Results

Multiple nuclear localization signals within hnRNP K

Previous work had identified a bipartite-basic type NLS in hnRNP K. This NLS (termed 'NLSI'-²¹KRPAED-MEEEQAFKRSR³⁷), comprising amino acids 21–37 of the human K protein, contains basic amino acids whose spacing matches the consensus sequence for classical bipartite-basic NLSs (Dingwall and Laskey, 1991) and also contains a PA dipeptide situated between the two basic clusters which has been shown recently to be important for bipartite-basic NLS activity (Makkerh *et al.*, 1996). Furthermore, this sequence confers complete nuclear localization onto cytoplasmic reporter proteins when expressed as a fusion protein (Michael *et al.*, 1995a,b). To ask if this NLS is solely responsible for nuclear localization of the K protein, an epitope-tagged deletion mutant which removes the first 37 amino acids, and therefore eliminates the bipartite-basic NLS from the protein, was constructed. We also produced an epitope-tagged derivative of the full-length K protein and compared the subcellular localization of these two proteins after transient transfection of HeLa cells. Removal of the classical NLS within K had no effect on the ability of the protein to localize completely to the nucleus (Figure 1, compare C and D), indicating that K contains at least one more NLS activity. We next analyzed the ability of this deletion mutant to localize to the nucleus in cells which had been treated with actinomycin D to inhibit pol II

transcription. As controls for transfection-induced over-expression, we also expressed epitope-tagged A1 and C1 as well as full-length K and found, as expected (Piñol-Roma and Dreyfuss, 1991), that A1 but not C1 accumulates in the cytoplasm of transcriptionally inhibited cells (Figure 1A, B, E and F). Surprisingly, and in contrast to full-length K, the K deletion mutant lacking the classical NLS accumulates in the cytoplasm of transcriptionally inhibited cells in a manner indistinguishable from A1 (Figure 1C, D, G and H). This result demonstrates that removal of the classical NLS in the K protein reveals an NLS that is dependent on ongoing pol II transcription for nuclear localization.

Visual examination of the K sequence (Matunis *et al.*, 1992) indicates that there are no good candidate sequences within the remainder of the protein (amino acids 38–463) which match either the bipartite-basic or SV40 large T-type NLS consensus sequences (Dingwall and Laskey, 1991) or the A1 M9 domain (Michael *et al.*, 1995a; Siomi and Dreyfuss, 1995). We therefore chose to delineate the activity or activities which provide nuclear localization to the K38–463 mutant. In order to accomplish this, more deletion mutants were constructed and their subcellular distribution was determined after transient transfection of HeLa cells. The structure of K is depicted in Figure 2C; the known functional domains of the protein include three KH domains (Siomi *et al.*, 1993; Burd and Dreyfuss, 1994) which are involved in RNA binding (Siomi *et al.*, 1994), NLSI and a proline-rich SH3-binding domain (SBD) which has been shown to interact *in vitro* with a subset of SH3-containing proteins including Src and Vav (Taylor and Shalloway, 1994; Weng *et al.*, 1994; Bustello *et al.*, 1995). The K38–277 mutant, which contains the first two KH domains and is missing the SBD and third KH domain, was distributed evenly between the nucleus and cytoplasm, indicating that it lacks a strong NLS activity (Figure 2A, panel K38–277). Although this protein does gain access to the nucleus, its relatively small size (<30 kDa) may allow it to enter the nucleus by diffusion, where it may then be retained selectively by association with a nuclear component. The other three deletion mutants all localized completely to the nucleus (Figure 2A, panels K277–463, K323–463 and K323–390; summarized in Figure 2C). The sequences common to these three fragments correspond to amino acids 323–390, a region of the K protein situated between the SBD and the third KH domain. We shall refer to the NLS activity contained within amino acids 323–390 as KNS.

To characterize KNS further, and to confirm that this signal is transferable, a series of expression vectors encoding pyruvate kinase (PK) fusion proteins were constructed. PK has long been used as a reporter protein for NLS identification because it is normally located exclusively in the cytoplasm but can localize to the nucleus when attached to a functional NLS (Richardson *et al.*, 1986). We found that fusion of K amino acids 323–390 to PK (PK–K323–390) produced a nuclear protein (Figure 2B), indicating that this NLS can be transferred to heterologous proteins. To determine more precisely the amino- and carboxy-terminal boundaries of KNS, PK fusion proteins containing portions of this sequence were produced and these showed that the smallest fragment which maintained full NLS activity, as determined by complete nuclear

localization of the relative PK fusion protein, corresponds to amino acids 323–361 (Figure 2B). Removal of an additional three amino acids from the C-terminus of this fragment abrogated complete nuclear localization as did removal of 15 amino acids from the N-terminus (Figure 2B). Figure 2D summarizes these results and displays the sequence of the minimal defined KNS domain. KNS contains no sequences with obvious similarity to either the bipartite-basic type or the SV40 large T-type NLSs nor is it homologous to the M9 domain from A1. Therefore, KNS is a novel type of NLS. Database searches with this sequence have so far failed to identify other proteins, beyond the K homologs in rat and mouse, which contain significant homology to KNS.

Evolutionary analysis of KNS

In order to learn more about this domain, the sequence of K homologs from divergent eukaryotes was examined. The organism with the greatest evolutionary distance from humans for which K sequence is available is the African clawed frog *Xenopus laevis* (Siomi *et al.*, 1993). The frog protein, at 47 kDa, is considerably smaller than the 68 kDa human K protein, largely because it lacks two regions which are contained within the central part of the human protein (Siomi *et al.*, 1993). Interestingly, the second of these regions deleted in the frog protein corresponds almost precisely to KNS (Figure 3A). This suggests that KNS has arisen sometime during the 300 million years which evolutionarily separate amphibian and mammalian lineages. Alternatively, amphibians may have lost the KNS domain from the K protein during the course of evolution. To confirm that KNS activity is unique to the mammalian K protein, the remnants of this domain found in the frog protein (amino acids 288–321, Figure 3A) were fused to PK and this fragment was found to lack NLS activity (data not shown). Previous work on characterization of hnRNP complexes from chicken cells had demonstrated that the apparent K homolog from avians migrates much faster on SDS–PAGE and may therefore, as in *Xenopus*, be a smaller protein than the mammalian counterpart (Matunis, 1992). To compare directly the size of hnRNP K from diverse organisms, extracts were prepared from human HeLa cells, quail QT6 cells and *Xenopus* XL-177 cells and analyzed by SDS–PAGE and immunoblotting with monoclonal antibody 3C2, which is specific for the K protein (Matunis *et al.*, 1992). As noted previously, the human K protein migrates at 68 kDa and the frog K protein at 45 kDa (Matunis *et al.*, 1992; Siomi *et al.*, 1994), and we found that the quail K protein runs in between the two at ~54 kDa (data not shown). To ask if the size difference between human and quail K protein again reflects an alteration in the KNS region, a partial K cDNA from the quail cell line QT6 was cloned, and the region between the second and third KH domains, where KNS is situated in the human protein, was sequenced. An alignment of the KNS region highlighting the similarities and differences between the human, quail and frog proteins is presented in Figure 3A. The quail protein contains a region with more extended homology to the human protein than that of the frog counterpart. We therefore asked if the region corresponding to KNS in the quail protein is a functional NLS by producing a PK fusion protein and determining its subcellular localization in both human

Fig. 2. Delineation of KNS. (A) HeLa cells were transfected with expression vectors encoding myc-tagged fragments of K protein. The numbers above each panel correspond to the amino acids within K which were expressed. Cells were processed as in Figure 1, control. (B) HeLa cells were transfected with expression vectors encoding myc-tagged PK fusion proteins. The numbers above each panel correspond to the amino acids within K which were fused to PK. Cells were processed as in Figure 1, control. (C) Summary of the intracellular localization of the K protein fragments displayed in (A). See text for details concerning the domain structure of K. (D) Summary of the intracellular localization of the PK fusion proteins displayed in (B). Also shown is the minimally defined KNS domain.

(HeLa) and quail (QT6) cells. We found that the quail fragment has no NLS activity in either cell type, whereas the analogous sequence from the human protein has NLS activity in both cell types (Figure 3B). These results demonstrate that, although the quail K protein does not contain an active KNS, the human signal still functions properly in quail cells. This in turn suggests that the K protein in higher organisms may have evolved to access

a pre-existing cellular structure which is likely to be present in quail cells. An example of such a structure could be the nuclear pc complex (NPC) itself (see Discussion).

KNS contains a nuclear export signal

The M9 domain of A1 has been shown recently to function as a NES (Michael *et al.*, 1995a). Because of the emerging similarities between M9 and KNS (both are non-canonical

Fig. 3. Evolutionary analysis of KNS. **(A)** Sequence alignment showing the homology between the human, quail and *Xenopus* K proteins. The sequences shown correspond to amino acids 318–382 in the human protein and amino acids 287–313 in the *Xenopus* protein. The sequence of the quail K protein has not been completed, therefore reference points for its sequence are unavailable. The boxed region in the human sequence corresponds to the minimally defined KNS region, and the identical amino acids between the three proteins are highlighted in gray. **(B)** PK fusion proteins containing either human K amino acids 323–376 or the corresponding region from the quail K protein were expressed in either human HeLa cells or quail QT6 cells and the cells were then processed as in Figure 1, control.

NLSs found in shuttling pre-mRNA-binding proteins) the possibility that KNS also encodes a NES was tested. To do so, we relied on an assay system which we had developed to study the M9 NES. Briefly, this technique is based on the fact that the NPC, when fused to a classical NLS such as NLSI from the K protein or the SV40 T NLS, is retained within the nucleus of cells (Michael *et al.*, 1995a,b). This was demonstrated by the use of interspecies heterokaryons, a very sensitive *in vivo* assay which can detect the ability of a given protein to not only enter, but also efficiently to exit or be exported from, the nucleus. Therefore, when NPC fused to the SV40 T NLS (NPC-T NLS) is analyzed in interspecies heterokaryons, little if any nuclear export activity can be detected (Michael *et al.*, 1995b). However, when peptides containing NESs are fused to NPC and then analyzed in interspecies heterokaryons, nuclear export of the fusion protein occurs and consequently the protein shuttles between the heterologous nuclei of the heterokaryon (Michael *et al.*, 1995a). When NPC-KNS (K amino acids 323–361) was examined in this assay, we found that, unlike NPC-T NLS, it rapidly shuttles in heterokaryons, indicating that KNS also has NES activity (Figure 4).

To determine if the NLS and NES activities of KNS are co-linear or if the NES corresponds to a subdomain of KNS, we made deletion fragments of KNS and tested their ability to activate export of NPC. Because these fragments lack NLS activity when fused to PK (see Figure 2), we included the SV40 large T NLS in these constructs

to ensure complete nuclear localization of the resultant fusion protein. The results are summarized in Figure 4B and show that, surprisingly, amino acids 338–361 represent the smallest fragment which is sufficient to maintain NES activity (Figure 4A, row NPC-T NLS-K338–361). Removal of nine amino acids from the carboxy-terminus of this fragment (Figure 4A, row NPC-T NLS-K338–352) as well as removal of eight residues from the amino-terminus (data not shown) severely inactivates the NES. These results demonstrate that while the NLS and NES activities of KNS overlap (Figure 4A), they may not be completely coincident (see Discussion).

Characterization of the nuclear transport pathway accessed by KNS

In order to begin to identify cellular factors which mediate KNS activity, we examined nuclear import of a KNS-containing protein in an *in vitro* import assay system (Adam *et al.*, 1990). The transport substrate used, which we refer to as GST-KNS, is GST fused to amino acids 323–463 of the human K protein. We were forced to use such a relatively large piece of K because GST fusion proteins containing only the minimal KNS region are unstable in *Escherichia coli* and therefore difficult to purify. When GST-KNS is combined with a cytoplasmic extract and an ATP-regenerating system and then incubated with digitonin-permeabilized cells, efficient nuclear uptake is observed (Figure 5A). Nuclear import of GST-KNS in this system is energy dependent, as omission of the ATP-regenerating system and inclusion of apyrase completely inhibits nuclear import of GST-KNS (Figure 5B). We next asked if KNS-mediated nuclear import is a saturable process by examining GST-KNS import in the presence of a large excess of competitor transport substrate. Excess NPC fused to the K bipartite-basic NLS (NPC-K NLSI, Pollard *et al.*, 1996) had little effect on GST-KNS nuclear import, whereas excess NPC-KNS completely inhibited import of GST-KNS (Figure 5C and D). This suggests that a KNS receptor is present in limiting amounts and is required for KNS-mediated nuclear import. Recently, a dominant-negative mutant of importin β has been described which inhibits most signal-mediated macromolecular transport through the NPC [e.g. import of both classical NLS- and M9-containing proteins as well as nuclear export of mRNA, U snRNA and NES-containing proteins (Kutay *et al.*, 1997)]. When GST-KNS import was examined in the presence of this importin β mutant, we found that import was completely inhibited (Figure 5E), demonstrating that KNS-mediated transport occurs through NPCs.

The fact that NPC-K NLSI did not compete with GST-KNS for nuclear import suggests that KNS, like the A1 M9 domain, does not require the importin NLS receptor complex for nuclear import. To address this issue directly, we assayed GST-KNS import in the presence of saturating amounts of the importin α IBB domain, a potent inhibitor of importin-mediated import (Görlisch *et al.*, 1996; Weis *et al.*, 1996). We found that excess IBB efficiently blocks import of a GST-IBB fusion protein but has no effect on GST-KNS import (Figure 6A). This result, in combination with the NPC-K NLSI competition experiment shown in Figure 5,

Fig. 4. KNS has nuclear export signal activity. (A) Expression vectors encoding the given NPc fusion proteins were transfected into HeLa cells. After expression of the transfected DNAs, the cells were fused with NIH3T3 cells to form heterokaryons and incubated in media containing 100 µg/ml cycloheximide for a period of 1 h. The cells were then fixed and stained for immunofluorescence microscopy with mAb 9E10 (anti-myc tag; panel Mab 9E10) to localize the proteins, and Hoechst 33258 (panel Hoechst) which differentiates the human and mouse nuclei within the heterokaryon (arrows identify the mouse nuclei). 'Phase' panel shows the phase contrast image of the heterokaryons with the cytoplasmic edge highlighted with either white or black dashed lines. (B) Summary of the results depicted in (A). The minimally defined KNS domain is shown below, with the sequences sufficient for NES activity highlighted in gray shading.

Fig. 5. Properties of the KNS import pathway *in vitro*. (A) GST-KNS (at 100 μ g/ml) was combined with rabbit reticulocyte lysate S100 extract, an ATP-regenerating system, transport buffer and digitonin-permeabilized HeLa cells for 30 min. Import of the GST fusion protein was detected by indirect immunofluorescence with an anti-GST monoclonal antibody (Santa Cruz Biotech.) (B) Same as (A) except the ATP-regenerating system was omitted and apyrase was included at 2.5 U/ml. (C) Same as (A) except recombinant NPc-KNS was included at 5 mg/ml. (D) Same as (A) except recombinant NPc-K NLSI was included at 5 mg/ml. (E) Same as (A) except recombinant importin β 45–462 (Kutay *et al.*, 1997) was included at 2 μ M.

Fig. 6. KNS accesses a novel nuclear import pathway. (A) Import of GST-IBB or GST-KNS under either standard conditions (control) or in the presence of excess IBB peptide (Weis *et al.*, 1996; panel 20 μ M IBB). (B) Import of GST-M9 or GST-KNS under either standard conditions (control) or in the presence of excess NPc-M9 (Pollard *et al.*, 1996; panel 50X NPc-M9). (C) Import of GST-M9 or GST-IBB under either standard conditions (control) or in the presence of excess NPc-KNS (panel 50X NPc-KNS).

demonstrates that KNS does not require the importin complex and therefore accesses a nuclear import pathway distinct from that used by classical NLS-containing proteins. The other known protein nuclear import pathway characterized thus far is the transportin-mediated

import of A1 (Pollard *et al.*, 1996). We therefore asked if KNS uses this pathway for nuclear import by performing competition experiments with excess M9-containing substrate. We have shown previously that excess NPc-M9 blocks import of M9-containing, but

Fig. 7. KNS does not require soluble transport factors for import *in vitro*. Import of GST fused to the T NLS, M9, IBB or KNS under either standard conditions (cytosol) or with an ATP-regenerating system and transport buffer alone (buffer).

not classical NLS-containing, proteins (Pollard *et al.*, 1996). Import of GST–KNS was therefore assayed under these conditions and found to be imported quite efficiently whereas, as expected, import of GST–M9 is blocked (Figure 6B). Figure 6A and B demonstrates that excess IBB and M9 do not inhibit import of GST–KNS. However, when the reciprocal competition experiments were performed, a surprising conclusion emerged. Excess KNS in the form of an NPC–KNS fusion protein was included in transport assays and import of either M9 or IBB was monitored. We found that excess KNS efficiently inhibits import of GST–M9 and severely reduces the import of GST–IBB to levels of ~30% of control reactions (Figure 6C). For this and other reasons (see later and Discussion), we suggest that KNS, unlike M9 and IBB, does not utilize a soluble transport receptor and may in fact directly contact the NPC.

KNS-mediated nuclear import in digitonin-permeabilized cells does not require exogenous factors

The results presented in Figures 5 and 6 demonstrate that KNS-mediated nuclear transport is a saturable, energy-dependent process which does not require either of the two known import receptors, importin and transportin. As a step towards the identification of mediators of KNS import, we began to fractionate cytoplasmic extract in order to purify this factor(s) biochemically. However, during initial assays, we found that import of GST–KNS in the *in vitro* system occurs without addition of any soluble cytoplasmic proteins. To demonstrate this more clearly, import reactions, containing an ATP-regenerating

Fig. 8. KNS does not require exogenous Ran for import *in vitro*. Import reactions containing an ATP-regenerating system and either: transport buffer alone, transport buffer plus recombinant importin β or transport buffer plus recombinant importin β and recombinant Ran along with the given transport substrate.

system and either cytoplasmic S100 extract or buffer alone, were performed and nuclear uptake of a variety of transport substrates was monitored. As expected, import of T NLS-, M9- and IBB-containing proteins absolutely requires addition of cytoplasmic extract, whereas import of the KNS derivative occurs efficiently in the reactions containing only buffer (Figure 7). Importantly, we do not observe any stimulation of GST–KNS import in the reactions containing cytoplasmic extract relative to those containing only buffer (Figure 7, compare panels 'cytosol' and 'buffer'). This result implies that the KNS receptor predicted by the competition experiment in Figure 5 is left behind in sufficient amounts when cells are treated with digitonin at a concentration known to release essential transport factors for classical NLS-mediated import (Adam *et al.*, 1990) and M9-mediated import (Pollard *et al.*, 1996). In order to ensure that the condition of the cells after digitonin permeabilization in our hands is similar to that seen by others, the fate of GST–IBB import in the presence or absence of recombinant transport factors was examined. We found, as expected (Görlich *et al.*, 1996; Weis *et al.*, 1996), that import of GST–IBB is completely dependent on the addition of both importin β and Ran to the reaction mixture (Figure 8). In contrast, and consistent with the results shown in Figure 7, import of GST–KNS occurs efficiently both in the absence and presence of exogenously added importin β or Ran (Figure 8). This result demonstrates that the permeabilization conditions employed here are sufficient to release Ran at levels required for GST–IBB import, and raises the interesting possibility that KNS-mediated import occurs along a Ran-independent pathway.

Discussion

In this work, we have extended our studies on the nuclear transport of shuttling hnRNPs by identifying and characterizing a novel transport signal in the hnRNP K protein which we have named KNS. KNS is a 38 amino acid domain from the human K protein which mediates bi-directional transport across the nuclear envelope.

Two different NLSs within human K protein

We have shown that human K protein contains both a classical, bipartite-basic NLS and the novel KNS domain. Why then should this protein contain two distinct types of nuclear targeting signals? One clue may be provided by the sequence of the amphibian and avian K homologs. Apparently, the KNS domain was acquired sometime after the divergence of the avian and mammalian lineages (see Figure 3), which suggests that the mammalian K protein may have acquired additional cellular functions for which KNS is necessary. Another clue comes from the studies with transcriptional inhibitors shown in Figure 1. Deletion of the classical NLS in K renders the protein dependent on continuous pol II transcription for complete nuclear localization. This result demonstrates that classical NLSs can override the requirement for pol II transcription when housed *in cis*, a point which is underscored by the observation that an hnRNP A1 fusion protein which contains the SV40 T NLS is no longer transcription dependent for nuclear localization (W.M. Michael and G. Dreyfuss, unpublished observation). These findings demonstrate that the K protein can utilize one of two pathways for nuclear entry, and we suggest that if K has more than one function in the nucleus then this choice may have consequences for targeting to different subnuclear domains where K can accomplish one or the other of these functions.

The transcription dependence of the K classical NLS deletion mutant is interesting for another reason. So far, five different hnRNPs have been characterized such that sequence information is known, the ability to shuttle has been documented and the transcription-dependent nature of nuclear localization has been determined (Piñol-Roma and Dreyfuss, 1991; Michael *et al.*, 1995b). From these studies, a clear pattern emerges: A1 and I proteins shuttle, show transcription-dependent nuclear localization and do not contain classical NLSs. Conversely, the C and U proteins do not shuttle, localize to the nucleus independently of pol II activity and contain classical NLSs. K deviates from this pattern because it shuttles yet is transcription independent and contains the classical NLS. We have shown here that K can in fact access the transcription-dependent nuclear import pathway and this then re-establishes the connection between transcription-dependent nuclear localization and nuclear export for the shuttling hnRNPs.

KNS is a shuttling domain

Deletion analysis and gene fusion experiments demonstrated that amino acids 323–361 of the human K protein are sufficient to direct nuclear import and nuclear export of reporter proteins. We note, however, that amino acids 338–361 are sufficient for nuclear export. It is possible that both of these activities are specified by the same amino acids (338–361), and that the reason amino acids 323–337 appear also to be required for NLS activity is that they provide a non-specific spacer function which allows amino acids 338–361, in the context of the PK fusion protein, to become more accessible to the nuclear import machinery. Two lines of evidence support this view. First, the quail K homolog is almost identical to the human sequence from position 323 to position 337 yet has no NLS activity, indicating that these sequences alone

cannot support even minimal NLS activity. Second, we have shown that PK fused to amino acids 338–361 from the human protein is completely cytoplasmic; however, if a 'random' 21 amino acid sequence encoded by the plasmid pSP72 polylinker is inserted between the C-terminus of PK and the human K338–361 fragment, this fusion protein is now evenly distributed between the nucleus and cytoplasm (data not shown). This suggests that simply increasing the distance between the PK fusion junction and human K amino acids 337–362 partially activates the NLS activity of the K337–362 fragment. This situation is reminiscent, therefore, of the spacing requirements for IBB NLS activity when fused to a reporter protein (Görlich *et al.*, 1996). In order to resolve this important issue, systematic mutagenesis will be required to analyze specific amino acid requirements for KNS NLS and NES activities, and such experiments are now in progress.

KNS is the third type of nuclear export signal to be identified. The others are M9 (Michael *et al.*, 1995a,b) and the leucine-rich NES identified in the PKI protein and HIV-1 Rev (Fischer *et al.*, 1995; Wen *et al.*, 1995), and more recently found in several other proteins. M9 and KNS differ from the leucine-rich NES in that they are coupled with NLS activities, whereas the leucine-rich signals specify export only. We suggest that, as this import/export signal relationship has now been found within two different shuttling hnRNPs, the mechanism which governs nucleocytoplasmic transport of shuttling hnRNPs is fundamentally different from that which governs import of classical NLS-containing proteins and export of leucine-rich NES-containing proteins. The M9 and KNS are shuttling domains in the sense that they specify entry into a pathway of continuous cycling between the nucleus and cytoplasm, as opposed to the unidirectional routes of transport specified by a classical NLS in one direction and a leucine-rich NES in the other direction. This continuous cycling is probably linked to the export of mRNA (Piñol-Roma and Dreyfuss, 1992, 1993), and this may explain why re-import into the nucleus requires continuous pol II transcription. This regulatory event might ensure that energy is not wasted to re-import shuttling hnRNPs into the nucleus if the nuclear pool of mRNA is low and hnRNPs are therefore not required to be there. The fact that M9-containing proteins use transportin, and not importin, for nuclear import already highlights one of the major differences between hnRNP import and most other nuclear protein import, and it is anticipated that identification of the transport effectors for KNS will provide a second example.

KNS-mediated nuclear import does not require known import receptors

We have begun to characterize the transport pathway accessed by KNS using the *in vitro* import assay. These experiments produced the surprising conclusion that KNS import occurs efficiently in the absence of additional soluble transport factors. Competition experiments presented in Figures 5 and 6 further support this result as saturating amounts of both IBB and M9 do not inhibit KNS import, demonstrating that neither the importin complex nor transportin, the two known NLS receptors, are required by KNS. Curiously, excess KNS does have a substantial effect on import of GST–M9 and, to a lesser

extent, GST-IBB. One explanation for this is that KNS may compete for binding sites on the NPC with transportin and importin β . It is known that digitonin permeabilization of HeLa cells releases nearly all of the cytoplasmic transportin (Pollard *et al.*, 1996) but only a portion of the cytoplasmic importin β (Chi *et al.*, 1995; Görlich *et al.*, 1995). If KNS competes with transportin and importin β for binding to the cytoplasmic side of the NPC then this would explain why excess KNS has a stronger effect on M9 import than IBB: the residual importin β left behind after digitonin treatment is associated with the nuclear envelope (Chi *et al.*, 1995; Görlich *et al.*, 1995) and therefore allows a slightly higher level of import under conditions of saturating KNS. These results suggest the possibility that the KNS sequences contained within mammalian K proteins evolved to allow direct access to the NPC which therefore circumvents the need for a soluble cytoplasmic receptor during import.

Another possibility is that a KNS receptor does exist in the cytoplasm but sufficient quantities of this factor are left behind in the cells after permeabilization to support import in the absence of exogenously added material. Precedence for this scenario can be found in the initial studies on the importin complex (Görlich *et al.*, 1994, 1995). Importin α is completely solubilized and lost from the cells after permeabilization whereas, as mentioned earlier, amounts of importin β are left behind sufficient to support a basal level of classical NLS import when importin α and Ran are added back to the system. However, in reconstitution experiments, classical NLS import is greatly enhanced when importin β is also added back to the reaction. We do not observe such an enhancement when GST-KNS is imported either with complete extract or with buffer alone, suggesting that if a soluble receptor is required it is either very abundant or almost completely insoluble under these permeabilization conditions. A third possibility is that the receptor is, at steady state, largely in the nucleus and functions in the *in vitro* import assay only after export from the nucleus. We do not favor this possibility, as preliminary experiments using nuclear extract as a source of transport factors in the *in vitro* assay system indicate that KNS import is not significantly enhanced (data not shown).

Finally, we have shown that addition of exogenous Ran is required in our experiments for IBB import but not for KNS import. It is possible, therefore, that KNS import requires Ran at a much lower concentration than IBB import and that the cells contain this smaller amount even after permeabilization. Alternatively, KNS import may not require Ran at all, a possibility which we are currently investigating.

Materials and methods

Plasmid construction

Eukaryotic expression vectors. For generation of pCDNA1/mycA, a double-stranded oligonucleotide encoding the mAb 9E10 epitope of the c-Myc protein (Evan *et al.*, 1985) was synthesized. The sequence of the sense strand is: 5'-CCATTGTGCTGGCCACCATGGGAGAGCAGAACTGATCTCTGAAGAAGACCTGAACCCGGGTGACGAATTC-CAGACAATGG-3'. The oligonucleotides were phosphorylated with T4 polynucleotide kinase and then annealed. pCDNA1 (Invitrogen) was digested with *Bst*XI, and the ends were then blunted with the Klenow fragment. The double-stranded oligonucleotide was then ligated to the

vector. This parental plasmid was then used to generate the following plasmids.

For myc-C1, primers were designed which included an *Eco*RI site in the 5' partner and an *Xho*I site in the 3' partner to amplify via PCR a fragment corresponding to amino acids 1–290 of the human C1 coding sequences using plasmid pHK12 (Swanson *et al.*, 1987) as template. This fragment was then digested with *Eco*RI and *Xho*I and subcloned into similarly digested pCDNA1/mycA. The *Eco*RI sites in the 5' primer were designed to fit in-frame with the *Eco*RI site present in the myc tag coding sequences of pCDNA1/mycA.

The plasmid myc-A1 has been described by Michael *et al.* (1995a). To create myc-K and deletion mutants, plasmid pHK5 (Matunis *et al.*, 1992) was used as template in a series of PCRs. Primers were designed which included an *Eco*RI site in the 5' partner and an *Xho*I site in the 3' partner to amplify fragments of the K coding sequences corresponding to amino acids 1–463, 38–463, 38–277, 277–463, 323–463 and 323–390. These individual fragments were then digested with *Eco*RI and *Xho*I and subcloned into similarly digested pCDNA1/mycA. The *Eco*RI sites in the 5' primers were designed to fit in-frame with the *Eco*RI site present in the myc tag coding sequences of pCDNA1/mycA.

For pCDNA1/mycB, a slightly different double-stranded oligonucleotide encoding the mAb 9E10 epitope of the c-Myc protein was synthesized. The sequence of the sense strand is: 5'-GGGGAAGC-TTCCATATGGGAGAGCAGAACTGATCTCTGAAGAAGACCTG-AACGGATCCGGGG-3'. This oligonucleotide was digested with *Hind*III and *Bam*HI and ligated to *Hind*III- and *Bam*HI-digested pCDNA1. This parental plasmid was then used to create the following PK fusion vectors.

To create myc-PK, plasmid RLPK (Dang and Lee, 1988) was used as template in a PCR reaction to amplify codons 12–443 of the chicken muscle pyruvate kinase cDNA. This PCR fragment, which contained a *Bam*HI site at the 5' end and a *Eco*RI site at the 3' end, was then subcloned into *Bam*HI- and *Eco*RI-digested pCDNA1/mycB. The *Bam*HI site in the PCR fragment was designed to create a continuous open reading frame from the myc tag on into the PK coding sequences.

For PK-K fusion constructs, plasmid pHK5 was used as template in a series of PCRs. Primers were designed which included an *Eco*RI site in the 5' partner and an *Xho*I site in the 3' partner to amplify fragments of the K coding sequences corresponding to amino acids 323–390, 323–361, 323–358 and 338–361. These individual fragments were then digested with *Eco*RI and *Xho*I and subcloned into similarly digested myc-PK. The *Eco*RI sites in the 5' primers were designed to fit in-frame with codon 444 of the PK coding sequence. Additionally, similar primers corresponding to amino acids 288–321 of the *Xenopus* K protein were used in a PCR with plasmid pXK1 (Siomi *et al.*, 1993) as template. This fragment was then digested with *Eco*RI and *Xho*I and subcloned into similarly digested myc-PK.

Plasmid NPC-T NLS has been described by Michael *et al.* (1995b). To create NPC-K323–361, the *Eco*RI-*Xho*I fragment from plasmid PK-K323–361 corresponding to K NLS II was isolated and subcloned into similarly digested NPC-T NLS. This procedure resulted in a replacement of K323–361 for the T NLS coding sequences.

For NPC-T NLS-K fusions, plasmid pHK5 (Matunis *et al.*, 1992) was used as template in a series of PCRs. Primers were designed which included an *Xho*I site in the 5' partner and an *Xba*I site in the 3' partner to amplify fragments of the K coding sequences corresponding to amino acids 338–361, 338–352 and 346–361. These individual fragments were then digested with *Xho*I and *Xba*I and subcloned into similarly digested NPC-T NLS. The *Xho*I sites in the 5' primers were designed to fit in-frame with the *Xho*I site present in the T NLS sequences of NPC-T NLS.

Prokaryotic expression vectors. Plasmids GST-M9, GST-T NLS, petNPC-M9, and petNPC-K NLSI have been described (Pollard *et al.*, 1996). Plasmid pHK5 was used as template for PCR to create GST-KNS. Primers were designed which included an *Eco*RI site in the 5' partner and an *Xho*I site in the 3' partner to amplify a fragment of the K coding sequence corresponding to amino acids 322–463. This fragment was then digested with *Eco*RI and *Xho*I and subcloned into similarly digested pGEX-5X-1.

To create GST-IBB, a PCR fragment encoding the first 55 amino acids of *Xenopus* importin α (Görlich *et al.*, 1994) was subcloned into pCRII (Invitrogen). This plasmid was then digested with *Bam*HI, the ends were repaired with the Klenow fragment and the DNA was then digested with *Eco*RI. The fragment encoding the IBB domain was then isolated and subcloned into *Eco*RI-*Sma*I-digested pGEX-5X-3 (Pharmacia).

For petNPC-KNS, the *Eco*RI-*Xho*I fragment from plasmid GST-KNS was isolated and subcloned into similarly digested NPC-T NLS. This

procedure resulted in a replacement of K322–463 for T NLS coding sequences. This plasmid was then digested with *Bam*HI and *Xho*I and subcloned into pET28A (Novagen).

Tissue culture

HeLa and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% penicillin–streptomycin (Gibco-BRL) in an incubator adjusted to 37°C with 5% CO₂.

Transfection

Transfection of cultured cells was performed as described (Michael *et al.*, 1995a).

Actinomycin D treatment

HeLa cells were transfected as described. After removal of the media containing the DNA precipitant, the cells were allowed to recover for several hours. After recovery, the cells were then incubated in media containing 5 µg/ml actinomycin D (Calbiochem) for 4–6 h prior to fixation for immunofluorescence.

Immunofluorescence

Cells were washed in phosphate-buffered saline (PBS) and fixed and stained for immunofluorescence as described (Michael *et al.*, 1995a).

Heterokaryon analysis

On day 1, subconfluent HeLa cells growing in 100 mM dishes were transfected as described. Twenty-four hours later the cells were trypsinized and transferred to 35 mm dishes containing 18 mm² glass coverslips. The cells were split such that they would be at 40% confluence on the morning of day 3 of the experiment. At this time, an equal number of NIH3T3 cells were seeded into the 35 mm dishes. The co-cultures were then incubated for another 4 h to allow the 3T3 cells to attach. Medium containing 100 µg/ml cycloheximide (Calbiochem) was then added and the co-cultures were incubated for another 30 min to inhibit protein synthesis. After this, the coverslips were rinsed in PBS and the cells were fused by inverting the coverslip onto a drop of polyethylene glycol 5000/PBS (Gibco-BRL) for 120 s. The coverslips were then rinsed in PBS and returned to fresh media containing 100 µg/ml cycloheximide for another 60 min prior to fixation for immunofluorescence. Immunofluorescence was performed as described except that Hoechst 33258 (Sigma) was included at 5 µg/ml during the secondary antibody incubations.

Recombinant protein expression and purification

All GST fusion proteins were expressed in *E.coli* strain BL21DE3 and then purified according to the Pharmacia manual. All His-tagged proteins were expressed in *E.coli* strain BL21DE3 and then purified according to the Novagen manual using buffers supplied by the manufacturer.

In vitro nuclear import assays

Import assays, including the competition experiments, were performed exactly as described (Pollard *et al.*, 1996). For the experiment presented in Figure 8, recombinant importin β and Ran were supplied to the import reactions in the following manner. Importin β or Ran expression vectors were transformed into *E.coli* strains M15[pREP4] and BL21DE3 respectively. Bacteria were induced, grown for another 3 h, and the cells were then pelleted and resuspended in 1/20 volume of transport buffer [20 mM HEPES, pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM MgOAc, 0.5 mM EGTA, 2 mM dithiothreitol (DTT), 1 µg/ml each aprotinin, leupeptin, pepstatin]. The cell suspension was then lysed by sonication and the lysate was spun at top speed in an Eppendorf microfuge for 20 min at 4°C. The supernatant was collected and spun again at 100 000 g for 30 min at 4°C. The resultant supernatant was then used directly in transport reactions without further purification. The amount of importin β supplied by the lysate was 100 µg/ml final concentration in the import reaction and the amount of Ran supplied by the lysate was 100 µg/ml final concentration as determined by SDS–PAGE fractionation and Brilliant Blue staining of the lysates. Control experiments demonstrated that neither M15[pREP4] nor BL21DE3 lysates support IBB import unless they express importin β or Ran respectively (data not shown).

Acknowledgements

We thank Dirk Görlich for the importin β 45–462 mutant, Alan Wolffe for the *Xenopus* XL-177 cell line, Paul Bates for the quail QT-6 cell

line, Angus Lamond for the IBB expression vector, Mikiko Siomi for the *Xenopus* IBB cDNA, Haruhiko Siomi for the importin β expression vector and Mark Rush for the Ran expression vector. Additionally, we thank Sara Nakielnny and Haruhiko Siomi for critical reading of the manuscript and members of our laboratory for helpful discussions throughout the course of this project. This work was supported by the Howard Hughes Medical Institute and by a grant from the National Institutes of Health.

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Received on December 20, 1996; revised on February 24, 1997