The importin-β P446L dominant-negative mutant protein loses RanGTP binding ability and blocks the formation of intact nuclear envelope

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Summary

Three of the four independently induced Ketel⁴ dominant-negative female sterile mutations that identify the Drosophila importin-β gene, originated from a C4114→T transition and the concurrent replacement of Pro446 by Leu (P446L). CD spectroscopy of representative peptides with Pro or Leu in the crucial position revealed that upon the Pro→Leu exchange the P446L mutant protein loses flexibility and attains most likely an open conformation. The P446L mutation abolishes RanGTP binding of the P446L mutant form of importin-β protein and results in increased RanGDP binding ability. Notably, the P446L mutant importin-β does not exert its dominant-negative effect on nuclear protein import and has no effect on mitotic spindle-related functions and chromosome segregation. However, it interferes with nuclear envelope formation during mitosis-to-interphase transition, revealing a novel function of importin-β.

Key words: Importin-β, Nuclear envelope formation, Ran, Dominant-negative mutations, Drosophila

Introduction

About 40% of the EMS-induced dominant female sterile (Fs) mutations isolated in our laboratory identify genes with essential functions during the commencement of Drosophila embryogenesis (Erdélyi and Szabad, 1989; Szabad et al., 1989). Ketel is one of the Fs-identified genes and encodes the Drosophila homologue of importin-β (or karyopherin-β) (Lippai et al., 2000; Tirián et al., 2000). Importin-β is a component of the nuclear protein import machinery (reviewed by Pemberton et al., 1998; Mattaj and Englmeier, 1998; Wozniak et al., 1998; Melchior and Gerace, 1998; Weis, 1998; Görlich and Kutay, 1999). Briefly, during a ‘classical’ import cycle importin-β interacts with importin-α and, through importin-α, interacts with the NLS-containing nuclear protein. The three molecules form a nuclear import complex called karyopherin (Radu et al., 1995). The import complex docks on the cytoplasmic side of a nuclear pore complex (NPC). During translocation through the NPC, importin-β interacts with a number of nucleoporins, constituent proteins of the NPC. Import of the NLS-containing nuclear protein is completed on the nuclear surface of the NPC, where the import complex disassembles following the interaction of importin-β with RanGTP. Ran is a Ras-related G protein (Azuma and Dasso, 2000). While the nuclear protein stays inside the nucleus, the importin-β-RanGTP complex returns to the cytoplasm. In the cytoplasm, importin-β is released from RanGTP following the interaction of RanGTP with RanGAP (RanGTPase-activating protein) and RanBP1 (Ran-binding protein) (Bischoff and Görlich, 1997; Azuma and Dasso, 2000). Upon activity of RanGAP and RanBP1, RanGTP is converted to RanGDP, which is subsequently imported to the nucleus where it is converted to RanGTP by RCC1 (regulator of chromatin condensation), the guanine nucleotide exchange factor for Ran. The driving force of the nuclear transport process is Ran: whereas its GDP-bound form is prevalently cytoplasmic, RanGTP is generally nuclear. The different forms of Ran in the cytoplasm and nucleus are caused by the nuclear localization of RCC1 and the cytoplasmic localization of RanGAP and RanBP1 (Görlich and Kutay, 1999).

In addition to nuclear protein import, importin-β was shown to have a role in the organization of microtubules by regulating spatial and temporal distribution of microtubule-associated proteins throughout the cell cycle (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001). Briefly, during interphase, proteins such as NuMA and TPX2 that are required for mitotic spindle assembly are retained inside the nucleus. During mitosis, following the disassembly of the nuclear envelope (NE), the aforementioned proteins diffuse into the cytoplasm where importin-β binds and thus keeps them away from the chromatin. RanGTP was shown to be responsible for releasing proteins such as NuMA and TPX2, in the vicinity of the chromatin, from importin-β or the importin-α/β heterodimer. Ran is also involved in NE assembly by attracting and/or binding membrane vesicles (Zhang and Clarke, 2000) and is
required for vesicle fusion around chromatin (Hetzer et al., 2000).

The association of importin-β with different types of molecules during nuclear protein import cycles suggests conformational changes and, in fact, importin-β was proposed to be a rather flexible molecule (Vetter et al., 1999; Lee et al., 2000). Importin-β is composed from 19 so-called HEAT (armadillo) repeats arranged in a Spanish collar type of superhelix. It binds (1) Ran at its N-terminal region; (2) the nucleoporins with a large middle section; and (3) the importin-β binding (IBB) domain of importin-α with its C-terminal region (Kutay et al., 1997; Wozniak et al., 1998). In fact, the spatial structures of importin-β, on its own (Lee et al., 2000) and in complex with RanGTP (Vetter et al., 1999), as well as that of the IBB domain of importin-α (Cingolani et al., 1999) have recently been elucidated.

Although the Ketel⁺⁺ eggs, deposited by the Ketel⁺⁺/+ females, appear normal and are fertilized, cleavage nuclei do not form inside. The Ketel⁺⁺ egg cytoplasm is very toxic: when injected into wild-type cleavage embryos it hinders formation of cleavage nuclei (Tirián et al., 2000). Surprisingly, however, when injected into wild-type cleavage embryos the Ketel⁺⁺ egg cytoplasm does not prevent nuclear protein import. Moreover, nuclear proteins are imported into nuclei of digitonin-permeabilized HeLa cells in the presence of ovary extracts of the Ketel⁺⁺/+ females (Lippai et al., 2000); thus revealing novel importin-β function.

In the present report we show that the replacement of Pro446 by Leu – in three of the four independently isolated Ketel⁺⁺ alleles – dramatically changes the function of importin-β. We propose (based on CD spectroscopy of model peptides) that the flexibility of importin-β is reduced upon replacement of Pro446 by Leu. We show that the P446L mutant protein has no detectable affinity to RanGTP but binds RanGDP. We present evidence that the Ketel⁺⁺-encoded protein does not prevent nuclear protein import carried out by normal importin-β but inhibits NE formation at the end of mitosis. We also report that replacement of Ser317 by Thr restores characteristic importin-β functions: Drosophila females that lack a functional Ketel gene but carry a transgene with both the S317T and the P446L mutations are fully viable and fertile.

Materials and Methods

The Ketel⁺⁺ alleles

The four EMS-induced Ketel⁺⁺ mutations were isolated in screens for F₅ mutations and were shown to be of dominant-negative nature (Szabad et al., 1989; Tirián et al., 2000). Ketel⁺⁺⁺ and Ketel⁺⁺⁺⁺ were induced on Ibv labeled isogenic chromosomes. Ketel⁺⁺⁺⁺ and Ketel⁺⁺⁺⁺ were induced on wild-type, Canton-S-derived chromosomes (Szabad et al., 1989; Erdélyi et al., 1997). The loss-of-function ketel alleles are recessive zygotic lethal mutations and were generated through mutagenesis in PCR reactions. The generated Bag-III-ClaI fragment replaced the corresponding DNA segment in a plasmid containing a 4.0 kb Xba-BamHI genomic fragment, which includes the Ketel promoter and the 5' segment of the Ketel coding region, combined with a 2.3 kb cDNA fragment representing the rest of the transcribed part of the Ketel gene [see figure 1b in Lippai et al. (Lippai et al., 2000)]. The above sequences were cloned into the CaSpeR vector with the mini-white reporter gene and germ line transfectants were generated by standard procedures (Thummel et al., 1988). Flies carrying the Ketel⁺⁺ transgene have orange eyes of different intensities on a white genetic background. Three independent Ketel⁺⁺ transgenic lines were recovered. The Ketel⁺⁺ transgenes are inserted into different positions of third chromosomes and are maintained as the third chromosome-linked F₅ mutations (Erdélyi and Szabad, 1989). The expression level of the Ketel⁺⁺ transgenes was characterized by measuring eye pigment content of w/++; Ketel⁺⁺/+ flies. Eye pigment contents were determined by extraction of eye pigments and photometry as described by Reuter and Wolff (Reuter and Wolff, 1981). The Ketel⁺⁺ transgenes were used for the generation of w/+; Ketel⁺⁺/+ females and ketel⁺⁺/+; Ketel⁺⁺/+ zygotes.

For production of transgenes with both the S317T and the P446L mutations, we replaced a Bag-III-BsrEI fragment in the vector containing the Ketel⁺⁺ transgene with the same fragment containing the S317T mutation coding DNA fragment.

Production of the Ketel⁺⁺-encoded protein in bacteria

We produced normal Ketel protein as described (Lippai et al., 2000). For production of the P446L mutant protein in E. coli, an expression vector containing full length Ketel cDNA with the P446L mutation was generated by replacing the Bag-III-ClaI section in the pQE30 expression vector that contains the wild-type Ketel cDNA. Construction of the pQE30 expression vector with the wild-type Ketel cDNA was described previously (Lippai et al., 2000).

The expressed proteins included an N-terminal His tag that allowed purification on a nickel-NTA agarose column. Unlike the normal Ketel protein, over 95% of the P446L mutant protein were present in inclusion bodies. To produce functional P446L mutant protein, we dissolved the inclusion bodies in 6 M guanidine hydrochloride in the TMN buffer (50 mM Tris pH 7.5, 300 mM NaCl, 5 mM MgCl₂ and 5% glycerol) as used during purification of the normal Ketel protein. Renaturation of the P446L mutant Ketel protein was achieved through a 6 to 0 M decreasing guanidine gradient in TMN buffer and eluted with 0 to 0.5 M imidazole gradient in TMN buffer. The eluted protein was dialyzed against 0.1× TMN buffer overnight. Two hundred μl aliquots of the dialyzed protein were lyophilized and stored at −70°C. When used, the aliquots were dissolved in 20 μl H₂O. To test
The nuclear protein import assay

Digitonin-permeabilized HeLa cells were prepared by a modified protocol (Adam et al., 1990). Briefly, HeLa cells were grown on coverslips to 50-80% confluence, washed in ice-cold permeabilization buffer (20 mM Hepes-KOH pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose and 0.5 mM EGTA) and permeabilized for 15 minutes in the same buffer containing 60 µg/ml digitonin. The coverslips were washed three times in permeabilization buffer without digitonin. Coverslips were incubated with each 20 µl of import reaction. The import buffer contained 2 mg/ml nucleoplasmic core (to block nonspecific binding), 20 mM Hepes-KOH pH 7.5, 140 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA. Where indicated, reactions were supplemented with an energy regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 µg/ml creatine kinase) and Ran mix [3 µM Ran-GDP, 150 nM RanGAP (Rna1p from yeast), 300 nM NTF2 and 150 nM RanBP1]. Nuclear import of the IBB core pentamer was monitored in optical sections. Import reaction samples contained 0.24 µM fluorescein-labeled IBB core pentamer, 1.2 µM wild-type or P446L mutant Ketel protein and, where indicated, Ran mix and an energy regenerating system. Reactions were stopped after 5 minutes by fixation in 3% paraformaldehyde (w/v) in PBS, washed in PBS and water, and mounted with 2 µl of vectorshield mounting medium (Vector).

Binding assays and immunoprecipitations

Binding assays were carried out as described (Hughes et al., 1998). Briefly, GST-Ran loaded with GDP or GST-RanQ69L (a RanGTP binding assays were carried out as described (Hughes et al., 1998). Binding assays and immunoprecipitations

Enzymatic assays

Labeling of Ran with [γ32P]GTP and GTPase assays were performed essentially as described (Görlich et al., 1996). Concentration of the proteins were as follows: Ran 0.3 µM, wild-type and P446L Ketel 1 µM, RanBP1 0.4 µM, Drosophila RanGAP 25 nM. Hydrolysis of Ran[γ32P]GTP to RanGDP and the release of [γ32P]P was measured in a liquid scintillation counter 2 minutes after bringing the components together. For measuring of nucleotide exchange activity of RCC1 on Ran, human Ran protein was loaded with [32P]GTP or [3H]GDP. Protein concentrations in the reactions were as follows: 0.3 µM Ran, 1 µM wild-type and P446L Ketel and 30 nM RCC1. The exchange of labeled GTP or GDP to unlabeled GDP was measured for 2, 3 and 4 minutes in a liquid scintillation counter after the components were brought together.

Structural analysis

As shown in Table 3, we synthesized two model polypeptides. (1) The first included helix B of HEAT repeat 10, the linker region plus helix A of the HEAT repeat 11 (Cingolani et al., 1999). The linker region, like the wild-type Ketel protein in the 446th position (441st in human importin-β), contained a proline (Table 3). (2) The second polypeptide differed from the first by one amino acid: the proline in the linker region was replaced by leucine.

The polypeptides were synthesized by standard solid phase technique using Boc (butyl-oxy-carbonyl) chemistry and an automated ABI 430A synthesizer (Merrifield, 1963). The crude peptides were purified by reverse-phase HPLC and characterized by mass spectrometry. The polypeptides were dissolved in (1) 100% trifluoro-ethanol (TFE); (2) a mixture of 67% TFE and 33% H2O; and (3) 33% TFE and 67% H2O. CD spectra of the solutions were recorded on a Jobin Yvon dichrograph Mark VI in a 0.02 cm cell. The concentration of the samples varied between 0.1-0.5 mg/ml. CD spectra were analyzed using a Convex Constrain Analysis Plus software.

Results

In three of the four KetelD alleles the same C4114→T transition leads to Pro446→Leu replacement

We isolated genomic DNA from KetelD– hemizygous second instar larvae and used the DNA as template in four overlapping PCR reactions. Altogether a 7869 bp segment including the promoter and the transcribed region of the Ketel gene was PCR amplified. The PCR fragments were cloned and sequenced. The complete sequence was subsequently compared with the wild-type nucleotide sequence of the Ketel gene. We noticed a C→T transition in position 4114. C4114 is part of a CC4114C code for Pro446 in the Ketel protein (Lippai et al., 2000) (see also the Ketel gene nucleotide sequence under accession no.
The CTC mutant sequence is part of the CTCGAG palindrome that is in fact an XhoI restriction site and hence the KetelD (K_D) transgenes act as the Ketel D mutations. To determine whether the C4114→T transition did indeed lead to formation of three of the four KetelD alleles, we generated three K_D transgenic lines: A, B and C, with the in vitro generated C4114→T transition inside. The K_D transgene became inserted into different sites on different third chromosomes. Three features of the K_D transgenes confirm that the KetelD-related phenotypes are consequences of the C4114→T transition in the Ketel gene.

The K_D transgenes completely or largely sterilize females. The +/-; K_D females (with two normal Ketel genes and one K_D transgene) are either sterile or their fertility is severely reduced (Table 1). Whereas the ‘C’ K_D transgenic line renders females completely sterile, as was described for the KetelD/+ females which, in addition to KetelD1, carried two normal (+) Ketel gene copies (Tirián et al., 2000), very low offspring production rates are characteristic for lines A and B (Table 1). The variability in the female-sterilizing ability of the three K_D transgenic lines can be best explained by the different expression levels of the transgenes. Since the mini-white marker gene is also included in the K_D transgenes, eye pigment content of the transgenic flies is the measure of the expression level of the transgenes (Table 1). While eyes of the line ‘C’ flies are dark orange and reveal intensive expression of the K_D transgene, eyes of the line ‘A’ flies are light orange and reflect a low expression level. However, even the low expression level renders the +/-; line ‘A’ K_D females almost completely sterile (Table 1).

Production of offspring by the +/-; K_D line ‘A’ females provided an opportunity to recover a few +/+; K_D/K_D females. As expected, they had dark orange eyes and were completely sterile and, as in case of the KetelD1/+ females, embryogenesis did not commence in their eggs.

The K_D transgenes, like the KetelD alleles, act as dominant-negative mutations. The KetelD mutations are of dominant-negative type (i.e. their female sterilizing effect can be slightly reduced by extra doses of wild-type Ketel alleles) (Tirián et al., 2000). To determine whether the mutation in the K_D transgene possesses dominant-negative features, we combined the line ‘A’ K_D transgene with three different K* transgene lines (‘N’, ‘J’ and ‘K’; Table 2), which carry a normal Ketel gene inside (Lippai et al., 2000). Results of the experiment are summarized in Table 2 and clearly show the dominant-negative nature of the mutation in the K_D transgene: offspring production of the +/-; K_D; K* females significantly exceeded those of the +/-; K_D ones (P<0.01; χ² test). The difference in the K_D-compensating effect of the K* transgenic lines correlates well with the expression level of the mini-white reporter gene: the more intensively the K* transgene is expressed, the more eye pigment flies have and

### Table 1. Effects of the K_D transgenes on female fertility

<table>
<thead>
<tr>
<th>K_D transgene line</th>
<th>Tested</th>
<th>Offspring</th>
<th>Test period*</th>
<th>Rate of offspring production†</th>
<th>Eye pigment content‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>437</td>
<td>130</td>
<td>25.1</td>
<td>11.9×10⁻³</td>
<td>0.169</td>
</tr>
<tr>
<td>B</td>
<td>76</td>
<td>3</td>
<td>7.6</td>
<td>5.2×10⁻³</td>
<td>0.245</td>
</tr>
<tr>
<td>C</td>
<td>77</td>
<td>0</td>
<td>14.0</td>
<td>–</td>
<td>0.469</td>
</tr>
</tbody>
</table>

*Average test period per female (days).
†Offspring/female×day. Control females with a K* transgene produce ~50 offspring/female×day.
‡Eye pigment content (OD₄₈₅ of eye pigment solutions; see Materials and Methods).

### Table 2. The combined effects of the K_D (line A) and the K* transgenes on female fertility

<table>
<thead>
<tr>
<th>K* transgenic line</th>
<th>Chromosomal location of the K_D transgene</th>
<th>Tested</th>
<th>Offspring</th>
<th>Test period*</th>
<th>Rate of offspring production†</th>
<th>Eye pigment content‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>437</td>
<td>130</td>
<td>25.1</td>
<td>11.9×10⁻³</td>
<td>0.169</td>
</tr>
<tr>
<td>N</td>
<td>X</td>
<td>111</td>
<td>40</td>
<td>8.2</td>
<td>43.9×10⁻³</td>
<td>0.287</td>
</tr>
<tr>
<td>J</td>
<td>3rd</td>
<td>24</td>
<td>10</td>
<td>9.3</td>
<td>44.6×10⁻³</td>
<td>0.313</td>
</tr>
<tr>
<td>K</td>
<td>4th</td>
<td>13</td>
<td>8</td>
<td>10.2</td>
<td>60.2×10⁻³</td>
<td>0.360</td>
</tr>
</tbody>
</table>

*Average test period per female (days).
†Offspring/female×day.
‡Eye pigment content (OD₄₈₅ of eye pigment solutions; see Materials and Methods).
the more effectively the \( K^+ \) transgene reduces \( K^D \)-imposed female sterility (Table 2).

The \( K^D \) transgenes do not support zygotic development

Efforts to construct \( ketel^{null/-} \); \( K^D \) flies, that lack functional \( Ketel \) gene and carry one of the three \( K^D \) transgenes, failed: not a single \( ketel^{null/-} \); \( K^D \) adult emerged among the well over 1000 offspring recovered in the case of each of the three \( K^D \) transgenic lines. The \( ketel^{null/-} \); \( K^D \) zygotes, like the \( ketel^{null/-} \) and the \( Ketel^{P/}\)– ones die during second larval instar. Unlike the \( K^D \) transgenes the \( K^+ \) transgenes, with normal Ketel gene in the transgene, completely rescue lethality associated with loss of Ketel gene function: the \( ketel^{null/-}; K^+ \) flies are fully viable and fertile (Lippai et al., 2000; Tirián et al., 2000). Apparently the \( P446L \) mutation in the \( K^D \) transgenes behaves as the \( Ketel^- \) alleles: when paternally derived it acts as the \( ketel^{null/-} \) recessive zygotic lethal mutations.

The \( P446L \) mutant Ketel protein inhibits formation of intact NE when injected into wild-type cleavage embryos

Cytoplasm of the \( Ketel^D \) eggs is exceedingly toxic: when injected into wild-type cleavage embryos the \( Ketel^D \) egg cytoplasm prevents formation of nuclei at the end of mitosis (Tirián et al., 2000). To determine whether the \( P446L \) importin-\( \beta \) molecules (produced and purified from \( E. coli \) cells) possess the same effect as the \( Ketel^D \) egg cytoplasm, we injected small volumes of the \( P446L \) protein solution – along with a fluorescent nuclear substrate – into wild-type cleavage \( Drosophila \) embryos. As illustrated in Fig. 1A and D, the nuclear substrate entered the cleavage nuclei irrespectively of whether wild-type or \( P446L \) mutant importin-\( \beta \) protein solutions were injected. It is important to note that the \( P446L \) protein did not disrupt the NE. Similarly, ovary extracts of the \( Ketel^{P/}\)+ females did not block accumulation of the fluorescently labeled import substrates into digitonin permeabilized HeLa cells, and nuclei remained intact for at least 4 hours in the presence of mutant \( P446L \) protein (Lippai et al., 2000). During the upcoming mitosis the fluorescent substrate was homogeneously distributed in the egg cytoplasm around the site of injection, which indicated disassembly [that is partial in [Fig. 1.](#)

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**Fig. 1.** Effects of the wild-type (A-C) and the \( P446L \) mutant importin-\( \beta \) (D-F) following their injection, along with a fluorescent nuclear substrate, into wild-type cleavage embryos. Arrows show the site of injection. Import of the fluorescent nuclear substrate into the nuclei was followed in a laser-scanning microscope. The A and D, the B and E and the C and F photographs were taken at roughly identical stages of the cleavage cycles. The few nuclei shown on F appeared following diffusion of the fluorescent substrate away from the site of injection. Bar, 100 \( \mu \)m.
spindles form normally and the spindle elongation and disassembly is not affected. However, the homogenous distribution of the GFP-tubulin (Fig. 3H) indicates that NE failed to assemble since GFP-tubulin is not excluded from the space where nuclei should have formed. Failure of the chromosomes to move apart during interphase is most likely the consequence of the failure of NE formation.

To elucidate the possibility of failure of NE assembly, we conducted two further sets of injection experiments. To test whether or not intact NE forms around the chromatin in the presence of P446L protein, we co-injected a high molecular weight red-fluorescent dextrane with the P446L protein into histone-GFP expressing cleavage embryos. If intact NE is assembled around the chromatin the dextrane is expected to be excluded from the nuclei. If, however, functional NE fails to form around the chromatin, the dextrane is expected to possess an almost homogenous distribution. Following co-injection of wild-type importin-β and the red-fluorescent dextrane, the red and the green (chromatin-derived) signals were clearly separated: the green signal originated from the inside of the interphase cleavage nuclei and the red signal from the cytoplasm, which shows the formation of cleavage nuclei and hence functional NE (Fig. 4A,B). In the case of P446L and red-fluorescent dextrane co-injections the dextrane-derived signal was basically homogeneously distributed (Fig. 4D) even though the histone-GFP highlighted chromatin resembled normal interphase chromatin (compare Fig. 4A,C). Results of the above experiment are in agreement with the failure of functional NE formation in the presence of the P446L mutant importin-β.

To visualize the effect of the P446L-protein-induced NE defect, we injected wild-type (as control) or P446L mutant importin-β solutions into cleavage embryos in which lamin-GFP highlighted the lamin lining of the internal NE surface. Most of the lamin is phosphorylated upon entry into mitosis and the residual lamin-GFP molecules faintly show the so-called spindle envelope. (Cleavage mitosis in Drosophila is an intermediate between closed and open mitosis.) Upon entering the upcoming mitosis the lamin molecules re-enter the nucleus and highlight the NE. It is to be expected that if the P446L mutant molecules prevent NE assembly there will be no lamin-GFP signal outlining the NE at the site of injection. As Fig.

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**Fig. 2.** Effects of P446L mutant importin-β on cleavage chromatin following its injection into wild-type cleavage embryos expressing histone-GFP. Approximately 200 picolitres P446L protein solution (1.2 μM, approximately the endogenous importin-β concentration) was injected into the posterior end of a wild-type cleavage embryo in which histone-GFP highlighted chromatin. Chromatin organization was followed in a laser-scanning microscope. Optical sections from the anterior (A-C) and the posterior (D-F) regions of the same embryo are shown. While the anterior section was devoid of P446L, the P446L mutant protein was present at the posterior region. A and D represent interphase chromatin following P446L protein injection. B and E show segregating chromosomes. C and F show chromatin during the upcoming interphase. Note that the nuclei doubled in number and the chromosomes segregate both at the anterior (control) and posterior (‘experimental’) regions of the embryo. Bar, 20 μm.

**Fig. 3.** Effect of P446L importin-β on cleavage mitotic spindle organization. Wild-type (A-D) or P446L mutant importin-β (E-H) solution was injected into cleavage Drosophila embryos expressing tubulin-GFP fusion protein. While mitotic spindle assembly, elongation and disassembly is not affected by the injected wild-type (A-C) and P446L (E-G) importin-β, the tubulin-GFP protein is homogeneously distributed in the site of P446L injection indicating the failure of NE assembly (H). Tubulin-GFP is excluded from the nuclei and appear as dark holes on the optical sections (D). Bar, 10 μm.
Dominant-negative importin-β mutations

5A-C shows, following the injection of wild-type importin-β the lamin-GFP molecules highlight the NE during the upcoming interphase. Upon entry to mitosis, most lamin molecules diffuse into the cytoplasm and only some remain attached to the spindle envelope (Paddy et al., 1996) (Fig. 5B). Following chromosome segregation the NE reassemble as pictured by formation of the green fluorescent lamin lining (Fig. 5C). When the P446L mutant protein is injected into the lamin-GFP-expressing cleavage embryos, the lamin disappears during mitosis as in the control experiment showing that mitosis is not affected until late anaphase (Fig. 5D-F). In the presence of the P446L molecules, however, the lamin lining never re-forms, which shows the failure of intact NE assembly.

The above injection experiments show that the P446L importin-β molecules interfere with the formation of intact cleavage nuclei and the defect is manifested during the mitosis-to-interphase transition through the prevention of intact NE assembly. The KetelD mutations possess dominant-negative action on NE assembly and impede function of the normal importin-β molecules and thus reveal a novel role of importin-β required during NE formation at the end of mitosis. The novel importin-β function is distinct from both its role in nuclear protein import and the recently described function in mitotic spindle assembly (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001).

The P446L mutant importin-β allows formation and docking on the NE of the import complexes but does not support nuclear protein import

As described earlier, the wild-type importin-β molecules support import of NLS-containing substrates into nuclei of digitonin-permeabilized HeLa cells (Lippai et al., 2000). Interestingly, ovary extracts of the KetelD/+ females, with both wild-type and P446L mutant protein inside, support nuclear protein import as efficiently as ovary extracts of wild-type (+/+ ) females (Lippai et al., 2000). As presented above, when injected into wild-type embryos the P446L mutant protein does not prevent import of a nuclear substrate into the cleavage nuclei. Two feasible possibilities seem to account for the above phenomena: (1) it may be that, although the P446L mutant protein molecules do not participate in nuclear protein import, they do not prevent function of the wild-type importin-β molecules to accomplish their function; or (2) perhaps P446L mutant importin-β supports nuclear protein import. To determine which of these two possibilities is true we analyzed behavior of the P446L mutant protein in the nuclear protein import assay. As illustrated in Fig. 6, in the presence of only the fluorescent nuclear substrate and importin-β the P446L mutant protein, although in reduced amounts, nuclear import complexes form and dock on the cytoplasmic surface of digitonin-permeabilized HeLa cell nuclei (Fig. 6A,C). The higher cytoplasmic background, in the case of P446L, is most likely the consequence of the altered structure of the P446L molecules (see below) leading to association of the import

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**Fig. 4.** Localization of the chromatin (as revealed by GFP-tagged histone; A,C) and the red-fluorescent 170 kDa TRITC-dextrane (B,D) in cleavage embryos injected with wild-type (A,B) or with P446L mutant importin-β (C,D). Following the injection of wild-type importin-β, the TRITC-dextrane is excluded from the nuclei that form following mitosis (B). However, following the injection of P446L, the TRITC-dextrane is not excluded from the region of the chromatin following mitosis (D), an indication of the absence of functional NE. Note that the chromatin morphology is hardly affected (C). Bar, 20 μm.

**Fig. 5.** Cleavage embryos expressing lamin-GFP were injected at the posterior end (arrows) with wild-type (A-C) or P446L mutant importin-β (D-F). A and D show localization of lamin-GFP during interphase following injection. The lamin-GFP molecules highlight the NE. Embryos are in metaphase in B and E, and the spindle envelopes are not affected (B,E). During the upcoming interphase, nuclear lamina re-forms in the embryos that were injected with normal importin-β (C). No nuclear lamina assembles at the site of injection of the P446L mutant importin-β revealing the failure of intact NE formation (F). Bar, 50 μm.
cargo/P446L importin-β with cytoplasmic structures, possibly membranes or microtubules. In the presence of normal importin-β and when further components of nuclear import are added (i.e. Ran, NTF2, RanGAP, RanBP1 and energy supply) the complexes are imported into the nuclei (Fig. 6B). In the case of the P446L mutant protein, however, import complexes do not form (Fig. 6D) as revealed by the absence of fluorescent signal in the HeLa cells.

Results of the above experiments show that the P446L mutant protein does not support nuclear protein import. However, as the above-mentioned injection experiments and nuclear import assays with ovary extracts of Ketel D/+ females revealed (Lippai et al., 2000; Tirián et al., 2000), they do not hinder nuclear import accomplished by the normal importin-β molecules. Apparently effects of the P446L mutant protein are manifested only during the mitosis-to-interphase transition in preventing intact NE formation.

**Fig. 6.** Nuclear import complexes form and dock on the NE of the digitonin-permeabilized HeLa cells following the addition of either wild-type (A) or P446L mutant importin-β (C) in the presence of the fluorescent IBB-nucleoplasmin fusion protein. Upon addition of the import mixture (the fluorescent IBB-nucleoplasmin fusion protein, Ran, NTF2, RanGAP, RanBP1 and energy supply) and the wild-type importin-β, nuclear import complexes form and enter the nuclei (B). However, when P446L mutant importin-β is added along with the import mixture, import complexes do not form and the HeLa cell nuclei are not highlighted by fluorescent signal (D). Bar, 10 μm.

Fig. 7. RanGDP removes higher amounts of importin-β from extracts of Ketel D eggs than from extracts of wild-type (WT) eggs. RanQ69L protein binds higher amounts of importin-β protein from WT egg extract than from extract of Ketel D eggs. GST protein was used as a negative control (A, left). RanQ69L protein removes high amounts of purified WT importin-β but not purified P446L mutant protein. At the same time RanGDP removes higher amounts of purified P446L importin-β compared with the purified WT importin-β (A, right). More Ran is precipitated with the anti-Ketel antibody from extracts of the Ketel D eggs than from extracts of WT eggs. (B, left). However, if an energy-regenerating system and 3 μM (10 times the endogenous importin-β concentration in the extract) purified wild-type or P446L mutant importin-β are added to WT egg extract, more Ran is precipitated from the extract supplemented with WT importin-β (B, right). Wild-type importin-β inhibits both exchange of the labeled GTP from Ran (C) and GTP hydrolysis (D), whereas P446L mutant importin-β has no effect on both nucleotide exchange and GTP hydrolysis. In C, the time course of nucleotide exchange is shown on a semi-logarithmic scale and D shows the results of the reactions performed in duplicate.
The P446L mutant protein loses affinity to RanGTP but binds RanGDP

Changes in the Ran-binding ability of the P446L protein are suggested by the fact that the P446L molecules are unable to accomplish nuclear import protein in the digitonin-permeabilized HeLa cells (Fig. 6D). To examine this possibility, we tested the binding of wild-type and P446L importin-β to different GST-Ran fusion proteins in solution binding assays (Fig. 7A). Apparently, RanGDP binds significantly higher amounts of importin-β from KetelD egg extracts than from wild-type Drosophila egg extracts. Since Ran is mainly in its GDP-bound form in cytoplasmic extracts, the above result suggests increased RanGDP binding affinity of the P446L protein. Conversely, RanQ69L loaded with GTP binds higher amounts of importin-β from wild-type egg extracts than that from KetelD extracts, suggesting reduced binding ability of P446L to RanGTP. As a negative control we used GST protein alone, which showed only background binding levels with both P446L and wild-type importin-β. Since the KetelD egg extracts contain 50% wild-type importin-β, the extracts are not suitable to examine the RanGTP binding ability of the P446L protein. To confirm the reduced affinity of P446L to RanGTP, we measured the amount of the pulled down importin-β proteins from solutions containing purified wild-type or P446L mutant importin-β. As shown in Fig. 7A, wild-type importin-β binds strongly to RanQ69L, but the P446L protein shows only background binding to RanQ69L. (RanQ69L is a GTP-loaded GTPase deficient mutant Ran protein.)

To support the altered binding of P446L to Ran, we carried out immunoprecipitations with the polyclonal anti-Ketel antibody. The amount of precipitated endogenous Ran was higher from KetelD egg extracts compared with wild-type egg extracts. However, if an energy regenerating system and purified wild-type or P446L importin-β is added to the wild-type extract, more Ran is precipitated from the extract supplemented with the wild-type importin-β (Fig. 7B). The shift in Ran binding ability following addition of an energy supply correlates with the ability of wild-type importin-β to bind RanGTP and the inability of P446L to do so. Results of the described experiments are further supported by the enzyme assays described below.

Importin-β has been known to inhibit both GTP hydrolysis on Ran and the exchange of RanGTP catalyzed by RCC1. We studied, in solutions, the effects of the purified importin-β and P446L proteins on both nucleotide exchange and GTP hydrolysis on Ran. The wild-type Ketel protein inhibits both GTP nucleotide exchange and GTP hydrolysis, whereas the KetelD encoded protein has no effect on the processes (Fig. 7C,D), which shows that the P446L mutant protein cannot bind to RanGTP. Neither wild-type nor P446L have significant effect on nucleotide exchange from RanGDP (data not shown). In conclusion, the failure of functional NE formation may be the consequence of the altered RanGTP binding ability of the P446L mutant importin-β.

The P446L mutation appears to increase helix content and reduce flexibility of the encoded protein

As described above, the C4114→T transition leads to replacement of a helix-breaking Pro by Leu in position 446. It may be that in the P446L mutant protein the Pro446→Leu exchange leads to fusion of two adjacent helices, namely the B helix of HEAT repeat 10 and the short helix in the linker region towards helix A of HEAT repeat 11. To test whether helix content of the P446L molecules is indeed increased compared with the wild-type importin-β molecules, we synthesized two model peptides representing the noteworthy region in importin-β. We then carried out CD spectroscopy of the peptides that were dissolved in the apolar solvent trifluoro-ethanol (TFE) or in mixtures of TFE and water (Fig. 8). CD spectroscopy has been known to be a sensitive technique to analyze protein structures: CD spectra recorded in different polarity molecular environments (i.e. in solvents with different dielectric constant) often reveals structural changes (Pericz et al., 1991; Pericz et al., 1992).

In 100% TFE, which provides low polarity and a membrane mimicking environment, both model peptides resulted in so-called C-type CD spectra, which refers to a mixture of β-turns (type I or III) and α- (or 310) helices (Pericz et al., 1991). Ordered structure of the peptides is revealed by (1) the presence of the two shoulders in the spectra at 209 and 224 nm (the π* and nπ* transitions, respectively) and also by (2) reaching the 209 nm shoulder lower θ values as the 224 nm shoulder (Fig. 8). CD spectra of the Pro-containing peptide – representative of the wild-type importin-β – reveal high α-helix content in both TFE and mixtures of TFE and water and the spectra changed significantly along with increase in water content of the molecular environment, reflecting flexibility of the peptide (Fig. 8A). Results of CD spectroscopy are in line with the previously published 3D structure of the aforementioned segment of importin-β (Cingolani et al., 1999; Lee et al., 2000) and are illustrated in Fig. 9B: amino acids that comprise HEAT repeats in 10B and 11A form α-helices and are interconnected with a β-turn. There is a short, three amino acid α-helix starting with Pro446 in the turn region adjacent to HEAT repeat 10B.

![Fig. 8. CD spectra of model peptides representing wild-type (A) and P446L mutant importin-β (B). The CD spectra were recorded in 100% trifluoro-ethanol (TFE, continuous lines), in a mixture of 66% TFE and 33% H2O (dashed lines), and in 33% TFE and 66% H2O (dotted lines).](image-url)
In contrast to the Pro-containing peptide, CD spectra of the Leu-containing model peptide, representing the P446L mutant protein, remained essentially unchanged on increasing the water content (i.e. the dielectric value) of the medium, which shows that the Leu-containing peptide, while keeping its predominantly helical organization, loses flexibility compared with the Pro-containing peptide (Fig. 8B). It appears that in the presence of water, helices of the Pro-containing peptide undergo a limited untwisting and/or rearrangement of the helix-turn-helix conformation and the Pro$\rightarrow$Leu exchange results in a mutant molecule that lost flexibility. An illustration of the altered molecular structure is shown in Fig. 9D, considering that the Pro$\rightarrow$Leu exchange increased $\alpha$-helix content of the peptide.

Computer modeling of the importin-$\beta$ and the P446L mutant importin-$\beta$ proteins

Based on results of the formerly described CD spectroscopy, we carried out computer 3D modeling of both the normal and the P446L mutant importin-$\beta$s. We took X-ray crystallographic data of human importin-$\beta$ associated with the IBB domain of importin-$\alpha$. [For atomic coordinates of importin-$\beta$ see access code 1QGK (Cingolani et al., 1999).] We assume that the human importin-$\beta$ 3D data used for the modeling is adequate because: (1) the size of human and Drosophila importin-$\beta$s are similar: 876 and 884 amino acids; (2) amino acid sequences of human and Drosophila importin-$\beta$s share 60% amino acid identity and 78% similarity (Lippai et al., 2000); and (3) The 10B HEAT repeat–linker–11A HEAT repeat region is evolutionary highly conserved: the amino acid identity and similarity are 64.1% and 92.3% (Table 3). Pro446 resides in the linker region between the B helix of HEAT repeat 10 and the A helix of HEAT repeat 11 at the beginning of a small 3-amino-acid-long $\alpha$-helix after HEAT repeat 10 (Fig. 9) (Cingolani et al., 1999). CD spectra of model peptides representing the wild-type and the P446L mutant proteins reveal loss of flexibility upon Pro446$\rightarrow$Leu replacement and the lost flexibility is most likely the consequence of the B helix of HEAT repeat 10 fusing with the short $\alpha$ helix in the linker region. Changing the $\Psi$ and $\Phi$ angles of Leu439 and Leu440 such that the two amino acids fit into a fused $\alpha$-helix, an open molecular conformation emerges (Fig. 9). As a consequence of opening the Spanish collar, the hydrophobic internal surface of the molecule becomes exposed to water. The consequence of the amino acid exchange may be less dramatic in the whole molecule due to its flexibility, but a significant conformational change is supported by the more hydrophobic behavior of the P446L mutant protein (i.e. it was much more difficult to express and purify compared with normal importin-$\beta$), and by the fact that the exchange of a distantly located residue suppresses the mutant defects caused by the P446L protein (see below).

S317T is an intragenic dominant suppressor mutation of P446L and restores Ketel gene function

Following molecular analysis of the ketel$^{R}$ alleles, which are revertant alleles of the Ketel$^{D}$ mutations and were induced

<table>
<thead>
<tr>
<th>Species</th>
<th>Helix A of HEAT 10</th>
<th>Linker</th>
<th>Helix B of HEAT 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>VVVRDTAATVGRICELLPEAIAINDYVAPLLQCLIEGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>VVVRDTAATVGRICELLPEAIAINDYVAPLLQCLIEGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>VVVRDTAATVGRICELLPEAIAINDYVAPLLQCLIEGL</td>
<td></td>
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</tbody>
</table>

Pro446, which is replaced by Leu in the P446L mutant protein, is highlighted in bold. The identity and the similarity levels for the depicted region between human and Drosophila are 24/39 and 36/39. The sources are as follows: human (Girlich et al., 1995), accession L38951, NID G893287; rat (Radu et al., 1995), accession L38644; Drosophila (Lippai et al., 2000), accession number AJ002729.
through second mutagenesis (Szabad et al., 1989; Erdélyi et al., 1997), we identified S317T, which is an intragenic dominant suppressor mutation of P446L. The S317T mutation originated through a single T→A transversion in the 3656th position and led to replacement of Ser317 by threonine. We generated five transgenic lines (labeled K317T&P446L), which included both the S317T and the P446L mutations. The +/+; K317T&P446L females with two wild-type Ketel alleles (+) and with the K317T&P446L transgene are fully fertile, which suggests that the S317T mutation annulled the P446L-imposed dominant female sterility. That the K317T&P446L transgene does indeed restore Ketel gene function is best shown by the fact that the kete111777; K317T&P446L zygotes are fully viable and fertile.

Discussion
Proline 446 is a functionally important residue in importin-β
The fact that in three of the four independently isolated Ketel<sup>D</sup> mutations the same C→T transition and the concurrent replacement of Pro446 by Leu is the basis of dominant female sterility underlines the importance of Pro446 in importin-β function. It is assumed that during a nuclear import cycle, whereas importin-β interacts with the NLS containing protein (directly or through importin-β), nucleoporins and RanGTP, the conformation of importin-β changes significantly (Vetter et al., 1999; Lee et al., 2000). In fact the region around HEAT repeat 10 was suggested, based on X-ray crystallography, to be a flexible point during switching between the IBB- and the Ran-bound forms. Pro446 resides in the linker region connecting HEAT repeats 10 and 11 and, as described here, plays a crucial role in enduring flexibility of importin-β. CD spectra of model peptides representing the wild-type and the P446L mutant proteins reveal loss of flexibility upon Pro446→Leu replacement. The lost flexibility is most likely the consequence of fusion of the small α-helix in the linker region with the α-helix of HEAT 10B. Computer 3D modeling of the P446L protein structure, based on results of CD spectroscopy, shows altered molecular structure: the P446L molecule takes on an open conformation such that its inner hydrophobic surface becomes exposed to water (compare Fig. 9B and D), explaining the reduced hydrophilic nature of the P446L protein.

The significant conformational change due to the exchange of Pro446 to Leu in the Ketel protein is further supported by the S317T suppressor mutation that restores Ketel gene function. In human importin-β the corresponding Ser311 (in the linker region between HEAT repeats 7 and 8) and Pro441 are 32.5 Å apart and yet the Ser→Thr exchange in the Drosophila homologue restores function of importin-β (Fig. 9). The 10 Å area surrounding serine is hydrophobic. The stronger hydrophobicity of threonine compared with serine does perhaps increase apolar interactions and bend the molecule back to its functional structure.

The P446L mutation changes Ran binding ability
Experiments with digitonin-permeabilized HeLa cells show that, to a reduced extent, the P446L proteins do participate in formation of the nuclear import complexes and in their docking to the cytoplasmic surface of the NE; however, they do not support import of the complexes into the nuclei in the presence of Ran, energy source, RanGAP and RanBP1. In fact, the import complexes do not form upon the addition of the latter components. Apparently the main structural domains of the P446L protein are intact (binds importin-α, NPC and Ran) but the interaction with Ran is altered. Indeed, we found that the binding of wild-type and P446L Ketel proteins to Ran are very different: the P446L cannot bind to RanGTP, to which the wild-type importin-β binds strongly, but shows elevated affinity to RanGDP, to which the wild-type protein shows very little affinity. It is noteworthy that a single amino acid exchange outside the classical Ran-binding domain can change Ran binding ability dramatically. The change in Ran-binding ability is most likely the source of the Ketel<sup>D</sup>-associated dominant female sterility. However, the Ketel<sup>D</sup>-associated dominant-negative effect is not manifested via nuclear protein import but rather through the prevention of cleavage nuclei formation: revealing a novel importin-β function.

The P446L mutant importin-β exerts its toxic effect at the end of mitosis
The injection experiments into wild-type cleavage embryos revealed that the P446L mutant protein does not inhibit nuclear protein import: when co-injected with P446L, a fluorescent nuclear substrate readily entered the nuclei. Furthermore, although the cleavage nuclei enter mitosis and the chromosomes segregate normally, intact NE never forms in the presence of P446L mutant importin-β. Failure of NE assembly in the presence of P446L is revealed by the following observations. First, the homogenous distribution of (1) a fluorescent nuclear substrate; (2) the high molecular weight dextrane; and (3) the GFP-tubulin. Second, the absence of the nuclear lamina lining. Thus the mutant P446L importin-β reveals a novel importin-β function required during the mitosis-to-interphase transition, a function distinct from the already known functions of importin-β in nuclear protein import and in mitotic spindle assembly (Görlich and Kutay, 1999; Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001).

The P446L mutant importin-β possesses altered Ran-binding properties: it does not bind RanGTP but shows elevated affinity to RanGDP. A series of experiments showed that altered RanGTP-RanGDP balance leads to a similar phenotype in yeast (i.e. arrest in mitosis-to-interphase transition) (Sazer and Nurse, 1994; He et al., 1998). Results of enzyme assays described in the present paper show that the altered Ran-binding ability of P446L importin-β does not interfere with the GTP hydrolysis and nucleotide exchange on Ran and thus it is unlikely that the Ketel<sup>D</sup>-related defects are consequences of distorted Ran metabolism. Most probably importin-β is a downstream effector of Ran during mitosis-to-interphase transition, as in nuclear protein import and mitotic spindle assembly.

Although several functions of Ran and importin-β during the cell cycle were described, the exact molecular mechanisms are still missing. Here we describe a novel function of Drosophila importin-β during mitosis-to-interphase transition where it is involved in the formation of intact NE. There seem to be three feasible explanations for the P446L-associated defects. First,
since the P446L importin-β shows higher affinity to RanGDP than wild-type importin-β, a possible explanation may be the depletion of significant amounts of RanGDP that is required for NE reassembly at the end of mitosis (Zhang and Clarke, 2000; Hetzer et al., 2000). Removal of RanGDP by P446L may lead to the failure of cleavage nucleus formation. We do not think this explanation is very likely for the following reasons. (1) Binding and nucleotide exchange assays revealed that the affinity of the P446L to RanGDP is low and hence depletion of a significant fraction of Ran from the cytoplasm is rather unlikely. (2) Interestingly, defects do not evolve in nuclear protein import or in spindle formation and chromosome segregation following injection of P446L despite the fact that both nuclear protein import and spindle formation have been shown to be Ran dependent. Ran’s involvement in NE assembly has also been described but since none of the aforementioned Ran-related processes were disturbed, the P446L protein does not seem to disturb the Ran cycle. A second possible explanation of the P446L-related defects is perhaps the inability of the P446L protein to bind RanGTP and, consequently, the inability to release factors required for proper chromatin decondensation and/or NE assembly. In this case the role of importin-β in the above processes would resemble its function in mitotic spindle formation, where it is thought to be required for the release of factors needed for spindle assembly [e.g. NuMA, TPX2 (Wiese et al., 2001; Nachury et al., 2001; Gruss et al., 2001)]. A third possibility is that the P446L-related defects are not associated with the change in Ran-binding ability. The P446L mutation may disturb the association of thus far unidentified factors (e.g. nucleoporins). In the case of the second and third possibilities the factor(s) required for the newly described importin-β-related functions remain to be identified.

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References
Dominant-negative importin-β mutations


