Distribution and functions of kinectin isoforms

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Summary
Kinectin is an integral transmembrane protein on the endoplasmic reticulum, binding to kinesin, interacting with Rho GTPase and anchoring the translation elongation factor-1 complex. There has been debate on the specific role(s) of kinectin in different species and cell types. Here we identified 15 novel kinectin isoforms in the mouse nervous system, constituting a family of alternatively spliced carboxyl-terminal variants. Isoform expression is subject to cell type- and developmental stage-specific regulation. We raised specific antibodies to the kinectin variants to characterise their differential intracellular localisation and discovered that certain kinectin isoforms are found in axons where kinectin was previously believed to be absent. We also demonstrated in vivo by overexpression and RNA interference assay that kinectin is selectively involved in the transport of specific types of organelles. A 160 kDa kinectin species is mainly concentrated in the endoplasmic reticulum, anchored via its transmembrane domain and is essential for endoplasmic reticulum membrane extension. A 120 kDa kinectin species is specifically associated with mitochondria, and its interaction with kinesin was found to influence mitochondrial dynamics. These findings contribute to a more unified view of kinectin function. They suggest that different cellular processes use specific kinectin isoforms to mediate intracellular motility and targeting by transient interaction with different motor proteins or other binding partners.

Key words: Kinesin receptor, Intracellular motility, Alternative splicing, Microtubule-mediated transport

Introduction
Diverse processes in eukaryotic cells, such as fast axonal transport, intracellular bidirectional transport of organelles between membrane compartments, morphogenesis of the endoplasmic reticulum (ER) membrane and force generation on the mitotic spindle, are microtubule (MT) based and mediated by motor proteins (for reviews, see Bloom and Goldstein, 1998; Hirokawa, 1998; Vale, 1999; Allan and Schroer, 1999; Goldstein and Yang, 2000; Karsenti and Vernos, 2001). Biochemical studies and cDNA cloning from different organisms have revealed the universal existence of motor protein superfamilies, which are broadly classified as kinesin and kinesin-like proteins (KLPs; mainly anterograde movement), and cytoplasmic dynein and related proteins (retrograde movement) (Bloom and Endow, 1995; Allan and Schroer, 1999).

Cargo binding of molecular motors by interaction with membrane anchors was first suggested by the discovery that the C-terminal tail of kinesin interacts with the 160 kDa integral ER membrane protein kinectin (Toyoshima et al., 1992). Based on its predicted amino acid sequence, the full-length kinectin comprises a short N-terminal domain embedded in the ER lumen, an adjacent single transmembrane region spanning the ER membrane and a large C-terminal coiled-coil-forming cytoplasmic part. A truncated 120 kDa kinectin species, lacking the initial 232 N-terminal residues, is still associated with membranes, probably via lipid linkages (Kumar et al., 1998). Kinectin is thought to exist as a dimer through coiled-coil interactions of the cytoplasmic tail (Yu et al., 1995; Futterer et al., 1995; Toyoshima and Sheetz, 1996). An antibody binding to the C-terminal domain of kinectin blocks motor binding to microsomes and dramatically reduces kinesin- and dynein-mediated organelle motility (Kumar et al., 1995). This led to the concept of kinectin as a potential regulator of organelle motility rather than a mere membrane anchor for kinesin (Sheetz, 1996). In yeast two-hybrid screens and in vitro peptide binding assays, kinectin interacts with small G proteins (Hotta et al., 1996; Alberts et al., 1998), putative regulators of MT-based organelle transport (Fullerton et al., 1998). Recently, Ong et al. (Ong et al., 2000) have corroborated the role of kinectin as a motor protein receptor by delineating the kinesin-interacting domain in the C-terminus of kinectin with yeast two-hybrid mapping, co-selection experiments and in vivo co-immunoprecipitation. This study demonstrated the effect of kinectin-kinesin interaction on lysosome dynamics and observed that the kinesin-binding domain of kinectin can significantly enhance the MT-stimulated ATPase activity of kinesin.

Despite such progress, our understanding of the kinectin function in organelle motility or intracellular targeting remains sketchy and controversial. Kinectin was not detected in axons of cultured neurons where kinesin is the major motor
responsible for fast anterograde transport (Toyoshima and Sheetz, 1996). Furthermore, the kinectin gene is not found in Caenorhabditis or Drosophila genomes, despite the presence of the conserved conventional kinesin heavy chain gene (Yang et al., 1989; Goldstein and Gunawardena, 2000). Additional membrane anchors of motor proteins have been discovered (Bowman et al., 2000; Verhey et al., 2001; Dorner et al., 1998; Kamal et al., 2000; Nakagawa et al., 2000; Takeda et al., 2000; Setou et al., 2000) and kinectin-deficient mice exhibited no defects in organelle motility (Plitz and Pfeffer, 2001).

Apparent contradictions surrounding kinectin might find their explanation in functional complementarity and redundancy of different motor proteins. A given cargo may be transported by different motors of the same directionality, as has been shown for KLP-mediated transport of mouse mitochondria (Nangaku et al., 1994; Tanaka et al., 1998).

Conversely, organelles can use motors with opposite directionality at different times, as was demonstrated for the transport of lysosomes (Oda et al., 1995; Nakata and Hirokawa, 1995). The emerging, more encompassing view recognises the role of integral organelle membrane proteins, organelle coat proteins, scaffold proteins and small GTPases as organelle-specific effectors of transport. Their function as binding and/or motility regulators and their interaction with specific binding partners may be under the influence of cellular microenvironments that may adjust binding and motility of activated motor complexes via reversible phosphorylation and other mechanisms (Okada et al., 1995; Lindersmith et al., 1995; Sheetz, 1999; Manning and Snyder, 2000; Klopfenstein et al., 2000; Gross, 2003). Such dynamic modularity of the transport machinery implies the presence of transport components with overlapping roles and may explain how lysosome and phagosome function can be maintained when kinectin is knocked out (Plitz and Pfeffer, 2001).

There is preliminary evidence to suggest the existence of different kinectin isoforms. In both chicken and human, at least two alternatively spliced forms have been detected (the N-terminally truncated species of 120 kDa being the most prominent), although formal proof of their origin and/or identity is still lacking (Yu et al., 1995; Futterer et al., 1995; Kumar et al., 1998). Five partial kinectin splice variants with alternative C-termini in spleen and testis were identified in mouse (Leung et al., 1996) and at least two isoforms have been observed in different fox tissues (Xu et al., 2002). Characterising the function and distribution of different kinectin isoforms will provide an insight into their individual roles and is paramount to our understanding of kinectin as a component of the transport machinery.

In this study we have identified 15 kinectin isoforms, constituting a family of kinectin-like proteins. This complexity appears to parallel the complexity of the previously described kinesin-like family of proteins in any given cell type/tissue. The identified kinectins are alternatively spliced isoforms with variable C-termini, isolated from mouse nerve tissue (embryonic/adult hippocampus and from cultured astrocytes). The expression of these kinectin isoforms was cell type-specific and subject to developmental regulation in the nervous system, with ubiquitous expression of at least one isoform in all mouse tissues tested. The intracellular localisation of isoforms also appears to be differential with some isoforms localized in the axon where kinectin was previously believed to be absent (Toyoshima and Sheetz, 1996). We demonstrated in vivo the role of the N-terminal domain of 160 kDa kinectin as an ER membrane anchor and showed a differential intracellular distribution of the 160 kDa and 120 kDa species, with the latter specifically associated with mitochondria. We examined the specific role of the kinectin-kinesin interaction in kinesin-driven motility by transient overexpression and RNA interference approaches and demonstrated that it is involved in ER extension and the translocation of mitochondria. These new data contribute to a more unified understanding of the functions and variation of kinectins intracellularly.

Materials and Methods

Cell cultures

Astrocyte primary cultures (see Levison and McCarthy, 1991) were derived from newborn mice and maintained in modified Eagle’s medium (MEM), containing 10% (v/v) horse serum, 2 mM glutamine and 1 ng/ml fibroblast growth factor at 37°C in 5% CO2. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2. PC12 cells were cultured in DMEM, containing 10% (v/v) horse serum and 5% (v/v) FBS at 37°C in 5% CO2. The neuronal phenotype was induced with nerve growth factor at 20 ng/ml.

Oligonucleotides

The following oligonucleotides were used: V0up, 5’TAAAGGAGTTGCACATCAGAGTTG-3’ and V0do, 5’TGAAGGTTGCTTACAGAGTTG-3’ for the simultaneous co-amplification of the entire variable C-terminal domain of all kinectin isoforms; V1up, 5’TGAACAGGAAATAGAGGT-3’ and V1do, 5’TCCAAGCTGGCGACTTCCA-T-3’ for the specific amplification of the C-terminal variable insert V1; V2up, 5’TGGAGGAAAACACTGGGAAG-3’ and V2do, 5’GTTCTGTGTTGACTTCCGTCCTG-3’ for the specific amplification of the C-terminal variable insert V2; V3up, 5’CAGTCTTCCATTTCTCAACGCTCTC-3’ and V3do, 5’ATTCTCTCATTTCTGTATT-3’ for the specific amplification of the C-terminal variable insert V3; V4up, 5’GCTGAAAGATCTGGTGACTG-3’ and V4do, 5’CTCTTCATCTGCTTACTG-3’ for the specific amplification of the C-terminal variable insert V4; V5up, 5’TCCGGAGGAGGAGGATGG-3’ and V5do, 5’ATTGTTGGTGGGATGATGTTGATT-3’ for the specific amplification of the C-terminal variable insert V5.

RNA extraction, RT-PCR for the identification of kinectin isoforms and diagnostic PCR

Polyadenylated RNA (polyA+ RNA) was affinity-purified from mouse astrocyte cultures (2x106 cells) or from isolated hippocampi (2–4 organs) at distinct stages of development, i.e. E15 embryos and adult mice (3 months old), using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). cDNA was synthesised with MMLV reverse transcriptase using equivalent amounts of polyA+ RNA from each source (10 ng), with the Advantage RT-for-PCR kit (Clontech).

Polymerase chain reactions (PCR) for the simultaneous co-amplification of the entire variable domain of putative kinectin isoforms were carried out using oligo pair V0up/V0do with the Expand Long Template polymerase system (Roche) and the following protocol: 2 minutes denaturation of template at 94°C, 33 cycles of a 10 second denaturation at 94°C, a 30 second annealing at 55°C and an extension of 3 minutes at 68°C, followed by a final 5 minute extension at 68°C. PCR products were cloned into vector pCR2.1 (T/A cloning kit; Invitrogen) and used for bacterial transformation. For the assessment of variable domain size in the clones, diagnostic PCRs were set up using AmpliTaq polymerase (Perkin Elmer) with selected oligo pairs and the same protocol as before, except that
extension was carried out at 72°C. Selected clones that generated PCR products of distinct size were sequenced on both strands with an ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Kit and an ABI PRISM® 377 DNA sequencer (DNA sequencing service; National University Medical Institutes). Standard molecular biology techniques were performed according to Sambrook and Russel (Sambrook and Russel, 2001).

Generation of kinectin and kinesin constructs
Cloning strategy was based on GenBank accession numbers Z22551 for human kinectin cDNA and X65873 for human ubiquitous kinesin heavy chain (uKHC) cDNA. ‘Variable inserts’ V1-6 in human kinectin form the ‘variable’ C-terminal domain and represent residues: 831-853 (insert V1); 894-911 (insert V6); 1030-1058 (insert V2); 1177-1200 (insert V3); 1229-1256 (insert V4); and 1316-1346 (insert V5). Kinesin domains KHC+ (residues 833-900), KHC- (735-801) and kinecin domains KN+ (1188-1288, containing variable inserts V3 and V4), KN- (1049-1146 without variable domain sequences), transmembrane (TM) domain (1-445) and non-TM domain (80-1356) were individually subcloned into pEGFP-C1 (Clontech) to generate N-terminally GFP-tagged constructs. (These constructs are shown later in Fig. 3.)

Transient transfections
HeLa cells were at least 60% confluent for transfection. Plasmid DNA (1.5 µg in TransFast transfection reagent, Promega) was added at 1:1 v/v ratio into OptiMEM reduced serum medium (Life Technologies) and incubated at room temperature for 5-10 minutes. The cell culture medium was removed and replaced by the transfection solution for 1 hour at 37°C, incubated by complete medium. Microscopic evaluation of the morphology and organisation of ER and mitochondria was carried out 48 hours post-transfection.

Isolation of mitochondria
HeP2 cells were harvested by trypsinisation, transferred to 4°C, washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold mitochondria lysis buffer (MB; 10 mM HEPES, 220 mM mannitol, 70 mM sucrose and 1 mM EDTA). The cell pellet was washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold mitochondria lysis buffer (MB; 10 mM HEPES, 220 mM mannitol, 70 mM sucrose and 1 mM EDTA). The cell pellet was centrifuged (3000 g, 15 minutes, 4°C), followed by pelleting of mitochondria (13,000 g, 15 minutes, 4°C). A portion of the supernatant, collected from the 3000 g spin, was saved as the post-nuclear supernatant fraction for SDS-PAGE analysis. The crude mitochondrial pellet obtained was resuspended in 0.9 volumes of MB, incubated on ice for 30 minutes and homogenized (100 strokes) with a pestle (2B; Kontes). Unbroken cells and nuclei were removed by centrifugation (3000 g, 10 minutes, 4°C), followed by pelleting of mitochondria (13,000 g, 15 minutes, 4°C). A portion of the supernatant, collected from the 3000 g spin, was saved as the post-nuclear supernatant fraction for SDS-PAGE analysis.

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Heat shock treatment and staining of mitochondria with Mitotracker
HeLa cells were transfected with GFP-tagged constructs at least 36 hours prior to heat shock treatment. For heat shock treatment, fresh culture medium supplemented with 10 mM HEPES was added and plates were incubated at 45°C for 15 minutes. One coverslip with live cells was then rinsed in warm DMEM and transferred to DMEM containing 250 mM Mitotracker Red (Molecular Probes), labelled for 15 minutes at 37°C, rinsed in warm DMEM three times and fluorescence was observed to check for disruption of mitochondrial organisation. The rest of the coverslips were incubated at 37°C for overnight recovery and then labelled with a mitochondria-specific antibody to assess recovery of mitochondrial distribution.

Western blot analysis
Proteins were transferred to PVDF membranes by wet electroblotting and processed for ECL detection (Amersham Pharmacia Biotech) with HRP-conjugated anti-mouse/rabbit IgG, as appropriate (1:2000; Amersham Pharmacia Biotech).

RNA interference (RNAi) assay
RNAi was carried out using the pSilencer™ 2.1-1 U6 hygro vector (Ambion) to express the relevant hairpin double-stranded sequence from the U6 RNA polymerase III promoter. A pair of oligonucleotides, targeting nucleotides 726-745 of human kinectin sequence Z22551, was designed (upstream 5’-GATCCCCGTGGTTCTCTGGATGATGAACCCCTCTCAGGAGC-3’ and downstream 5’-AGCTTTTCAAAATTGCTTCGATGATGACCCCTCTCAGGAGGAGCGAGAGGGA-3’ and its specificity was confirmed by a BLAST search against the NCBI databases. The pair of oligonucleotides was annealed and cloned into pSilencer vector. Transfection (TransFast transfection reagent, Promega) was then carried out on HeLa cells. Stable kinectin knockdown clones were generated by selecting the transfected HeLa cells with hygromycin. Untransfected HeLa and stable cells harbouring empty pSilencer vector were used as controls.

cDNAs from stable clones were reverse transcribed from RNA as described in the previous section using the pair of oligonucleotides flanking the targeting region. G3PDH primers were used as internal controls. For the preparation of protein extracts, HeLa cells were lysed using RIPA buffer (Futterer et al., 1995) supplemented with protease inhibitors. 10 µg of proteins were resolved by SDS-PAGE on 10% gels and followed by western blotting for detection of kinectin using CT-1 monoclonal antibody. Anti-actin polyclonal antibody (Sigma) was used as a loading control.

Polycional antibody production
Polyclonal antibodies against mouse kinectin insert V2 (mIn2) (residues 989-1018) (CDLREKNNWAMEALASTEKMLQDRVNKT-SK, N-terminally conjugated to KLH) and mouse kinecin insert V3 (mIn3) (residues 1136-1160) (CMQSSFTASERECLRQENKDMEN, N-terminally conjugated to KLH), were generated commercially (Genemed Synthesis, Inc.). Antibodies were affinity-purified using glutathione S-transferase fusions with mIn2 or mIn3 proteins, coupled to cyanoen bromide-activated sepharose 4B.

Antibodies
Mitochondria were specifically labelled using anti-Mn superoxide dismutase polyclonal antibody (anti-Mn SOD; StressGen Biotechnologies Corp) at 1:500 (immunofluorescence) or 1:2500 dilution (western blots). ER was detected with anti-calreticulin (StressGen Biotechnologies Corp) at 1:200 (immunofluorescence) or 1:1000 (western blots) and anti-protein disulfide isomerase monoclonal antibodies (anti-PDI; StressGen Biotechnologies Corp) at 1:200 for immunofluorescence. Microtubules were labelled using
anti-β-tubulin monoclonal antibody (mAb) (Sigma) at 1:1000 for immunofluorescence.

Kinectin was detected using CT-1 monoclonal antibody raised against the C-terminus of human kinectin (a kind gift from Dr Martin Krönke, University of Cologne, Cologne, Germany) at 1:2 dilution and also polyclonal anti-mouse insert V2 antibody (mIn2) at 1:25 dilution and polyclonal anti-mouse insert V3 antibody (mIn3) at 1:25 dilution for immunofluorescence. For western blots, monoclonal antibody CT-1 and anti-mIn2 were used at 1:2 and 1:100 dilutions, respectively.

Immunolabelling of cultured cells
Cells grown on coverslips were rinsed in warm PBS, fixed with 3.7% paraformaldehyde and permeabilized with 1% and 0.1% Triton X-100 for mitochondria and ER staining, respectively. Fixed cells were blocked with 10% FBS in PBS, incubated with appropriate primary antibodies for 2 hours, washed three times over 15 minutes with blocking buffer and an appropriate secondary antibody, i.e. TRITC-conjugated goat anti-rabbit/anti-mouse IgG (Sigma), at 1:150 dilution for 1 hour. Coverslips were washed extensively with blocking buffer and mounted in FluorSave™ (Calbiochem) for microscopic examination.

Confocal microscopy
Cells were observed with a Zeiss LSM510 confocal microscope. An excitation wavelength of 458 nm and an LP590 nm emission filter were used for the visualisation of Mitotracker Red and TRITC and an excitation wavelength of 488 nm and emission filter BP515-525 nm were used for the visualisation of GFP fusion proteins. An excitation wavelength of 543 nm and an emission filter were used for the visualisation of DAPI. The microscope. An excitation wavelength of 405 nm and BP420-480 nm were used for the visualisation of DAPI. The microscope. An excitation wavelength of 405 nm and BP420-480 nm were used for the visualisation of DAPI.

Results
Identification of a family of kinectin isoforms with C-terminal sequence variation
We first tested the hypothesis that kinectin C-terminus variants might impart selectivity to different intracellular functions in the nervous system where kinectin functions have been rigorously debated. Previous analysis of kinectin cDNAs in some species had suggested the existence of further kinectin isoforms.

In order to identify and analyse potential novel kinectin cDNAs expressed in the nervous system, an RT-PCR strategy was designed attempting to co-amplify multiple isoforms simultaneously. The strategy employed a pair of oligonucleotide pairs to conserved sequences flanking the entire variable C-terminal domain of mouse kinectin (Fig. 1A: nucleotides 2407-4182 of mouse kinectin L43326). RT-PCR of isolated mouse embryonic E15 hippocampal mRNA resulted in the simultaneous amplification of several products, ranging in size between 1.3-1.7 kbp (at least five distinct, closely migrating bands can be resolved in Fig. 1B,arrowheads). This mixed product was subcloned and 100 recombinant clones were subjected to diagnostic PCR with the same pair of oligonucleotides, to distinguish clones with different insert sizes. To differentiate clones with apparently identical insert sizes, these were further analysed with pairs of oligonucleotides specific for each of the variable domain inserts. This analysis revealed eight distinct products (Fig. 1C), corresponding to new kinectin isoforms with different combinations of the C-terminal variable domain ‘inserts’ or ‘exons’ 1-5 (see Leung et al., 1996). One isoform contained no variable inserts (mKNT1) and the other seven comprised different combinations of one to three variable inserts; the exact permutations of variable domain inserts are given in Table 1.
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To determine whether the expression of the kinectin gene and its post-transcriptional processing are subject to developmental regulation, the RT-PCR strategy described in the previous section was used with adult mouse hippocampal mRNA. The mixed PCR product obtained (not shown) was subcloned and 75 recombinant clones were analysed for different electrophoretic mobility or variable insert combinations, following a second, diagnostic PCR. This led to the identification of six distinct kinectin isoforms from adult mouse hippocampus (Fig. 1C). Three of these were common with developmental stage E15, specifically mKNT1, containing no variable inserts, mKNT11, including variable insert V5 and mKNT12 with variable inserts V2 and V3. The other three corresponded to novel, previously unknown isoforms comprising one or three insert permutations (Table 1).

We examined with the same experimental methodology whether the pattern of kinectin isoform expression also exhibited cell-type specific variability of expression. Six distinct kinectin isoforms were identified out of 80 recombinant clones derived from pure glial astrocyte cultures established from newborn mice (Fig. 1C). Two of the glial isoforms were common with E15 hippocampal stage (including the non-variable insert isoform mKNT1) and four were additional novel isoforms with combinations of two, three and four variable inserts, respectively (Table 1).

In total, our results revealed the existence of 15 kinectin isoforms in the mouse nervous system, containing 11 entirely novel and four isoforms previously tentatively reported in other mouse tissues (testis and spleen) (Leung et al., 1996). This brings the current number of kinectin isoforms known in mouse to 16, constituting the ‘kinectin-like family of isoforms’ (Table 1).

### Table 1. Novel kinectin isoforms in distinct cell types and developmental stages in mouse hippocampus

| Isoform  | E15 embryonic hippocampus | Adult hippocampus | Glia (astrocytes) | Spleen/Testis* | Variable domain inserts (V1-V5) |
|----------|--------------------------|-------------------|------------------|---------------|---------------------------------|
| mKNT1    | 1                        | 1                 | 1                | 1             | None                            |
| mKNT2    | 1                        | 1                 | 1                | 1             | V4                              |
| mKNT3    | 1                        | 1                 | 1                | 1             | V3, V5                          |
| mKNT4    | 1                        | 1                 | 1                | 1             | V1, V3, V4                      |
| mKNT5    | 1                        | 1                 | 1                | 1             | V1, V3                          |
| mKNT6    | 1                        | 1                 | 1                | 1             | V2, V4, V5                      |
| mKNT7    | 1                        | 1                 | 1                | 1             | V2, V5                          |
| mKNT8    | 1                        | 1                 | 1                | 1             | V1, V2                          |
| mKNT9    | 1                        | 1                 | 1                | 1             | V3, V4, V5                      |
| mKNT10   | 1                        | 1                 | 1                | 1             | V2, V3, V4, V5                  |
| mKNT11   | 1                        | 1                 | 1                | 1             | V5                              |
| mKNT12   | 1                        | 1                 | 1                | 1             | V2, V3                          |
| mKNT13   | 1                        | 1                 | 1                | 1             | V2                              |
| mKNT14   | 1                        | 1                 | 1                | 1             | V1, V2, V5                      |
| mKNT15   | 1                        | 1                 | 1                | 1             | V3                              |
| mKNT16   | 1                        | 1                 | 1                | 1             | V2, V3, V4                      |

Novel kinectin isoforms mKNT1-15 (mouse KNT 1-15), identified in this study were submitted to the EMBL database with accession numbers AJ517365-AJ517384.

*For comparison, the table incorporates known sequence information of isoforms identified in spleen and testis (Leung et al., 1996), some of which were partial so the full-length insert permutation over the variable domain region has not been definitively established.

Immunolocalisation of kinectin isoforms using insert-specific antibodies

Our results revealed the existence of multiple kinectin isoforms that appear to be subject to cell-specific and developmental regulation. To address the question of whether these variable isoforms exhibit distinct intracellular localisation, we have successfully

Fig. 2. Kinectin intracellular localisation in PC12 cells as revealed by anti-kinectin antibodies. (A) Staining using anti-CT1, reactive to all kinectin isoforms, reveals reticular cytoplasmic labelling. (B) Staining with anti-insert V2 antibody, reactive to insert V2-containing isoforms, results in cytoplasmic reticular labelling as in A, plus distinct labelling of the tips of neurite-like processes (arrow heads). (C) No labelling occurs using pre-immune serum. (D1-D3) Double immunofluorescence localisation of kinectin isoforms and the ER. (D1) Labelling using anti-insert V3 antibody, reactive to insert V3-bearing isoforms, again results in cytoplasmic reticular staining plus staining of neuritic processes (arrowheads); (D2) anti-PDI antibody to visualise the ER; (D3) overlay of the two images. Bars, 5 μm.
generated two antibodies specific to variable inserts V2 and V3 of mouse kinectin, respectively, and used them in parallel with anti-CT1 mAb, reactive to all kinectin isoforms, for immunolabelling of differentiated PC12 cells. Anti-insert V2 and V3 antibodies are highly specific to kinectin when tested by western immunoblotting using crude cell lysates. Insert V2 is included in eight of the 16 kinectin isoforms and insert V3 in another subset of eight. Three of these isoforms contain both inserts V2 and V3. Probing PC12 cells with anti-CT1 resulted in reticular cytoplasmic labelling, compatible with an ER distribution for kinectin (Fig. 2A), which was also concurrently labelled by anti-insert V2 antibody (Fig. 2B,C). In addition to the ER staining, anti-insert V2 also revealed distinct labelling of the neurite-like processes of differentiated PC12 cells with pronounced immunoreactivity concentrating at the tips (Fig. 2B, arrowheads). The same results were obtained with the use of anti-insert V3 antibody (Fig. 2D1), which in addition to the cytoplasmic reticular staining, confirmed as ER by concurrent anti-PDI antibody labelling (Fig. 2D2), also intensely stained the tips of neuritic processes (Fig. 2D1, D3, arrowheads). These results revealed neuritic processes as an additional, novel localisation for a subpopulation of kinectin isoforms, containing inserts V2 and V3.

Functional assays using kinectin and kinesin constructs in cultured cells

The discovery of an extended family of kinectin species with differential patterns of developmental, tissue-specific and intracellular localisation was suggestive of the possibility of differential effects in kinesin-driven intracellular function. To probe in more detail the possible role of kinectin-kinesin interactions in the dynamics and morphogenesis of membranous compartments, a series of GFP fusions with distinct kinectin and kinesin domains was constructed for overexpression in HeLa cells. Human kinectin incorporates six variable inserts in its cytoplasmic C-terminal domain (inserts V1-6). The minimum kinesin-interacting domain of kinectin is within residues 1188-1288, shared between inserts V3 and V4 (Ong et al., 2000).
has been mapped between residues 1188-1288, shared between inserts V3 and V4, with insert V4 possessing the higher proportion of such residues (Ong et al., 2000). Our constructs (Fig. 3) comprised different combinations of variable domain inserts: KNT+, containing the kinesin binding domain only (inserts V3, V4), K1 (inserts V3, V4, V5), K2 (inserts V4, V5) and K3 (inserts V2, V3, V5). We also tested the TM construct, including the transmembrane domain of kinectin, KHC+, including the kinectin binding domain of kinesin, and two negative controls, KNT− and KHC−, incorporating regions of the molecules that are not involved in the kinectin-kinesin interaction (Fig. 3).

The kinectin transmembrane domain is involved in ER membrane dynamics

On the basis of sequence analysis, immunolocalisation and transient expression, the 160 kDa kinectin includes an N-terminal single TM domain embedded in membranes and a long, coiled-coil forming C-terminal cytoplasmic domain that includes the kinesin-interacting site (Futterer et al., 1995; Yu et al., 1995; Toyoshima and Sheetz, 1996; Ong et al., 2000). Transient expression of GFP-fused TM kinectin domain generated an ER network-like localisation pattern (Fig. 4Aa).

In contrast, expression of a 120 kDa non-TM domain (lacking the trans-membrane domain residues) showed organelle-like dots in the cytoplasm and only occasional, weak ER network-like localisation (Fig. 4Ab). Forty-eight hours following transfection, HeLa cells overexpressing the TM-GFP fusion protein, but not the non-TM fusion protein, exhibited a pronounced effect on ER network distribution, with ER staining accumulating in the perinuclear region instead of its previous reticular pattern (Fig. 4Baa, see also Fig. 5B). This phenotype was specific to the ER and was not observed with redistribution of mitochondria following heat shock recovery (Fig. 4Bbb). Thus, the maintenance of the ER network, but not mitochondria dynamics, seems to involve the kinectin transmembrane domain.

Kinectin-kinesin interaction is important for ER extension

The overexpression of constructs KNT+, K1 and K2, all harbouring the entire or part of the kinesin interacting domain, had dramatic and comparable effects on ER structure. In particular, overexpression of these three constructs induced a pronounced collapse of the ER network, observed in 65.7%, 71% and 77.7% of transfected cells, respectively, compared to only 15.7% of KNT− controls (Fig. 5A,B). Similarly, overexpression of KHC+, bearing the kinectin interacting domain of ubiquitous human kinesin, resulted in a marked reduction in ER extension in 64.3% of cells (Fig. 5B). This reduction was detected in only 13.3% of KHC− expressing cells (Fig. 5B). In all cases, ER branching, as visualised by labelling with anti-calreticulin antibody as a specific ER marker, was decreased and occupied a smaller surface area of the cytoplasm, compared to controls. Accumulation of ER staining towards the cell centre was often observed in KHC+, KNT+, K1 and K2-expressing cells (Fig. 5A).

An interesting result was obtained with the K3 construct that only includes part of the kinesin interacting domain in insert V3 but lacks insert V4, which represents the largest part of the minimum kinesin-binding sequence. The ER network in K3-overexpressing cells remained largely unaffected with 89% of cells exhibiting the normal morphology, whereas the percentage of cells that showed evidence of ER collapse (11%) was comparable to GFP-only control (15.7%) and non-

**Fig. 5.** Effects of overexpression of kinectin-kinesin binding domains on ER distribution in HeLa cells. (A) Typical images. Anti-calreticulin mAb was used to label the ER network (red). Transfected cells were identified by GFP expression (green). The network was normally distributed in a and a′, representing the largest part of the minimum kinesin-binding sequence. The ER network in K3-overexpressing cells remained largely unaffected with 89% of cells exhibiting the normal morphology, whereas the percentage of cells that showed evidence of ER collapse (11%) was comparable to GFP-only control (15.7%) and non-
transfected cells (7.7%) (Fig. 5B). The comparison of these results with construct K2, which also harbours an incomplete kinesin interacting domain lacking insert V3 but containing insert V4 in its entirety, underlined the higher importance of insert V4 compared to insert V3 in the interaction with kinesin.

Furthermore, overexpression of the TM domain of kinctin caused ER network reduction in 86.7% of cells (Fig. 5B). ER morphology was normal in 92.3% of non-transfected cells (Fig. 5B), which was at the same levels as in the GFP-only control (84.3%), KNT+ (84.3%) and KHC− (86.7%) overexpressing cells (Fig. 5B).

The results of these functional assays confirm previous evidence (Ong et al., 2000; Ong et al., 2003) indicating that the essential kinesin-binding domain of human kinctin is in the region of residues 1188-1288 and lend strong support to the idea that ER assembly and morphogenesis is kinctin-dependent via its interaction with kinesin.

120 kDa kinctin is specifically associated with mitochondria and kinctin-kinesin interaction influences mitochondria dynamics

In chicken and human, at least two different forms of kinctin have been observed, the 160 kDa species and a shorter, N-terminally truncated version of 120 kDa. Although it is not clear how these two forms are generated at the transcriptional level, they appear to be associated with organelle subpopulations of different density (Kumar et al., 1998).

To address the possible role of kinctin-dependent motility in mitochondria dynamics, we first proceeded with biochemical isolation of a mitochondria-enriched fraction through density gradient sedimentation. Fig. 6A shows prominent immunoreactivity in the mitochondrial fraction using an anti-Mn SOD antibody as a specific mitochondrial marker, compared to the post-nuclear supernatant. The mitochondrial and post-nuclear supernatant fractions were probed with anti C-terminal monoclonal antibody (CT-1), raised against the carboxyl terminus of human kinctin and recognising both the 160 kDa and the 120 kDa forms of kinctin (Futterer et al., 1995). In the post-nuclear supernatant both the 160 kDa and 120 kDa species were detected, whereas in the mitochondrial fraction only the 120 kDa band was observed (Fig. 6B). The same results were obtained with the use of anti-insert V3 antibody, raised against the mouse kinctin domain in the variable region between residues 1136 and 1160. This antibody is highly specific to kinctin in western blotting using crude cell lysates (Fig. 6C). Again, although both 160 kDa and 120 kDa bands were visible in the post-nuclear supernatant, only the 120 kDa band was detectable in the mitochondria-enriched fraction (Fig. 6C').

There was negligible ER protein contamination in the mitochondria-enriched fraction (lane 2). The effects of heat shock are reversible when cells are returned to normal temperature (Vale et al., 1985). MT-based anterograde transport of mitochondria is dependent on one or more kinesin-like proteins (Nangaku et al., 1994; Tanaka et al., 1998). For example, genetic inactivation of KIF5B (ubiquitous KHC, uKHC) in mouse results in perinuclear clustering of mitochondria (Tanaka et al., 1998).

We reproduced this reversible dynamic behaviour in HeLa cells with the heat shock regime described in the Materials and Methods, visualising mitochondria by vital dye staining with MitoTracker Red and immunofluorescence using anti-Mn SOD antibody. The effects of kinesin and kinctin overexpression were examined after overnight recovery.

Overexpression of KHC+ domain resulted in perinuclear clustering of mitochondria, a phenotype strikingly reminiscent of that in KIF5B-deficient cells. After overnight recovery from heat shock, 54.7% of KHC+ overexpressing cells still maintained a pronounced mitochondrial clustering, compared to only 1.6% in KHC− overexpressing cultures and 3.5% in

![Fig. 6.](image)

Association of 120 kDa kinctin isoform with mitochondria in HeLa cells. (A) Western blot of post-nuclear supernatant (lane 1) and mitochondria-enriched fraction (lane 2) of equal concentrations (7 mg/ml), probed with the mitochondrial marker anti-Mn SOD (25 kDa). (B) CT-1 was used to probe the post-nuclear supernatant (lane 1) and mitochondrial fractions (lane 2) of equal concentration (7 mg/ml). 160 kDa and 120 kDa species were detected in lane 1. Only a 120 kDa species was detected in lane 2. (C) The specificity of the affinity-purified anti-insert V2 and V3 antibodies was tested by immunoblotting of a crude cell lysate (concentration 2 mg/ml) Coomassie Blue-stained lysate (lane 1), purified anti-insert V2 and V3 antibodies recognized the prominent 160 kDa form of kinctin (lanes 2 and 3, respectively). (C') Anti-insert V3 antibody was used to probe the post-nuclear supernatant (lane 1) and mitochondrial fractions (lane 2) of equal concentration (7 mg/ml). 160 kDa and 120 kDa kinctin were detected in lane 1 whereas a 120 kDa band was detected in lane 2. (D) An ER marker, anti-calreticulin (63 kDa), was used to probe the post-nuclear supernatant (lane 1) and mitochondrial fractions (lane 2) of equal concentration (0.76 mg/ml). ER protein was not enriched in lane 2.
non-transfected cells (Fig. 7B). This clearly indicated that mitochondria of KHC* overexpressing cells were unable to resume their normal distribution, in contrast to control cells.

To assess the involvement of kinectin in mitochondrial transport, constructs KNT*, K1, K2, K3 and KNT– were tested. KNT* and K1, both containing the entire kinectin-kinesin interacting domain, and also K2, which comprises a large fragment of the interacting domain, caused retention of mitochondrial clustering after the recovery period in 25.7%, 42.3% and 32.6% of transfected cells, respectively (Fig. 7A,B). In contrast, KNT– overexpressing cells exhibited a normal, dispersed cytoplasmic distribution of mitochondria in 86.3% of cells with only 13.7% of cells retaining mitochondrial clustering after overnight recovery (Fig. 7B). The latter was comparable to cells transfected with TM domain (8.3%), GFP-only control (11.6%) and non-transfected cells (3.5%) (Fig. 7B). Again, cells transfected with K3, with the largest part of the kinesin interacting domain missing (i.e. insert V4), displayed a behaviour that paralleled that of KNT*, KHC* and GFP-only controls, with the vast majority of cells (90.5%) being able to resume a normal mitochondrial distribution after recovery from heat shock and a small number (9.5%) still retaining a clustered phenotype. Taken together, these data strongly suggest that kinectin is required for kinesin-mediated attachment and transport of mitochondria along microtubules and that interaction occurs through the characterised C-terminal domain of kinectin.

Kinectin involvement in ER and mitochondria motility is confirmed by RNAi studies

To corroborate the results obtained from our overexpression studies, we induced a knockdown of kinectin expression using RNA interference technology. The choice of the oligonucleotide targeting sequence corresponded to part of the constant domain of kinectin that is common to all isoforms thus aiming for the simultaneous interference with all kinectin transcripts. We observed that RNAi reduced native kinectin mRNA and protein by more than 50% in stable kinectin knockdown HeLa cells, as determined by semi-quantitative RT-PCR and western blots (Fig. 8A,B respectively). Furthermore, endogenous kinectin immunofluorescent staining appears significantly less pronounced compared to its wild-type pattern and mainly localised near the perinuclear region (Fig. 8C,a). Interestingly, there is a marked collapse of both the ER and mitochondria networks, as detected with specific markers for these compartments, characterised by accumulation at the cell centre (Fig. 8C,a). In contrast, untransfected HeLa and vector alone control cells exhibited normal ER and mitochondrial distribution (Fig. 8C,b-c). RNAi had no detectable effect on the microtubule network in transfected or control cells (Fig. 8C,a-c). Taken together, both the overexpression and RNAi studies demonstrate that kinectin is involved in kinesin-mediated motility of ER and mitochondria.

Discussion

Recent discoveries have begun to illustrate that proteins with diverse cellular functions may serve as receptors for motor proteins, such as kinesin-like proteins. These were shown to interact with organelle coat proteins (API clathrin, spectrin and fodrin), large complexes of scaffold proteins, small GTPases (Rab6), other motors (myosin V) and transmembrane proteins including kinectin and Sunday driver (SYD), thus functionally coupling MT-based transport with processes such as membrane

Fig. 7. Effects of overexpression of kinectin-kinesin binding domains on anterograde distribution of mitochondria in HeLa cells after heat shock treatment. (A) Typical images. Mitochondria were labelled by anti-Mn SOD Ab, followed by TRITC-conjugated secondary antibody (red). Transfected cells were identified by GFP expression (green). Normal, radially distributed mitochondria, similar to those observed in non-transfected cells, appeared in GFP transfected cells (a,a’). KNT* transfected cells showed pronounced clustering of mitochondria at the cell centre (b,b’). Bar, 20 μm. (B) Quantification. The values shown represent the mean±s.e.m of three experiments. A total of 90 cells were analysed per construct.
trafficking, organelle formation/fusion and signal transduction (reviewed by Klopfenstein et al., 2000). Further scope for variability of interactions and regulation of intracellular transport is created by variation within a single class of receptor, for example by the expression of different isoforms and their differential intracellular localisation.

To address the possibility that additional kinectin-like isoforms may exist in the mammalian nervous system we devised an experimental strategy that allowed us to clone a large number of novel additional kinectin isoforms. We then proceeded to investigate the likely mechanism by which the interaction between these kinectins and the range of available motors in a single cell type is determined.

Fifteen kinectin isoforms with variable C-termini, probably products of alternative splicing, were identified in the mouse nervous system, bringing the total known number of isoforms in this organism to 16 (Table 1). The 16 kinectin isoforms (mKNT1-16) include one no-insert-form, four one-insert, five two-insert, five three-insert and one four-insert form out of the 32 theoretically possible zero- to five-insert permutations. The least common insert is V1, appearing only in four out of 16 isoforms, whereas inserts V2, V3 and V5 appear with about the same frequency. Insert V4 is also abundant and present in six of the 16 isoforms but interestingly, is uniquely excluded from all adult hippocampus isoforms.

The smallest, no-insert isoform (mKNT1) appears ubiquitous in all cell types and developmental stages examined. Other isoforms are developmental stage- or cell type-specific. For example, isoforms mKNT5-8 are expressed only in the embryonic hippocampus and not in later developmental stages, nor are they present in astroglial cells. mKNT8 however, seems not to be nervous system-specific as it has also been identified in spleen/testis (tissues that have the same embryogenetic origin as the nervous system nevertheless). Isoforms mKNT11-14 can be considered as neuron-specific as they are absent both from glia, spleen and testis. Furthermore, isoforms mKNT13-15 are clearly adult-specific, although mKNT15 is not uniquely of hippocampal origin as it has been detected in testis and spleen. One

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**Fig. 8.** Effects of kinectin RNAi on anterograde distribution of ER and mitochondria in HeLa cells. (A) mRNA levels in untransfected cells (lane 1), vector alone stable cells (lane 2) and kinectin knockdown stable cells (lane 3) were determined by semi-quantitative RT-PCR. (B) 10 μg of whole cell lysates as in (A) subjected to western blotting for kinectin and actin proteins. (C) Silencing of kinectin protein. Untransfected HeLa cells (a1-4) and HeLa cells transfected with pSilencer vector control siRNA (b1-4), or with kinectin siRNA (c1-4). Kinectin knockdown stable cells showed distinct disruption in kinectin (c1), ER (c2) and mitochondria (c3) distribution. By contrast, vector control cells (b1-3) and untransfected cells (a1-3) show normal staining patterns. Each cell type was stained for kinectin (anti-CT-1) (red); ER (anti-calreticulin) (red); mitochondria (anti-Mn SOD) (red) and DNA (DAPI) (blue). Microtubule (anti-β-tubulin) (red) distribution appeared normal for all cells type (a4-c4). Bars, 20 μm.
isoform, mKNT10, is uniquely present in glial cells and can therefore be considered genuinely glia-specific, whereas mKNT9 is excluded from neurons but expressed in glia, spleen and testis. Finally, the cell-specificity of isoforms common to the hippocampus and pure glial cultures cannot be assigned, as hippocampal samples contain both neurons and glia (at an approximate ratio of 1:10).

The identification of a total of 16 kinectin isoforms reveals the long-suspected existence of a family of kinectin isoforms with a complexity that parallels that of the kinesin-like family of motor proteins in the nervous system of mouse (currently over 30 known anterograde motors in mouse alone) (Nakagawa et al., 1997; Miki et al., 2001). This finding strengthens the hypothesis that membrane anchors may be selected ‘à la carte’ to induce (via their variable C-termini) transient interactions with a multitude of motor proteins, thus determining the directionality of transport and/or modulating motility of motor-membrane complexes.

In mouse, the rod-shaped C-terminal domain extends to the cytoplasm and is composed of 18 putative coiled-coil regions, formed by contiguous heptad repeats (Leung et al., 1996). It is within this domain that the sequence variability, determined by the five variable insert permutations, is observed. This domain is involved both in homo- and hetero-dimerisation (Kumar et al., 1998) and harbours the kinesin-binding site (Ong et al., 2000). The positions of the five variable C-terminal inserts do not correlate with the boundaries of the 18 heptad-containing coiled coils, but the C-terminal sequence variability can be assumed to have important structural and functional implications for the kinectin isoforms. Although no functional motifs are readily discernible in the five variable inserts, it is likely that small C-terminal sequence variations may be the basis for selective motor interactions. For example, insert V5 encodes a highly hydrophobic, uncharged region of 18 residues that although unlikely to function as a second trans-membrane domain (it lacks the characteristic KKXX retention motif), may be involved in interactions with hydrophobic surfaces. Intriguingly, six of the 16 kinectin isoforms lack both insert V3 and insert V4, another two bear no insert V3 and an additional four have no insert V4, bringing the total number of isoforms lacking the whole or part of the kinesin-binding domain (mapped within inserts V3 and V4) to 12 of 16. Notably, the only ubiquitous kinectin isoform is the one lacking all C-terminal variable inserts.

The absence of a functional kinesin-binding domain in the majority of the kinectin isoforms raises the question whether binding sites to motors other than conventional kinesin may exist outside the delineated kinesin-binding domain. It also points to the possibility that kinectin may bind non-motor partners through binding domains residing outside the conventional kinesin binding motif or through non protein-protein interactions, such as lipid attachments to membranes. Recent evidence substantiates this possibility by demonstrating with genetic, in vivo and biophysical methods the interaction of a kinectin isoform with translation elongation factor 18 subunit (Ong et al., 2003). This kinectin isoform lacks a large portion of the kinesin-binding domain. Our recent BLAST search of the mouse genome revealed that both chromosomes 14 and 18 encode a kinectin gene, with the latter missing the entire translation elongation factor 18 binding site and partial kinesin binding site. If such gene duplication is not a result of sequencing artefacts, it might provide the redundancy that is important in safeguarding critical cellular functions. It is plausible that the repertoire of kinectin isoforms and binding partners is larger and its functional role in intracellular motility and protein targeting wider and more significant than originally perceived.

The role of kinectin-kinesin interaction in intracellular transport of ER and lysosomes is well documented (Kumar and Sheetz, 1995; Ong et al., 2000). In the present study, the reduction in the ER network observed in cells overexpressing kinectin constructs, containing either the intact or partial kinesin-binding domain, strongly suggests that kinectin-kinesin interaction is essential for maintaining ER integrity. The effect on ER structure varied in intensity in proportion to the number of residues abolished in the partial constructs. These results are in agreement with previous findings on lysosomal motility (Ong et al., 2000) and underline the greater importance of residues contained in variable insert V4 in the kinectin-kinesin interaction.

Previous work indicated that human, mouse and chicken kinectins (Toyoshima et al., 1992; Yu et al., 1995; Futterer et al., 1995; Leung et al., 1996; Kumar et al., 1998) are associated with ER membranes involving a hydrophobic N-terminal transmembrane sequence (TM). In the present work, transient transfection of the TM sequence revealed a reticular, perinuclear pattern of expression, consistent with previous reports using anti-kinectin antibodies and transient expression of kinectin lacking the N-terminus (Futterer et al., 1995; Yu et al., 1995; Toyoshima and Sheetz, 1996). Interestingly, overexpression of TM caused a specific disruption of maintenance and perinuclear clustering of the ER network without affecting anterograde transport of mitochondria. The drastic disruption of the ER network might imply some hitherto undiscovered interaction between TM and other molecules in the ER membrane that are important for ER distribution. Alternatively, dimerisation of TM with the native kinectin might be inducing a dominant negative effect.

Kinecin-kinesin interaction has a multifaceted role in intracellular motility. In addition to its involvement in ER maintenance it enhances MT-stimulated ATPase activity of kinesin and influences the movement of lysosomes upon recovery from acidification (Ong et al., 2000). Here we identify mitochondria as an additional target of kinesin-kinectin interaction. Conventional kinesin (KIF5B) null mutant cells show perinuclear clustering of mitochondria, demonstrating that the motor protein is required for their translocation in intact cells (Tanaka et al., 1998). Cells overexpressing the KHC+ domain also showed perinuclear clustering, highly reminiscent of this phenotype. Overexpression of the kinectin KHC+ domain therefore mimics the genetic inactivation of KIF5B. We further observed that overexpression of other kinectin constructs containing the intact or partial kinesin binding domains resulted in inability of mitochondria to redistribute to the cytoplasm after recovery from heat shock. This indicated that kinectin is required in anterograde mitochondrial motility. Again, the intensity of the effect was proportional to the integrity of the kinesin binding site, with the residues of insert V4 being the most important. One alternative interpretation of results from these in vivo assays with kinectin constructs could be that overexpression causes its effects via kinesin mislocalisation/sequestering. It is not
a trivial matter to address this experimentally by immunolocalisation of kinesin following transfection because intracellular kinesin is both tightly bound to membranes (e.g. it is resistant to carbonate washing) and soluble, and its interaction with kinesin is weak. Furthermore, it is impossible, on the basis of localisation, to discriminate active from inactive motor complexes. More importantly, this explanation seems highly unlikely given that the association of kinesin with mitochondria was also confirmed biochemically. The 120 kDa kinesin species co-purified specifically with the mitochondrial fraction upon density gradient sedimentation.

The assays here provided the first evidence on the functional association of kinesin with mitochondria. How the 120 kDa form serves as a functional protein in mitochondria motility is unknown; however, the 120 kDa isoform has previously been reported to have numerous potential sites for phosphorylation (Kumar et al., 1998) and is also shown to be myristylated (Burkhardt, 1996). The myristylation of the 120 kDa kinesin may suggest that post-translational modifications could provide a mechanism for attachment to the mitochondrial membranes even without the transmembrane domain.

Results from RNAi were consistent and corroborate the conclusions derived from transfection experiments on the role of the kinesin-kinecin interaction with both the ER and mitochondrial motility. Significant downregulation in the levels of global kinecin transcription and translation led to marked changes in ER and mitochondrial organisation and positioning, not dependent on the cytoskeleton as the microtubule network remained intact. These results seem to contradict the lack of phenotype in the kinecin knock-out mice (Plitz and Pfeffer, 2001). It is possible that kinecin functions were compensated by other molecules in the knock-out mouse model just like many other essential proteins (reviewed by Pearson, 2002). In the in vitro studies however, the selection for compensatory mechanisms might not be as amplified as the selection in vivo. The functional significance of kinecin, in organelle motility and other intracellular processes, remains to be elucidated thoroughly in vivo.

Kinecin was previously thought to be absent from axons (Toyoshima and Sheeit, 1996). The detection of kinecin immunoreactivity in neurit processes, as revealed by both insert V2- and V3-specific antibodies, is thus a novel and intriguing finding that may be indicative of differential patterns of intracellular localisation for certain isoform subsets or may reflect localisation that is dependent on the state of cellular differentiation or responsive to external stimui. Alternatively, it may result from a higher sensitivity of our insert-specific antibodies, compared to the pan-kinecin antibody used so far. None of the known target organelles of kinecin (the ER, lysosomes and mitochondria) extend to the tips of neuritic processes, which are sites of active membrane trafficking and exchange. The possibility remains that kinecin is involved in a range of interactions with additional motors, thus affecting intracellular motility or targeting to different sites. The kinecin isoform population that appears, on the basis of immunolocalisation, to be concentrated to these sites, may be involved in the interaction with motors that participate in processes such as neuronal signal conduction and transmission.

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