Role of Xklp3, a Subunit of the *Xenopus* Kinesin II Heterotrimeric Complex, in Membrane Transport between the Endoplasmic Reticulum and the Golgi Apparatus

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Abstract. The function of the Golgi apparatus is to modify proteins and lipids synthesized in the ER and sort them to their final destination. The steady-state size and function of the Golgi apparatus is maintained through the recycling of some components back to the ER. Several lines of evidence indicate that the spatial segregation between the ER and the Golgi apparatus as well as trafficking between these two compartments require both microtubules and motors. We have cloned and characterized a new *Xenopus* kinesin like protein, Xklp3, a subunit of the heterotrimeric Kinesin II. By immunofluorescence it is found in the Golgi region. A more detailed analysis by EM shows that it is associated with a subset of membranes that contain the KDEL receptor and are localized between the ER and Golgi apparatus. An association of Xklp3 with the recycling compartment is further supported by a biochemical analysis and the behavior of Xklp3 in BFA-treated cells. The function of Xklp3 was analyzed by transfecting cells with a dominant-negative form lacking the motor domain. In these cells, the normal delivery of newly synthesized proteins to the Golgi apparatus is blocked. Taken together, these results indicate that Xklp3 is involved in the transport of tubular-vesicular elements between the ER and the Golgi apparatus.

Key words: Kinesin II • microtubules • ER • Golgi • IC

In eukaryotic cells, membranous organelle components and secretory proteins are synthesized and distributed through a complex network of vesicles and tubules that include the endoplasmic reticulum (ER) and the Golgi apparatus. Proteins and lipids synthesized in the ER are translocated in transport intermediates (tubular-vesicular structures) towards the Golgi apparatus, where they are processed and sorted to specific subcellular destinations. The steady-state size and function of the Golgi apparatus depends on anterograde transport as well as on efficient recycling to the ER of some components that have entered the secretory pathway. In higher eukaryotes, the ER and Golgi apparatus are spatially segregated. The ER extends from the nuclear envelope throughout the cytoplasm towards the cell periphery whereas the Golgi apparatus is concentrated at the cell center, around the centrosome. Several lines of evidence indicate that the spatial segregation between the ER and the Golgi apparatus as well as trafficking between them requires both microtubules and motors (for review see Lippincott-Schwartz, 1998).

It is now clear that a minus end–directed motor, dynein or a dynein related protein, is required for positioning the Golgi apparatus at microtubule minus ends (Theulaz et al., 1992; Burkhardt et al., 1997; Corthesy-Harada et al., 1998). A plus end–directed motor like conventional kinesin or a kinesin-related protein, appears to be involved in the formation of tubules that emerge from the Golgi, and extend towards the ER upon brefeldin (BFA)[1] treatment. This observation is compatible with the idea that plus end–directed motors may drive the retrograde transport of vesicles from the Golgi to the ER (Lippincott-Schwartz et al., 1995; Sciaky et al., 1997). Among the large number of kinesin-like proteins found associated with membranes or vesicles, two have been shown to be associated with the Golgi apparatus. Rab–kinesin6 was identified as a Rab6

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1. *Abbreviations used in this paper:* AMP-PNP, 5′-adenylyllimido-diphosphate; BFA, brefeldin A; GFP, green fluorescent protein; GST, glutathione-S-transferase; HA, hemagglutinin; HP, Helix pomatia; IC, intermediate compartment; IFT, intraflagellar transport; KLP, kinesin-like protein; MBP, maltose-binding protein; MT, microtubules; PDI, protein disulfide isomerase; PNS, postnuclear supernatant.

interacting protein but its role in the spatial organization and dynamics of the Golgi has not been fully determined yet (Echard et al., 1998). The mouse KIF1C, has recently been described as a motor important for Golgi to ER vesicular transport induced by BFA treatment (Dorner et al., 1998).

Here we report the characterization of Xklp3, a kinesin-like protein previously identified in *Xenopus* (Vernos et al., 1993). Xklp3 sequence and biochemical features are characteristic of the Kinesin II subfamily (for review see Scholey, 1996). Kinesin II is a plus end–directed motor formed by the association of three proteins: two closely related kinesin-like proteins that interact through their coiled-coil region and a third nonkinesin protein associated with their COOH terminus (Cole et al., 1993; Rashid et al., 1995; Yamazaki et al., 1995, 1996; Wedaman et al., 1996). Different members of this subfamily have been shown to localize on vesicles and to be required for vesicular transport in the nervous system of the mouse (KIF3A/KIF3B/KAP3) (Kondo et al., 1994), *Drosophila* (KLP64/68) (Kondo et al., 1994; Pesavento et al., 1994) and *Caenorhabditis elegans* (OSM3) (Tabish et al., 1995), in ciliogenesis in sea urchin (KRP85/95/KAP115) (Morris and Scholey, 1997), and flagella formation in *Chlamydomonas* (FLA10) (Cole et al., 1998; Vashishtha et al., 1996). We find that Xklp3 is associated with Golgi membranes, more precisely with tubular-vesicular structures, where it colocalizes with recycling compartment components. Our data indicate that its activity is required for normal trafficking between the ER and the Golgi apparatus.

Materials and Methods

Isolation and Sequencing of Xklp3 cDNA

Partial Xklp3 cDNA clones were isolated from a *Xenopus* oocyte cDNA library (Vernos et al., 1993). DNA was sequenced on both strands using the T7, T3, and internal primers. The full-length cDNA for Xklp3 was reconstructed from three overlapping clones as follows: a Bluescript plasmid (Stratagene, La Jolla, CA) carrying the 5′ end sequence of Xklp3 cDNA was cut at a BclI internal site and EcoRI and ligated with a 730-bp internal fragment obtained by restriction digest of the second clone with BclI-EcoRI and a 3′ end fragment obtained from the third clone by digestion with EcoR1. The final construct was sequenced. Sequence analysis and comparisons were done with the DNASTAR program (London, UK) and coiled-coil predictions according to Lupas et al. (1991).

Production of Xklp3 Fusion Proteins and Antibodies

Fragments of Xklp3 corresponding to either the motor domain (Xklp3-M, nucleotides 217–1,248, amino acids 1–344) or part of the stalk and the full tail (Xklp3-T, nucleotides 1,684–2,446, amino acids 489–744) were generated by PCR using Pwo polymerase (Boehringer Mannheim, Indianapolis, IN) and inserted into the pGEX (Stratagene) and pMAL-c2 (New England Biolabs, Beverly, MA) vectors for expression in bacteria as recombinant proteins with the glutathione-S-transferase (GST) and the maltose-binding protein (MBP), respectively. The purified GST fusion proteins (Smith and Jonshon, 1988) were used to immunize rabbits following standard procedures (Harlow and Lane, 1988). Antibodies were affinity purified against the corresponding MBP fusion protein (Boleti et al., 1996) bound to CNBr-activated Sepharose (Pharmacia Biotech, Piscataway, NJ). The MBP-tail fusion protein (amino acids 489–744) was used to immunize mice and a monoclonal antibody (mAb X3T-3A6) was prepared following standard methods (Harlow and Lane, 1988).

Expression Constructs for Eukaryotic Cells

A PCR fragment corresponding to the full stalk and tail domains of Xklp3 (amino acid 345–744) was generated by PCR using the Pwo polymerase, and cloned into the XhoI-BamH1 sites of pEGFP-C3 (Clontech, Palo Alto, CA) to generate pEGFP-Xklp3-ST. The construct HA-Xklp3-ST was generated by cutting out the green flourescent protein (GFP) epitope from pEGFP-Xklp3-ST with Nhe1-Sca1 restriction enzymes and replacing it with a 100-bp HA epitope generated by PCR from the vector pCMU-IV (Nilsson et al., 1989). The full-length GFP-Xklp1 was cloned in pEGFP-C3 and a fragment of the stalk and tail (nucleotides 2,370–3,740) was amplified by PCR and cloned in the vector pCDNA3.1 (Invitrogen, Carlsbad, CA) to generate Xklp1-ST-myc. The stalk region of GalT, GalNacT2, or Mannosidase II were fused to the NH_2 terminus of GFP in the following way. DNA fragments encoding the stalk regions were generated by PCR: (*a*) a 361-bp fragment (amino acids 1–120) from human GalT cDNA (GenBank/EMBL/DDBJ accession number X55415), (*b*) a 338-bp fragment (amino acids 1–114) from human GalNAcT2 cDNA (GenBank/EMBL/DDBJ accession number X85019), and (*c*) a 348-bp fragment (amino acids 1–116) from mouse α-mannosidase II cDNA (GenBank/EMBL/DDBJ accession number X61172). The PCR fragments were digested and cloned in pEGFP–N1 (Clontech) to generate pGalT–GFP, pGalNacT2–GFP, and pMannII–GFP. Vesicular stomatitus virus glycoprotein (VSV-G)–GFP was constructed by fusing GFP to the COOH terminus of the full-length VSV-G ts045 protein. A DNA fragment encoding the VSV-G ts045 protein (GenBank/EMBL/DDBJ accession number M11048) was generated by PCR and cloned in pEGFP-N1 to generate pVSV-G–GFP. All constructs were sequenced and did not contain any mutation altering the amino acid sequence.

Cell Lines and Transfection

Two frog cell lines were used, XL177 (Miller and Daniel, 1977), and A6 (Rafferty and Sherwin, 1969). Cells were grown at 23°C in L-15 medium with glutamine and 15% FCS. For transfection, A6 confluent cells were split 1:5 onto coverslips placed in 24-well plates, at least 20 h before the experiment. Cells were transfected with the Superfect reagent (QIAGEN, Santa Clarita, CA) according to the manufacturer's instructions. In short, 5 μg of plasmid DNA (Maxiprep; Qiagen) were used and the Superfect-DNA complex was left on the cells for 3 h before washing twice with 70% PBS and then twice with complete medium. Cells were grown for an additional 12–20 h before observation.

Stable cell lines expressing Golgi markers were generated by transfecting A6 cells at 30% confluency on a 10-cm Petri dish with 10 μg of DNA. Transfected cells were selected by adding 1 mg/ml of G418 (Geneticin; GIBCO BRL Life Tech, Paisley, Scotland, UK) to the culture medium. The GFP-positive cells were selected after 2 wk by flow cytometry on a FACSort® (Becton Dickinson, Palo Alto, CA).

Antibodies and Reagents

Rhodamine- or FITC-conjugated lectin from Helix pomatia (Sigma Chemical Co., St. Louis, MO) were resuspended in PBS and kept at 4°C. Nocodazole, cycloheximide, and BFA (Sigma Chemical Co.) were resuspended in DMSO, water, or methanol respectively, and kept at −20°C. Nocodazole was used at a final concentration of 5 μM and BFA at 5 μg/ml. The following antibodies were used: a mouse monoclonal anti-protein disulfide isomerase (PDI) directed against the KDEL motif of the PDI protein, from S. Fuller (European Molecular Biological Laboratory [EMBL], Heidelberg, Germany); a sheep polyclonal anti–KDEL-receptor from J. Füllekrug, (EMBL) and I. Majoul, (Max Planck Institute, Göttingen, Germany); a polyclonal anti-GFP from K. Sawin (Imperial Cancer Research Fund, London, UK); a rabbit polyclonal anti-HA, from T. Nilsson (EMBL); a monoclonal anti-KRP85 and a polyclonal anti KAP-115 from J. Scholey, (University of California, Davis, CA); a monoclonal anti–α-tubulin purchased from Sigma Chemical Co. FITC-, Texas red-, cy3-, AMCA-conjugated secondary antibodies specific for rabbit or mouse were purchased from Dianova (Hamburg, Germany). Gold-coupled protein A was purchased from J.W. Slot, (Utrecht University, Utrecht, The Netherlands).

Immunofluorescence and Light Microscopy

For immunofluorescence, fully confluent cells were split 1:2 onto coverslips and allowed to grow 24 h before fixation. Cells were washed in 70% PBS, fixed either in methanol at −20°C for 10 min or with glutaraldehyde as described in Boleti et al., (1997). The antibodies were diluted in PBS with 2% bovine serum albumin (BSA) and 0.1% Triton X-100. Primary antibodies were applied for 20 min at room temperature. The coverslips

were washed three times for 5 min and secondary antibodies were then applied for 15 min. The coverslips were washed three times for 5 min and mounted in Mowiol (Hoechst, Frankfurt, Germany).

Confocal images were acquired using a confocal laser microscope Leica TCS-NT (Deerfield, IL) equipped with an Ar/Kr laser triple line. Confocal series were collected and projections were made using the TCS-NT software. For triple labeling experiments, we used a LSM-510 (Carl Zeiss Inc., Thornwood, NY) confocal microscope equipped with a Ar/Kr and a UV laser. In that case, single planes were collected. Image analysis was performed using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Electron Microscopy

Cells were grown on a 10-cm Petri dish and fixed by adding to the medium an equivalent volume of 4% paraformaldehyde in 0.2 M sodium phosphate buffer, pH 7.4, for 1 h. The medium was then replaced with a fresh solution of 2% paraformaldehyde in 0.1 M sodium phosphate buffer and incubated for one more hour. Cells were collected by careful scraping and processed for cryosectioning according to Slot et al., (1991). The cryosections were retrieved with a 1:1 solution of 2.3 M sucrose and 2% methylcellulose according to Liou et al. (1996). Double immunogold labelings were performed as described previously (Slot et al., 1991). The monoclonal antibody X3T-3A6 was used in combination with a rabbit anti–mouse linker antibody, and then 10 nm of protein A–gold. The polyclonal anti–Xklp3-tail antibody was combined with 10 nm of protein A–gold. The polyclonal anti–KDEL-receptor or the polyclonal anti-GFP were combined with 5 nm of protein A–gold. Sections were stained, embedded in 2% methylcellulose/ 0.3% uranyl acetate, and then viewed in a Phillips CM120 electron microscope (Eindhoven, The Netherlands) at 80 KV.

The labeling densities of Xklp3 over the KDEL-receptor structures, the Golgi stacks (GalT GFP-positive), and the ER (PDI-positive) and the cytoplasm, were determined by the point-hit method (Weibel, 1979). 20 images were analyzed per marker. The KDEL-receptor compartment was defined as the tubular-vesicular structures areas marked by the erd-2 antibody, the Golgi stacks as membrane structures containing three or more cisternae and positive for the GalT–GFP, and the ER structures as the membranes marked by the anti-PDI antibody. The cross-section surface area for each compartment was measured using a square-lattice grid with a spacing $d = 12.5$ mm to count the points P corresponding to the grid line intersections comprised within the boundaries of the respective compartment. The surface area is then given by $\Sigma P d^2/\text{mag}^2$ (in μm^2). The labeling density of Xklp3 in each compartment was calculated by dividing the number of gold particles that fall into the boundaries of each type of structure by the respective surface area. To evaluate Xklp3 density in the cytoplasm, we positioned a box corresponding to 10 points ($d = 12.5$ mm) on randomly selected areas of cytoplasm deprived of any membrane structures. We are conscious that this method might overestimate Xklp3 density in the cytoplasm, and for this reason, we counted a greater number of micrographs ($n = 30$). Background labeling was calculated by counting the number of gold particles detected on the mitochondria. It was usually $\sim$1 gold particle per μm^2 both for the polyclonal anti–Xklp3-tail and the mab-X3-T-3A6.

Extracts and Immunoprecipitations

Interphase or cytosolic factor-arrested extracts from *Xenopus* eggs were prepared as described by Murray (1991). Motor proteins were prepared from egg extracts as described by Vernos et al. (1995). Whole cell extracts were prepared according to Boleti et al. (1996). 10 μg of anti–Xklp3-tail antibody coupled to 25 μl of Affiprep Protein A beads (Bio-Rad) were used for immunoprecipitation from 100 μl of egg extract (Walczak et al., 1996). Immunoprecipitations from transfected cells were performed in the following way. One 10-cm Petri dish of cells transfected 20 h before the experiment was incubated on ice with lysis buffer (25 mM Tris-Cl, pH 7.6, 200 mM NaCl, 1% Triton X-100, protease inhibitors). Cells were collected by scraping and spun at 12,000 *g* for 10 min. The supernatant was incubated for 1 h at 4°C with anti–GFP-coupled protein A–Sepharose (Pharmacia). The immunoprecipitate was washed three times (25 mM Tris-Cl pH 7.6, 200 mM Nacl, 0.1% Triton X-100) and resuspended in sample buffer before loading on SDS-PAGE and transfer on nitrocellulose for Western blot analysis.

Biochemical Fractionation

Biochemical fractionations were done in the following way. A 10,000 *g* interphase egg extract was diluted three times with acetate buffer (Reinsch and Karsenti, 1997) and spun at 100 Kg for 30 min to obtain a membrane and a cytosol fraction. The membranes were resuspended in 2 M of sucrose in acetate buffer and loaded at the bottom of a sucrose step gradient (1.3/0.86/0.5/0.25 M sucrose in acetate buffer). The gradient was centrifuged overnight in a SW60 rotor (Beckman Instrs., Palo Alto, CA) at 300 Kg. The four layers were collected, centrifuged through a sucrose cushion, and then the pellets resuspended in one-tenth of the initial volume.

Cytosol fractionation from cells was performed in the following way. Cells grown on six 500 cm^2 plates were washed, scraped in 70% PBS, and then incubated in cytochalasin D. After centrifugation they were resuspended in KHEM (35 mM KCl, 50 mM Hepes, 5 mM EGTA, 1 mM $MgCl_2$, 3 μg/ml cycloheximide and 1 μM DTT, and protease inhibitors). Cells were broken by 10–15 passages through a ball-bearing homogenizer (8.0004-μm clearance) and centrifuged at 2,000 *g* to remove nuclei, heavy mitochondria, and unbroken cells. The postnuclear supernatant (PNS) was loaded at the top of a sucrose step gradient (0.25/0.5/0.86/1.3 M in KHEM) in SW40 tubes, and then centrifuged for 4 h at 200 Kg. 1-ml fractions were collected. The proteins contained in 200 μl of each fraction were precipitated with methanol/chloroform and loaded on SDS-PAGE followed by immunoblotting as described previously (Vernos et al., 1996).

Results

Xklp3 cDNA and Protein Sequences

Using a PCR-based approach, four kinesin-like proteins (Xklp1–Xklp4) were previously identified in *Xenopus* eggs (Vernos et al., 1993). We screened a *Xenopus* oocyte cDNA library with a probe corresponding to the motor domain of Xklp3. We obtained a 3,762-bp sequence from three overlapping clones containing an initiation codon at position 215, a stop codon at position 2,447, and a polyadenylation signal at position 3,716. The 2232-bp open reading frame encoded a protein of 744 residues with a calculated M_r of 85 kD.

The deduced protein sequence showed that Xklp3 had a conventional kinesin-like protein (KLP) organization with the NH_2-terminal motor domain, an α-helical region predicted to be involved in coiled-coil interactions and a globular COOH-terminal tail domain (Fig. 1 *A*). Alignment of the Xklp3 motor domain sequence with other KLP sequences had previously shown that Xklp3 was closely related to *Drosophila* KLP4 and KLP5 (renamed KLP64D and KLP68D), both members of the KIF3/KRP85/95 or Kinesin II subfamily (Vernos et al., 1993; Pesavento et al., 1994; Scholey, 1996). Xklp3 full-length sequence showed that it also shared a high degree of similarity to these KLPs outside the motor domain. The highest scores were obtained with KIF3B (Yamazaki et al., 1995), with 90% amino acid similarity in the motor domain, 85% in the stalk, and 74% in the tail (Fig. 1 *B*). This high level of conservation suggests that Xklp3 is probably the *Xenopus* counterpart of mouse KIF3B.

Xklp3 Is Part of a Trimeric Complex

To characterize Xklp3 we raised two polyclonal antibodies: one against the motor domain (anti–Xklp3-M) and another against the COOH-terminal tail domain (anti–Xklp3-tail). Both affinity-purified antibodies, as well as a monoclonal antibody, mAb X3T-3A6, recognized a single band with an apparent M_r of 95 kD on immunoblots of extracts from *Xenopus* eggs and from two different *Xenopus* cell lines, XL177 and A6 (Fig. 2 *A*). We then looked at Xklp3 ATP-dependent microtubule-binding activity. In

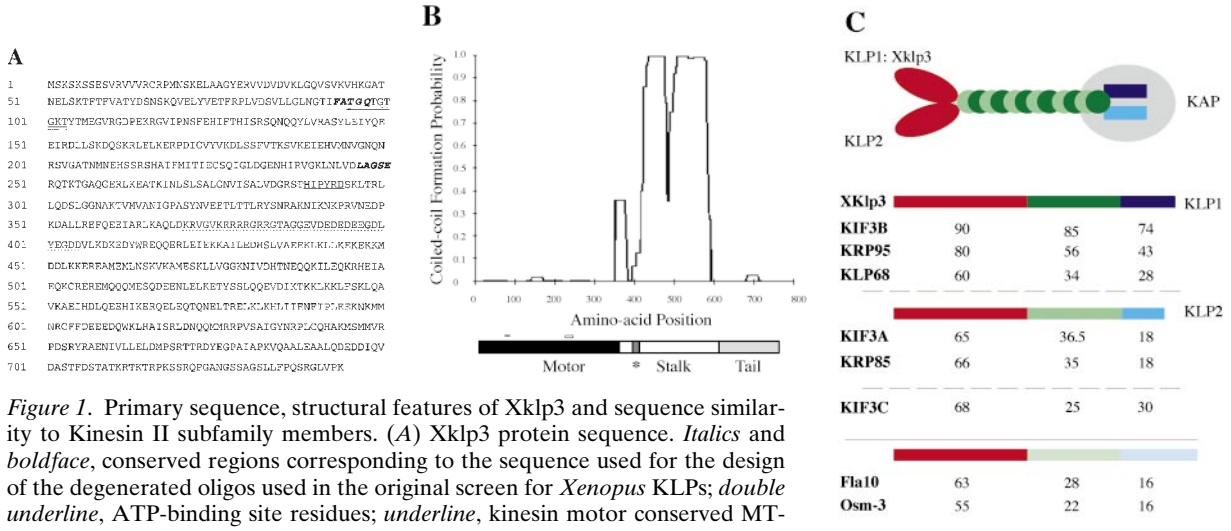


Figure 1. Primary sequence, structural features of Xklp3 and sequence similarity to Kinesin II subfamily members. (*A*) Xklp3 protein sequence. *Italics* and *boldface*, conserved regions corresponding to the sequence used for the design of the degenerated oligos used in the original screen for *Xenopus* KLPs; *double underline*, ATP-binding site residues; *underline*, kinesin motor conserved MT-binding site; *dotted underline*, region rich in charged amino acids that is present in other members of the family. This region has been proposed to favor heterodimerization over homodimerization. These sequence data are available from GenBank/EMBL/DDBJ under accession number AJ009839. (*B*) Output of the pepcoil program showing the probability of formation of coiled-coil interactions. Below a schematic representation of Xklp3 is shown, with regions predicted to be coiled coil (*white*). *Asterisk*, region rich in charged amino acids that interrupts the coiled-coil predicted domain and is present in other members of the family. The ATP (*dark line*) and MT binding sites (*white rectangle*) in the motor region are indicated. (*C*) Similarity between protein sequences of Xklp3 and the other Kinesin II family members. Schematic representation of the Kinesin II heterotrimeric complex, composed of two closely related kinesins (*Klp1* and *Klp2*) and a third nonmotor subunit (*KAP*). Each kinesin-like protein has three subdomains: motor (*red*), stalk (*green*), and tail (*blue*). The third nonmotor subunit (i.e., KAP115 in sea urchin, KAP3 in mouse) is represented as a *gray oval* interacting with the tails. Xklp3 sequence is taken as a reference and the numbers correspond to the percentage of similarities found in the different domains between Xklp3 and the corresponding KLP. The first group comprises KLPs more closely related to Xklp3, the second group comprises the second KLP subunit from the complex. In the third subgroup, Xklp3 sequence is compared with the mouse KIF3C, a motor able to heterodimerize with KIF3A but not with KIF3B. We place other members from this subfamily in the fourth group.

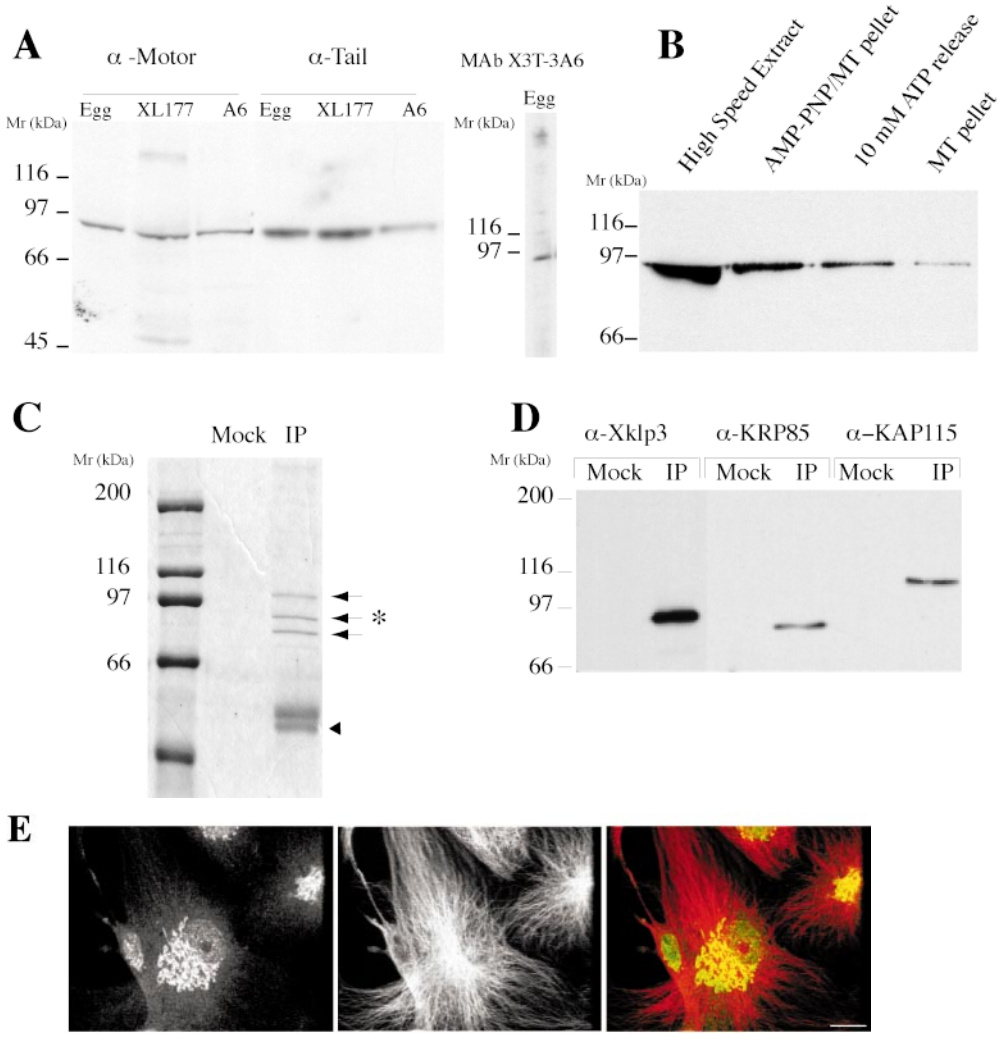


Figure 2. Xklp3 is a subunit of the Kinesin II heterotrimeric complex and is associated to Golgi membranes. (*A*) Characterization of antibodies against Xklp3. Antibodies raised against the NH_2-terminal (*α-Motor*) and the COOH-terminal (*α-Tail*) region of Xklp3 recognize a single band of ~95 kD on Western blots of *Xenopus* low-speed egg extract, XL177, and A6 cell extracts. The monoclonal anti-tail, mAb X3T-3A6, recognizes a single band at 95 kD in low-speed egg extract. (*B*) Xklp3 cosediment with microtubules in the presence of AMP-PNP, and is released from the MT by addition of ATP. *Xenopus* egg extract (*High Speed Extract*) was used to prepare a fraction enriched in motor proteins (Materials and Methods). The high speed extract, the MT pellet in presence of AMP-PNP, the motor proteins released from MT by ATP, and the MT pellet after ATP elution were analyzed by Western blot and probed with the anti–Xklp3-tail antibody. Most of Xklp3 is released from the microtubules by 10 mM ATP. (*C* and *D*) Xklp3 is a subunit of a het-

egg extracts, Xklp3 copelleted with endogenous taxol-stabilized microtubules in the presence of 5′-adenylyllimidodiphosphate (AMP-PNP) and was released from the microtubule pellet by 10 mM ATP (Fig. 2 *B*), similar to the behavior of kinesin and other KLPs (Bloom and Endow, 1994).

Most members of the Kinesin II subfamily form heterotrimeric complexes (Scholey, 1996). To determine whether this was also the case for Xklp3, we used the anti–Xklp3-tail antibody to immunoprecipitate the complex from *Xenopus* egg extracts. Three proteins of 95, 80, and 100 kD coimmunoprecipitated in equimolar amounts as estimated by SDS-PAGE gel (Fig. 2 *C*, *arrows*). Immunoblots of the immunoprecipitated material showed that the 95-kD band was Xklp3. The 80-kD band was recognized by an antibody against sea urchin KRP85 (the second motor subunit, Cole et al. [1993]) and the 100-kD band by an antibody against kinesin associated protein KAP115 (the third nonmotor subunit, Wedaman et al. [1996]). We conclude that Xklp3 is part of an heterotrimeric complex in *Xenopus* that is homologous to sea urchin Kinesin II and mouse KIF3A/KIF3B/KAP3 complexes (Fig. 1 *B*). Since in sea urchin and mouse, Kinesin II (Cole et al., 1993; Yamazaki et al., 1995) is a plus end–directed motor, it is likely that this is also the case for *Xenopus* Kinesin II.

Xklp3 Is Associated with Membranes from the Golgi Network

We first determined the expression pattern of Xklp3 by Western blotting of different *Xenopus* tissues. We found Xklp3 in oocytes, eggs, testis, brain, XL177, and A6 cells. It was also present, although in lower amounts, in kidney and stomach and at very low levels in heart, lung, and muscle (data not shown). These data are consistent with the ubiquitous tissue distribution found for Kinesin II in mouse (Yamazaki et al., 1995).

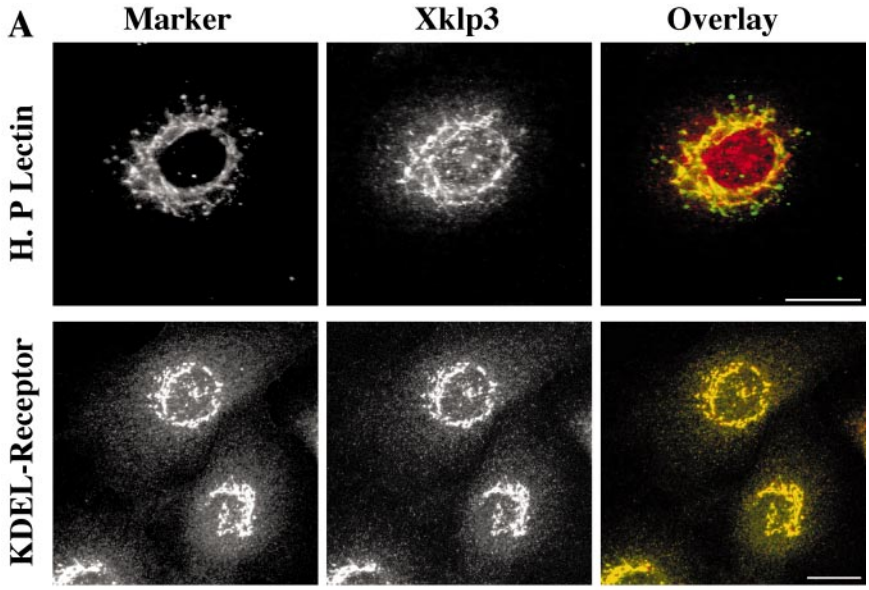
We then examined the subcellular localization of Xklp3 in XL177 and A6 *Xenopus* cells. Immunofluorescence analysis of interphase XL177 cells with the anti–Xklp3-tail antibody revealed a bright staining concentrated in a perinuclear or juxtanuclear position (Fig. 2 *E*). The structure stained by the antibody appeared as a network of interconnected tubules reminiscent of the Golgi apparatus. In addition to this bright network, we could detect in some cells a light labeling of vesicle-like structures scattered throughout the cytoplasm. In mitotic cells, the labeled network disappeared and was replaced by a punctuate staining in the cytoplasm enriched around the spindle poles (data not shown). This staining pattern was similar to that observed in *Xenopus* dividing embryos (data not shown). We did not see any enrichment of labeled structures at the spindle equator in anaphase embryonic cells, as it has been reported for sea urchin Kinesin II (Henson et al., 1995).

To determine if the interphase network stained by the anti–Xklp3-tail antibody corresponded to the Golgi apparatus, we performed double immunofluorescence labeling on XL177 cells with the anti–Xklp3-tail antibody and different markers of Golgi membranes. Xklp3 staining colocalized extensively with the Helix pomatia (HP) lectin that binds *N*-acetyl-galactosamine residues generated at the first step of O-linked glycosylation (Pavelka and Ellinger, 1985; Roth, 1984), in the Golgi apparatus (Fig. 3 *A*). It colocalized perfectly with the KDEL-receptor (revealed by an anti–KDEL-receptor antibody, Fig. 3 *A*), an itinerant Golgi protein which recycles KDEL-containing ligands from the Golgi back to the ER (Lewis and Pelham, 1992). The KDEL-receptor is usually localized in the *cis*-Golgi and the intermediate compartment (Tang et al., 1993). We also observed a perfect colocalization of Xklp3 with β-COP a component of the COP I complex implicated in transport steps between the ER and the Golgi (data not shown).

To further define the localization of Xklp3 and because of the lack of cross-reacting antibodies, we prepared three stable lines of *Xenopus* A6 cells expressing GFP-tagged versions of Golgi resident enzymes: mannosidase II–GFP (MannII–GFP), galactosyl-*N*-acetyl transferase (GalNacT2–GFP), and galactosyltransferase (GalT–GFP). In mammalian cells it has been shown that addition of the GFP tag does not interfere with the targeting of these proteins to the Golgi apparatus (for review see Lippincott-Schwartz et al., 1998). We checked by immunofluorescence with FITC conjugated HP and the anti–KDEL-receptor antibody that all the GFP-tagged proteins localized to the Golgi network in *Xenopus* cells as well (data not shown). In addition, in vivo time-lapse confocal microscopy (data not shown) and EM (see below) also confirmed that the behavior of these proteins in *Xenopus* cells was similar to what has been described for mammalian cells (Sciaky et al., 1997; Shima et al., 1997). By immunofluorescence, Xklp3 colocalized with all GFP-tagged markers in the juxtanuclear network (GalNacT2–GFP, GalT–GFP [Fig. 3 *B*] and MannII–GFP [data not shown]); in addition, Xklp3 was present on dimly labeled vesicular structures throughout the cytoplasm.

To determine whether Xklp3 was also present in the ER, we stained cells with the anti–Xklp3-tail antibody and an anti-PDI, generated against the KDEL motif of lumenal ER resident proteins (Tooze et al., 1989). Anti–Xklp3-tail and anti-PDI antibodies did not stain the same network of membranes (Fig. 4). To examine whether Xklp3 was present on ER-to-Golgi or TGN to plasma membrane

erotrimeric complex, homologous to the Kinesin II complex. (*C*) Coomassie-stained gel of an immunoprecipitation from low-speed *Xenopus* egg extract using the anti–Xklp3-tail antibody. Two proteins (80 and 110 kD, *arrows*) coimmunoprecipitate with Xklp3 (*asterisk*). A control was done using protein A beads alone (*Mock lane*), similar results were obtained using nonspecific rabbit IgG. *Arrowhead*, lower bands that represent the antibodies' heavy chains. (*D*) Western Blot of the immunoprecipitated fraction. The 95-kD band is recognized by the anti–Xklp3-tail antibody (*α-Xklp3*). The 85-kD band is recognized by an antibody against SpKRP85, the second KLP subunit of Kinesin II (*α-KRP85*), and the 110-kD band by an antibody against SpKAP115, the third nonmotor subunit (*α-KAP115*). (*E*) Double immunofluorescence on *Xenopus* XL177 cells with the anti–Xklp3-tail (*left panel*, *green*) and an anti-tubulin antibody (*middle panel*, *red*). Xklp3 localizes to an extended juxtanuclear network. The nuclear pattern observed here is not representative of Xklp3 staining (see other figures where it is not present). Bar, 10 μm.



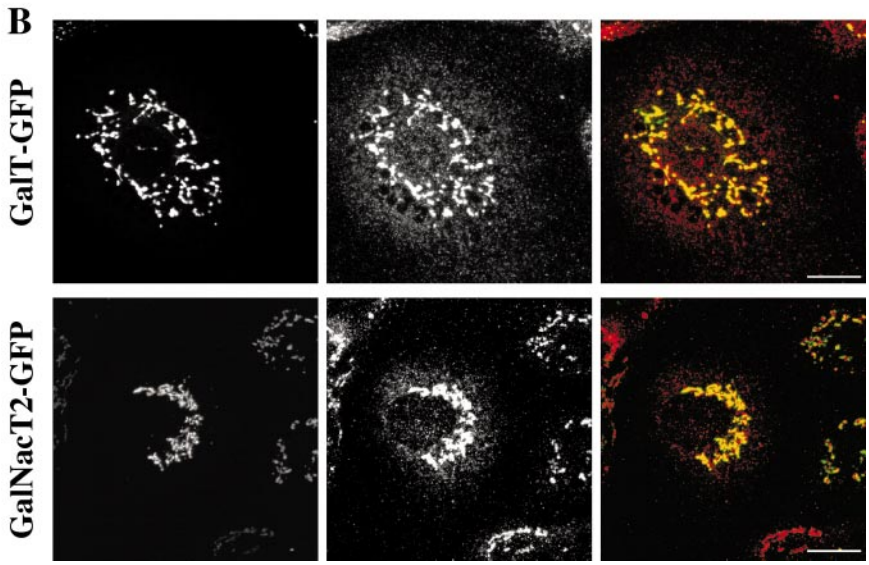


Figure 3. Xklp3 colocalization with different Golgi markers. (*A*) Double immunofluorescence on XL177 cells with the anti–Xklp3-tail antibody and antibodies against different markers for the Golgi apparatus, as indicated. Xklp3 colocalizes partially with the lectin HP and completely with the KDEL-receptor. (*B*) A6 cells stably expressing GalT–GFP or GalNacT2–GFP were fixed and processed for immunofluorescence with the anti–Xklp3-tail antibody. Xklp3 colocalizes with both markers. *Red*, Xklp3; *green*, corresponding marker. Bars, 10 μm.

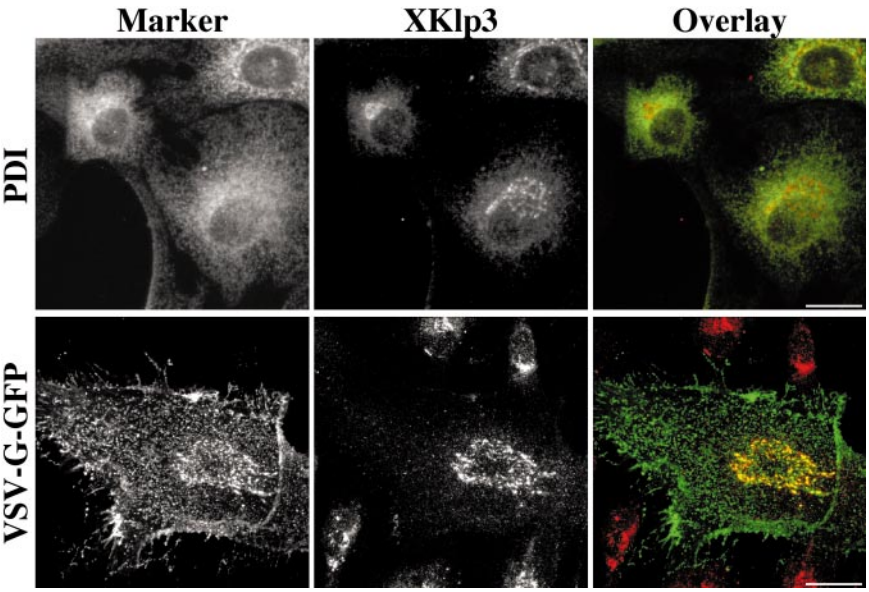


Figure 4. Xklp3 does not colocalize with ER the PDI or with post-Golgi structures en route to the plasma membrane. (*Top*) XL177 cells stained with the anti–Xklp3-tail antibody and an anti-KDEL signal antibody (anti-PDI). (*Bottom*) A6 cells transiently transfected with VSV-G–GFP and stained with the anti–Xklp3-tail antibody. Xklp3 colocalizes with VSV-G–GFP in the Golgi apparatus but not in structures at the vicinity of the plasma membrane, thus indicating that Xklp3 is not associated with post-Golgi membranes. *Red*, Xklp3; *green*, corresponding marker. Bar, 10 μm.

vesicles, we transiently transfected A6 cells with a VSV-G–GFP. VSV-G–GFP is exported from the ER to the Golgi apparatus where it is processed before being included in post-TGN membrane-bound structures and transported towards the plasma membrane (Arnheiter et al., 1984; Kreis and Lodish, 1986). Xklp3 and VSV-G–GFP colocalized extensively in the Golgi region, but no colocalization was seen in the peripheral (i.e., ER or post-TGN) vesicle-like structures or at plasma membrane extensions.

Altogether these results indicated that in A6 and XL177 *Xenopus* cell lines, Xklp3 was associated with Golgi membranes, but did not reveal to which specific Golgi subcompartment.

Upon BFA Treatment of the Cells, Xklp3 Does Not Behave As a Golgi Stack Protein

In a first attempt to determine whether Xklp3 was associated with Golgi stacks, we examined its relocalization after BFA treatment. BFA is used to discriminate secretory pathway subcompartments. This drug prevents the binding of peripheral coatomer proteins type 1 (COPI) proteins to

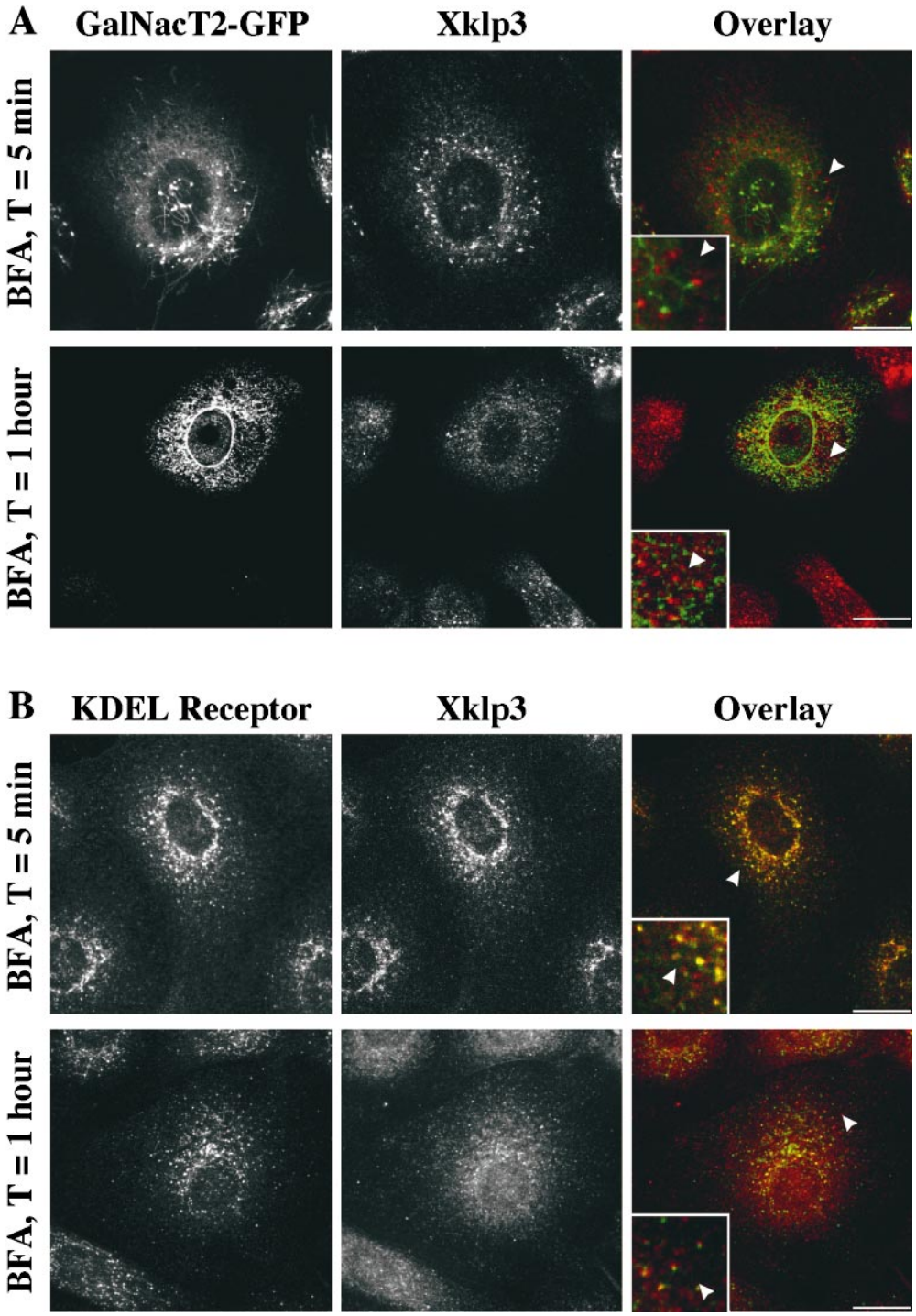


Figure 5. Xklp3 and the KDEL receptor have a similar behavior upon BFA treatment. (*A*) A6 cells expressing Gal NacT2–GFP constitutively were incubated with 5 αg/ml of BFA for 5 min or 1 h and then stained with the anti–Xklp3-tail antibodies. After 5 min of BFA treatment, GalNacT2–GFP starts to be redistributed into the ER often appearing on tubular structures. The anti–Xklp3-tail antibody gives a punctuate staining dispersed in the cytoplasm, often associated to but not overlapping with the GalNacT2–GFP staining (enlarged region). After 15 min, the structures stained by GalNacT2–GFP and the vesicular structures stained with the anti–Xklp3-tail antibody are completely distinct (*1 hour*, enlarged region). (*B*) XL177 cells were incubated with 5 μg/ml of BFA for 5 min or 1 h and double stained with the anti–Xklp3-tail and the anti–KDEL-receptor antibodies. The KDEL-receptor and Xklp3 colocalize on vesicular structures at early time points (enlarged region, overlay, *5 min*). After 1 h of treatment, vesicular structures positive for both Xklp3 and the KDEL-receptor are still seen, but part of this colocalization is lost (enlarged region, overlay, *1 hour*). *Red*, Xklp3; *green*, corresponding marker. Bar, 10 μm.

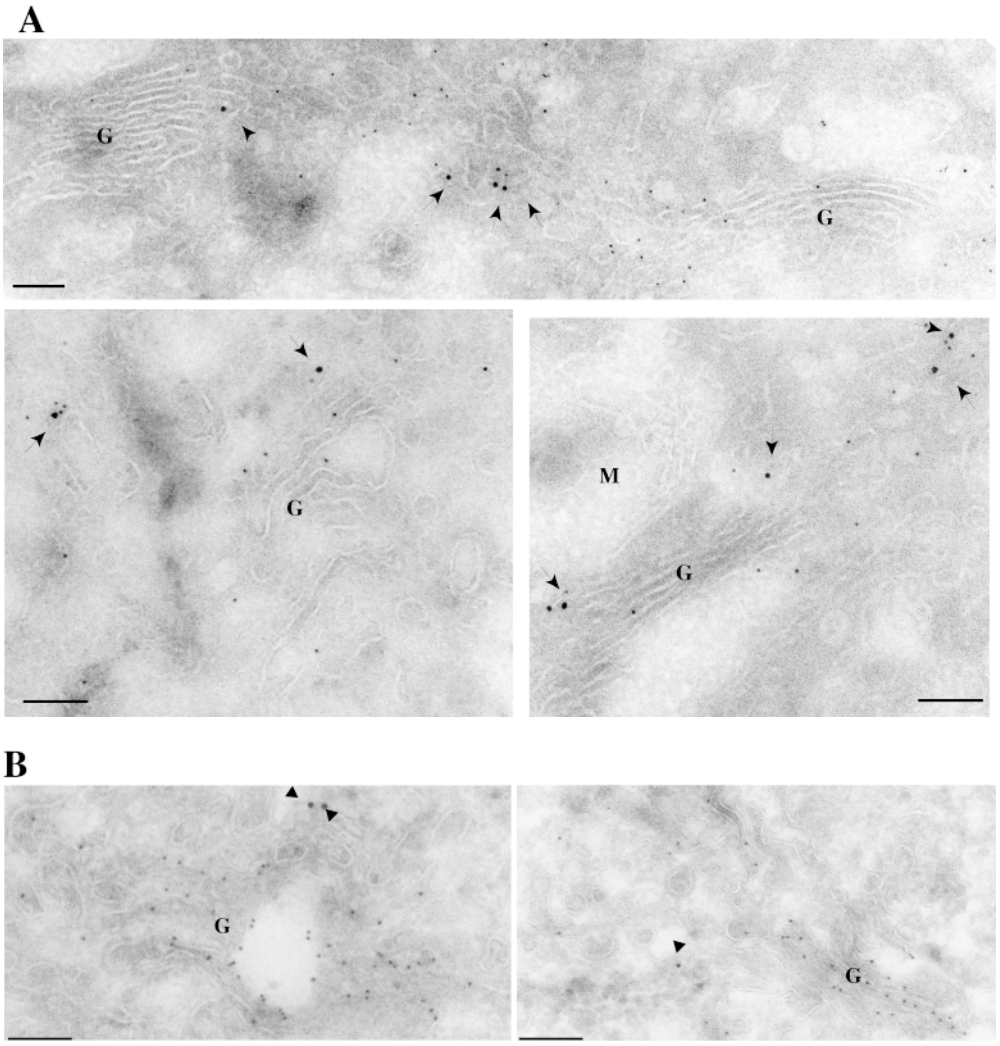
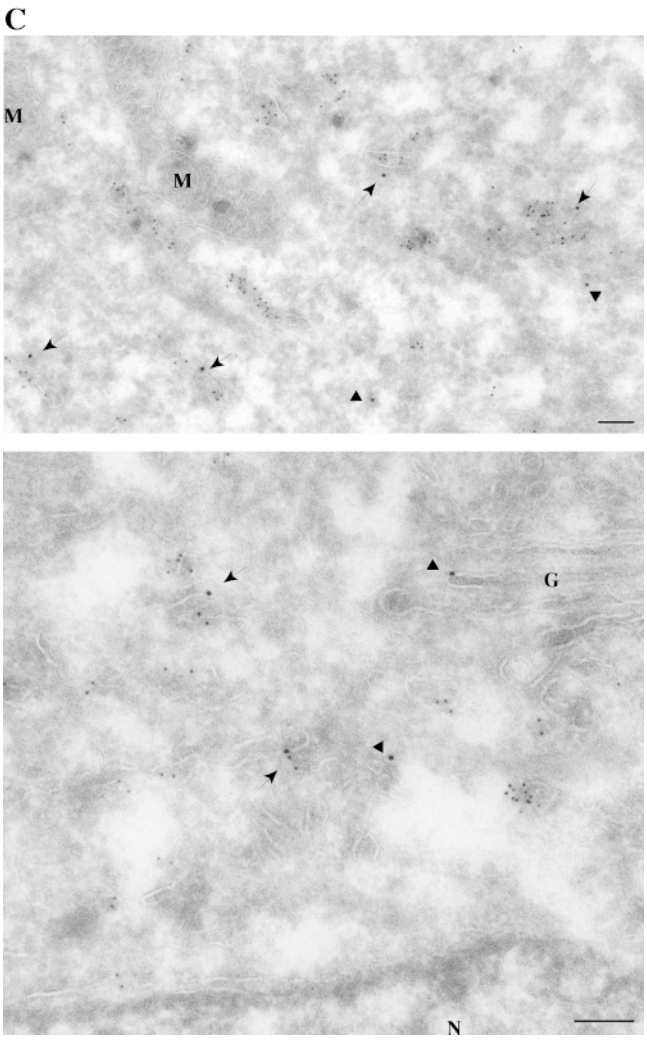


Figure 6. Xklp3 localizes to tubular-vesicular structures between the ER and the Golgi. (*A*) Double immunogold labeling of Xklp3 (mAb X3T-3A6, 10-nm gold) and the KDEL-receptor (polyclonal anti-erd2, 5-nm gold) on cryosections from A6-GalT–GFP cells. Xklp3 is present on tubular-vesicular structures (*arrows* and *arrowheads*) around the Golgi-cisternae (*G*) where it colocalizes with the KDEL-receptor (*arrows*). Similar results are obtained with the polyclonal anti-tail (see Table I for quantification). (*B*) Double immunogold labeling of Xklp3 (monoclonal anti–Xklp3-tail, mAb X3T-3A6, 10-nm gold) and the GalT–GFP (polyclonal anti-GFP, 5-nm gold). GalT–GFP is localized exclusively to the Golgi cisternae and there is no clear colocalization with Xklp3 (*arrowheads*) When Xklp3 is found on the Golgi stacks, it is always at its periphery, on budding-like structures (*A–C*). Similar results are obtained with the polyclonal anti-tail (see Table I for quantification). (*C*) Double immunogold labeling of Xklp3 (polyclonal anti–Xklp3-tail, 10-nm gold, *arrows* and *arrowheads*) and PDI (monoclonal anti-PDI, 5-nm gold). The PDI is found in cisternae throughout the cytoplasm. Xklp3 is often seen on one side of these ER membrane structures (*arrows*) (see Table I for quantification). Bar, 100 nm.

Golgi membranes leading to membrane tubulation and redistribution of Golgi resident proteins into the ER (Doms et al., 1989; Scheel et al., 1997). However, some recycling proteins such as the KDEL-receptor, do not redistribute into the ER but are dispersed into vesicular structures throughout the cytoplasm (Tang et al., 1995; Fullekrüg et al., 1997). We verified that BFA treatment of *Xenopus* A6 and XL177 cells did result in a cytoplasmic redistribution of β-COP, a component of the COPI complex (data not shown). After 5 min of BFA treatment, Xklp3 was still present in some perinuclear structures and appeared in punctuate structures dispersed throughout the cytoplasm whereas the GalNacT2–GFP was associated with long tubular processes (Fig. 5 *A*). After 1 h of treatment, Xklp3 was associated with dispersed peripheral vesicular structures while GalNacT2–GFP had completely redistributed into the ER (Fig. 5 *A*). The lack of redistribution to the ER at later time points suggested that Xklp3 was not truly associated with the Golgi stacks. We then compared Xklp3 distribution to that of the KDEL receptor in BFA-treated cells (Fig. 5 *B*). During the first 30 min of BFA treatment, Xklp3 and the KDEL receptor colocalized on some vesicular structures (Fig. 5 *B*, *5 min*, enlarged region). After 1 h of treatment, some of the vesicles stained by the anti-Xklp3 antibody were still positive for the KDEL receptor (Fig. 5 *B*, *1 hour*, enlarged region) although a fraction of them were not. These results suggested that Xklp3 was associated with membrane structures distinct from the Golgi stacks and somehow related to the recycling compartment.

Xklp3 Is Localized on Tubular-vesicular Elements between the ER and the Golgi

To identify the nature of the Golgi elements to which Xklp3 was associated, we examined thin frozen sections of cells using immunogold EM. Previous EM studies have shown that the Golgi apparatus is composed of stacks of flattened cisternae enriched in glycosylating enzymes, and tubulovesicular structures associated with the rims of the stacks probably involved in specific transport steps (Rambourg and Clermont, 1996). We first looked at the relative localization of Xklp3 and the GalT–GFP chimera using



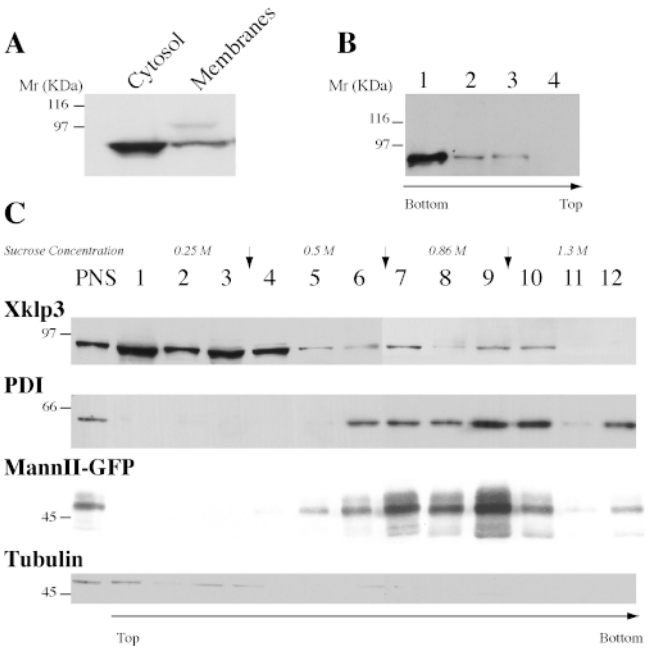


Figure 7. Xklp3 is associated to heavy membranes. (*A*) Xklp3 is found in a membrane fraction prepared from egg extracts. Western blot of a high-speed cytosol and the corresponding membrane fraction with the anti–Xklp3-tail antibody. Equivalent volumes from each fraction were loaded on each lane. (*B*) The membrane fraction shown in *A* was resuspended in 2 M sucrose, loaded at the bottom of a sucrose step gradient, and then centrifuged to equilibrium. Equivalent volumes of the different fractions were loaded on PAGE and transferred to nitrocellulose. The Western blot was probed with the anti–Xklp3-tail antibody. (*1*) 2 M/1.3 M sucrose (*Bottom*); (*2*) 1.3 M/0.86 M (heavy membranes, ~ER); (*3*) 0.86 M/0.5 M (membranes ~heavy Golgi); (*4*) 0.5 M/0.25 M (light membranes). (*C*) A fraction of Xklp3 is associated to heavy membranes from A6 cells extract. Cytosol (*PNS*) was prepared from A6 cells expressing GFP–mannosidase II constitutively as described in Materials and Methods. The PNS was then loaded on a sucrose step gradient (sucrose concentrations are written above fraction numbers; *arrows*, interface between two sucrose concentrations). Fractions were collected and run on PAGE. The Western blot was probed sequentially with the following antibodies: anti–Xklp3-tail, anti-PDI (as an ER marker), anti-GFP (to detect the Golgi marker mannosidase II–GFP), and anti-tubulin (as a nonmembrane-associated molecule). Xklp3 is present in soluble fractions *1–4*. In addition, there are two peaks of Xklp3 in fractions where heavy membranes sediment: one around fraction *7* corresponding to membranes enriched in mannosidase II–GFP, and another around fraction *9–10* corresponding to membranes enriched in PDI.

the monoclonal anti–Xklp3-tail (mAb X3T-3A6) and a polyclonal anti-GFP antibody followed by detection with protein A coupled to 10-nm (Xklp3) and 5-nm (GFP) gold, respectively. GalT–GFP was only found in the stacked cisternae of the Golgi complex as expected (Fig. 6 *B*, 5-nm gold), whereas Xklp3 was associated with tubular-vesicular structures sometimes in the proximity of the stacks (Fig. 6, *A* and *B*, *arrows* and *arrowheads*). A similar experiment using a polyclonal anti–KDEL-receptor and the same anti Xklp3 antibodies revealed that Xklp3 (10-nm gold) was found on tubulovesicular structures positive for the KDEL-receptor (Fig 6 *A*, *arrows*) (Table I, quantification). The rest of Xklp3 was localized partly in the cytoplasm or associated with unlabeled membranous elements some of which were morphologically similar to the ER. A double-labeling experiment with an anti-PDI antibody showed Xklp3 was localized as well on structures bearing PDI (Fig. 6 *C*, *arrows*) (Table I, quantification). EM studies have shown that the KDEL-receptor is distributed among the *cis*-Golgi, tubular-vesicular elements of the intermediate compartment and ER membranes (Griffiths et al., 1994). Thus the distribution of Xklp3 is similar to that of an intermediate compartment protein.

Table I. Quantification of Xklp3 Labeling on Different Membrane Structures

Compartment	Polyclonal anti–Xklp3-tail gold/μm^2 membrane	Monoclonal X3T-3A6 gold/μm^2 membrane
KDEL-receptor structures	11.6 (± 1.8)	11.6 (± 1.6)
Golgi stacks (GalT–GTP)	2.9 (± 0.6)	2.8 (± 0.6)
ER structures (PDI)	6 (± 1)	—
Cytoplasm	3.7 (± 0.8)	3.5 (± 0.7)

These data represent the average of 20 micrographs for each structure ($n = 30$ for the cytoplasm). Numbers in parentheses give the standard error of the mean.

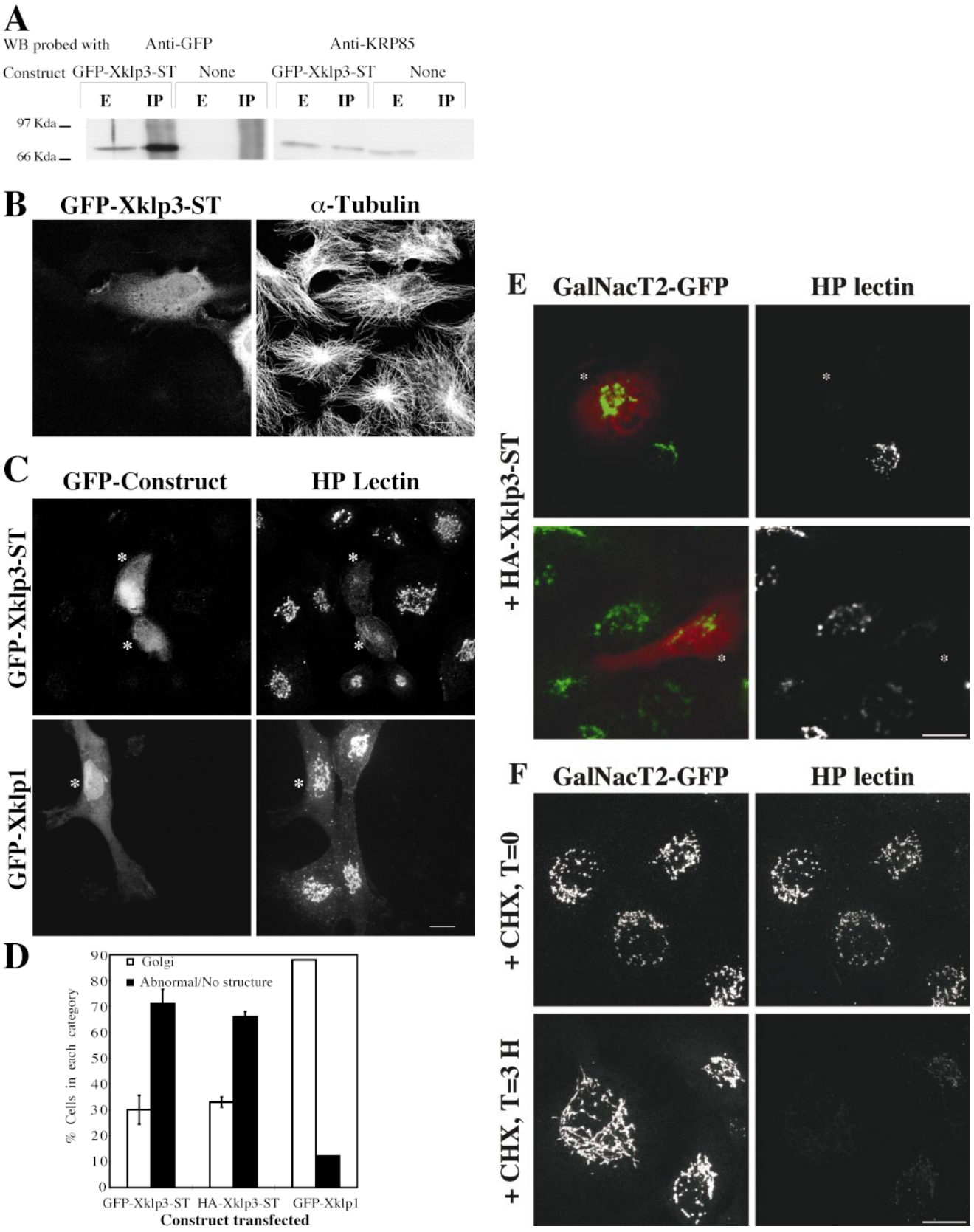


Figure 8. Effect of the expression of Xklp3–ST on Golgi staining by HP lectin. (*A*) The Xklp3–ST construct is able to bind to the endogenous Xklp3– KLP partner. A6 cells were transiently transfected with GFP–Xklp3-ST. The cytosol was used for immunoprecipitation with an anti-GFP antibody (lanes *GFP–Xklp3-ST*). The immunoprecipitate was probed with the anti-GFP and the anti-KRP85 antibodies. As a control we repeated this experiment with nontransfected cells (lanes *None*). Lane *E* is the crude extract fraction and lane *IP* the immunoprecipitate (loaded five times more than the extract). In transfected cells the anti-GFP antibody immunoprecipitates

Xklp3 Is Associated with Heavy Membrane Fractions

Membranes from the Golgi stacks, the ER, and recycling compartments behave differently upon sedimentation or floatation on sucrose gradients. We first separated the membranes from cytosolic proteins in egg extracts using a one step centrifugation, and further fractionated them by floatation. Approximately 20–25% of Xklp3 cosedimented with the membrane fraction (Fig. 7 *A*). We found Xklp3 associated with membranes that floated at the 1.3 M–0.86 M sucrose interface (like ER membranes) and at the 0.86 M–0.5 M sucrose interface (like Golgi membranes, Fig. 7 *B*). To further characterize the membrane fractions with which Xklp3 cosedimented, we prepared PNS from A6 cells stably transfected with MannII–GFP. The homogenates were then loaded on a sucrose step gradient (Fig. 7 *C*). Xklp3 was found in large amounts at the 0.25 M–0.5 M sucrose interface, probably corresponding to the soluble form of the trimeric complex. In addition, Xklp3 was found in two heavier fractions, at the 0.5 M–0.86 M sucrose interface which corresponded to a peak of Golgi membranes as revealed by the presence of MannII–GFP (Fig. 7 *C*, fraction 7, Xklp3, MannII–GFP) and at the 0.86 M–1.3 M sucrose interface which corresponded to a peak of ER/intermediate compartment as revealed by the presence of PDI. These results indicated that part of Xklp3 was associated with heavy membranes from both Golgi and ER/intermediate compartment.

Xklp3 Is Required for Normal Trafficking between the ER and the Golgi

The previous results pointed to a role for Xklp3 in the movement of tubular-vesicular elements between the ER and Golgi apparatus. To investigate the function of Xklp3, we transfected *Xenopus* cells with a plasmid expressing a truncated form of Xklp3 with either GFP (pEGFP–Xklp3-ST) or a hemagglutinin (HA) tag (pHA–Xklp3-ST) replacing the whole motor domain. Immunoprecipitation experiments with an anti-GFP antibody showed that the mutant protein could inhibit Kinesin II function by competing with the endogenous Xklp3 for the binding to the 85-kD subunit of the Kinesin II heterotrimeric complex (Fig. 1 *B* and Fig. 8 *A*). By immunofluorescence, both fusion proteins were mostly found distributed throughout the cytoplasm and not associated with the Golgi apparatus (Fig. 8, *B* and *C*). This could be explained in two ways: either the mutant protein could not be targeted to the vesicles to which the wild-type protein binds normally or the mutant protein prevented the localization of the Kinesin II complex to its target vesicles. In either case, this mutant clearly acted as a poison for the endogenous Kinesin II complex. Indeed, a similar construct has been used successfully to study Kinesin II dependent movement of the melanosomes in *Xenopus* melanophores (Tuma et al., 1998).

We first tried to detect an effect on one of the Golgi functions, glycosylation. The lectin from HP binds specifically to *N*-acetyl-galactosamine residues added at the beginning of the O-glycosylation. In A6 cells, it labels the Golgi apparatus, thus indicating that this step is occurring in the Golgi or in its direct vicinity. We stained the transfected cells with fluorescently labeled HP lectin and quantified the effect by scoring cells exhibiting a normal staining of the Golgi versus cells with no Golgi structure stained. Strikingly, the HP lectin no longer labeled the Golgi apparatus in 60–70% of Xklp3-ST–transfected cells (Fig. 8 *C*, *asterisks*; Fig. 8 *D*, quantification). This effect was similar in cells transfected with either tagged version of the mutant (Fig. 8 *C*). Transfected cells had a normal distribution of microtubules (MT) (Fig. 8 *B*), ER, mitochondria, and γ-tubulin (data not shown). As a control, we transfected cells with a plasmid expressing GFP-tagged Xklp1, a nuclear *Xenopus* kinesin-like protein involved in mitosis. Only 12% of the transfected cells had an abnormal HP lectin staining (Fig. 8 *C*, *arrow*; Fig. 8 *D*, quantification).

The effect of the Xklp3 mutant on lectin staining pattern suggested a defect in Golgi glycosylation function. The Xklp3 mutant could either affect directly the general structure of the Golgi or more specifically, the localization of the O-glycosylating enzymes. Alternatively, the mutant protein could affect the global transport of proteins from the ER to the Golgi.

To examine the morphology of the Golgi apparatus in the absence of Kinesin II function, we transfected cells

GFP–Xklp3-ST and a protein recognized by the anti-KRP85 antibody, probably the KLP partner for Xklp3. (*B*) Expression of Xklp3–ST has no effect on microtubules. A6 cells transiently transfected with GFP–Xklp3-ST were fixed and processed for immunofluorescence with an anti-tubulin antibody. Cells expressing Xklp3-ST have a normal microtubule network focused at the MTOC. (*C*) Suppression of Golgi staining by the lectin from Helix pomatia in cells expressing Xklp3–ST. A6 cells were transiently transfected with GFP–Xklp3-ST (*asterisks*), fixed, and then stained with rhodamine-labeled HP lectin. A projection of six sections taken with a LSCM is shown. In cells transfected with Xklp3–ST, the lectin no longer recognizes any identifiable Golgi structure. In contrast, the Golgi apparatus of non transfected cells is strongly stained. As a control we transfected A6 cells with GFP–Xklp1 (a *Xenopus* mitotic motor, *asterisk*). In these cells, the Golgi apparatus was normally stained by the HP lectin (*bottom*). (*D*) Quantification of the defective Golgi staining by the HP lectin. A6 cells were transiently transfected by GFP–Xklp3-ST, HA–Xklp3-ST, or GFP–Xklp1. 50–100 transfected cells were counted for each experiment. Bars correspond to the percentage of cells in each category. The standard deviations are shown ($n = 4$ for GFP–Xklp3-ST, HA–Xklp3-ST and $n = 1$ for GFP–Xklp1). *White bars*, normal Golgi HP lectin staining; *black bars*, abnormal or lack of staining. Note that 60–70% of Xklp3-ST–transfected cells have an abnormal Golgi staining, whereas this happens only in 10% of GFP-Xklp1–transfected cells. (*E*) The lack of Golgi staining by the HP lectin in cells expressing Xklp3–ST may reflect a defective transport from the ER to the Golgi. A6 cells expressing GalNacT2–GFP constitutively were transiently transfected with HA–Xklp3-ST (*asterisks*). Cells were fixed and double stained with a polyclonal anti-HA antibody, then visualized by an AMCA conjugated anti-rabbit antibody, and with rhodamine-labeled HP lectin. *Left*, *red*, HA–Xklp3-ST; *green*, GalNacT2–GFP. *Right*, HP lectin staining. The Golgi apparatus appears normal but is not stained by the rhodamine-labeled HP lectin. (*F*) In A6 cells, the lectin from HP binds to proteins en route along the secretory pathway. A6 cells expressing GalNacT2–GFP constitutively were treated for 3 h with cycloheximide, fixed, and then stained with rhodamine-labeled HP lectin. As above, the fluorescent lectin does not stain the Golgi apparatus. Bars, 10 μm.

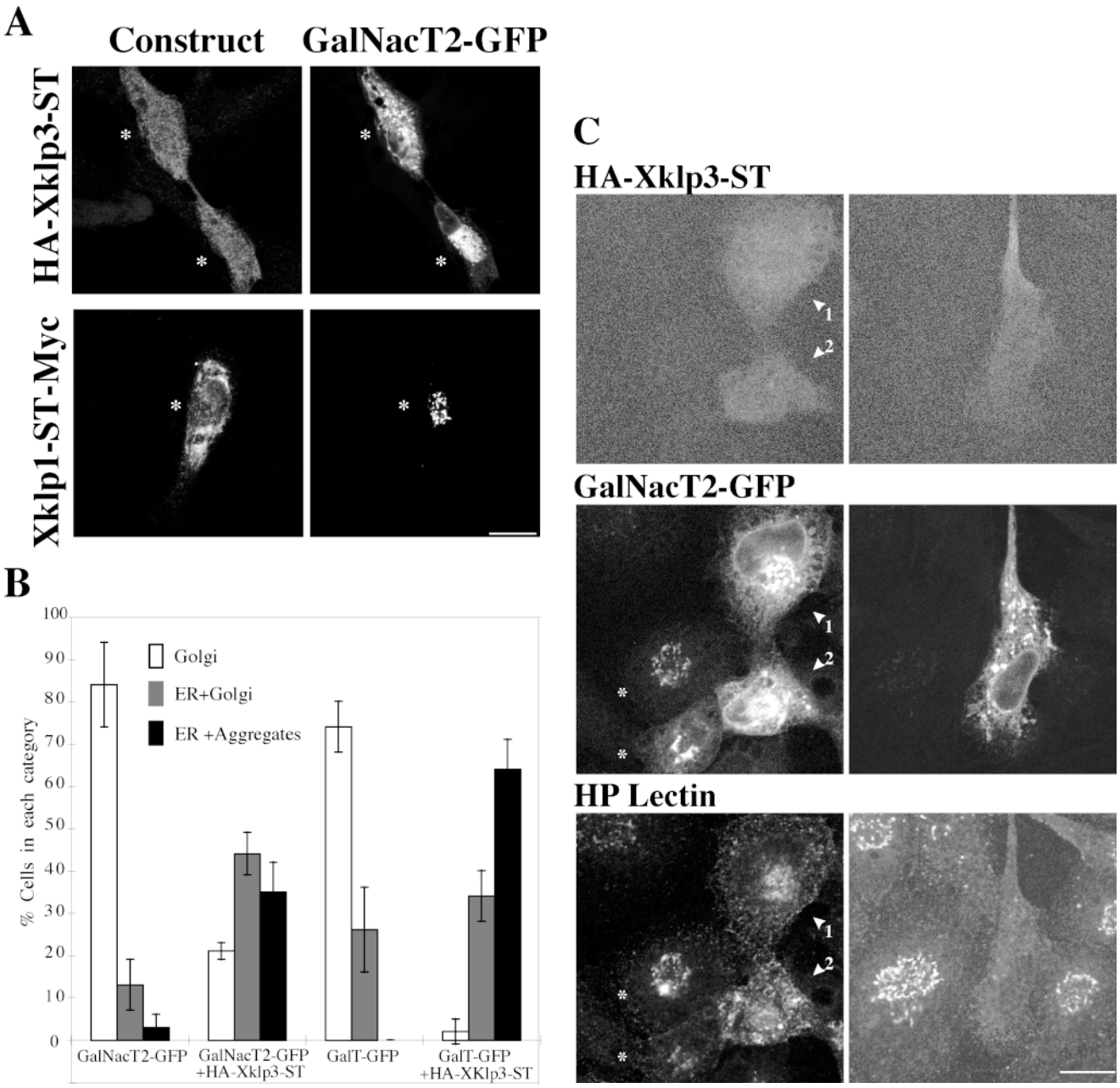


Figure 9. Newly synthesized GalNacT2–GFP and GalT–GFP proteins do not reach the Golgi apparatus in cells expressing Xklp3–ST. (*A*) A6 cells were transiently cotransfected with HA–Xklp3-ST and GalNacT2–GFP. Transfected cells (*asterisks*) were detected by immunofluorescence and confocal series were taken. A projection of six sections is presented. In cotransfected cells, GalNacT2–GFP is seen in aggregates in the cytoplasm and in the ER. In control cells, cotransfected with Xklp1-ST–myc and GalNacT2–GFP, the GFP marker localized to the Golgi apparatus. Bar, 10 μm. (*B*) Quantification of the defect of Golgi–GFP markers localization. A6 cells were transiently transfected with the different combinations indicated. Cells were scored as either having the GFP Golgi marker localized normally to the Golgi, to the ER and Golgi, or to the ER and aggregates. Bars show the percentage of cells in each category and the error bars show the standard deviation ($n = 3$). (*C*) Cells transiently cotransfected with HA–Xklp3-ST and GalNacT2–GFP exhibit an abnormal lectin staining. GalNacT2-GFP–expressing cells are detected by the GFP tag fluorescence. Cells were double labeled with a rabbit anti-HA antibody (then visualized by an AMCA conjugated anti-rabbit antibody) and the fluorescent lectin HP. Single confocal planes are presented. *Left*, note that two cells transfected only with GalNacT2–GFP (*asterisks*) show normal labeling of the Golgi by the HP lectin. Cotransfected cells show GalNacT2–GFP either in ER and Golgi (*left panel*, *arrow 1*) or in ER and aggregates (*left panel*, *arrow 2*; *right panel*). Bar, 10 μm.

stably expressing Golgi–GFP markers with the Xklp3 mutant. In these cells, the Golgi apparatus remained intact although the GFP staining was often weaker than in nontransfected cells (Fig. 8 *E*, *asterisks*). In the transfected cells, the HP lectin did not recognize any structure (Fig. 8 *E*, *top*) or very weakly the Golgi apparatus (Fig. 8 *E*, *bottom*). This suggested that the normal trafficking of proteins through the Golgi complex was altered. To test

the hypothesis that the HP lectin detected *N*-acetylgalactosamine residues of cargo proteins en route along the secretory pathway during their transit through the Golgi apparatus, we blocked protein synthesis in cells expressing constitutively GalNacT2–GFP with cycloheximide to prevent the delivery of newly synthesized proteins to the Golgi apparatus. Under these conditions, the HP lectin did not label the Golgi apparatus although the Golgi was structurally intact as indicated by the GalNacT2–GFP fluorescence (Fig. 8 *F*). This result was very similar to the one we obtained in cells transfected with the mutant Xklp3 and suggested that the mutant form of Xklp3 was interfering with the delivery of newly synthesized proteins to the Golgi apparatus.

To examine whether the mutant form of Xklp3 blocked transport between the ER and the Golgi, we cotransfected cells with HA–Xklp3-ST and GalNacT2–GFP or GalT–GFP. The GFP markers localized exclusively to the Golgi in more than 80% of control cells. Cells expressing both HA–Xklp3-ST and one GFP Golgi marker showed a strong defect in the localization of the GFP marker to the Golgi (Fig. 9, *A–C*). Interestingly, the mislocalization of the Golgi GFP marker following co-transfection with the Xklp3-ST was associated with an aberrant HP lectin staining (Fig. 9 *C*). The phenotype observed could not be due to a general effect on the MTs because the cells still had a normal MT network focused at the MTOC (data not shown). As a control, we repeated the co-transfection experiment using a plasmid expressing a fragment of Xklp1 lacking the motor domain (Xklp1-ST–myc) similar to HA–Xklp3-ST. The Xklp1-ST–myc lacks also the NLS, and as a consequence remains in the cytoplasm, as HA–Xklp3-ST does. In cells expressing both Xklp1-ST–myc and GalNacT2–GFP, the GFP marker localized properly to the Golgi apparatus (Fig. 9 *A*).

Therefore, the results obtained with the dominant-negative form of Xklp3 indicate that Kinesin II is required for the proper transport and localization of newly synthesized proteins, to the Golgi apparatus.

Discussion

In this study, we report the cloning and characterization of a subunit of the *Xenopus* heterotrimeric Kinesin II. Localization and functional data indicate that *Xenopus* Kinesin II plays an important role between the ER and the Golgi.

Evidence for an Association of Xklp3 with the Intermediate Compartment

By immunofluorescence, we show that Xklp3 is associated with the Golgi apparatus. We see a extensive colocalization with GFP chimeras targeted to the stack or to the *trans*-face of the Golgi, as well as with the KDEL-receptor, a recycling molecule enriched on the *cis*-face of the Golgi. On the other hand, we find that Xklp3 does not colocalize with markers for the ER nor with VSV-G–labeled peripheral structures en route along the secretory pathway. Several experiments suggest that Xklp3 is actually present on membranes between the ER and the Golgi, on the so-called intermediate compartment (IC). First, upon treatment with BFA, Xklp3 remains associated with vesicles that disperse throughout the cytoplasm in a way similar to some IC markers (Tang et al., 1993; Griffith et al., 1994). Second, biochemical fractionation of the cytosol shows that Xklp3-associated membranes are found in fractions containing Golgi and ER markers. This behavior has been reported for several intermediate compartment proteins (Tang et al., 1993; Griffith et al., 1994). Third, by electron microscopy, Xklp3 is present mainly on tubular-vesicular structures that are sometimes closely associated with the Golgi stacks and are often stained by an anti–KDEL-receptor antibody. From all these data we conclude that, in *Xenopus* fibroblasts, Kinesin II is associated with a specific subset of membranes localized between the ER and the Golgi.

Xklp3 and Golgi Function

Using a mutant form of Xklp3 that functions as a dominant negative, we show that the *Xenopus* Kinesin II is involved in an essential aspect of Golgi function. When the mutant is overexpressed, the HP lectin no longer detects *N*-acetyl-galactosaminyl residues on proteins in the Golgi apparatus. This indicates that Xklp3 is required for the normal localization of the O-glycosylating enzymes and/or for the normal delivery of their substrates. In fact, Xklp3 is required for the localization of newly synthesized proteins to the Golgi apparatus. When the Xklp3 mutant is coexpressed with GFP–Golgi markers, these are mislocalized to the ER or to aggregates in the cytoplasm and do not reach the Golgi apparatus. Strikingly, in this case the staining of glycosilated residues by the HP lectin is also aberrant. This indicates that there is a correlation between the delivery of newly synthesized proteins to the Golgi and the defect in lectin staining. This interpretation is further supported by the finding that the inhibition of protein synthesis results in the absence of Golgi staining by the lectin. These results lead us to propose that Xklp3 function is required for the transport of newly synthesized proteins from the ER to the Golgi apparatus.

Mode of Action for Xklp3 between the ER and the Golgi

Given the association of Xklp3 with membranes localized between the ER and the Golgi apparatus, the effect of the mutant on the transport of proteins from the ER to the Golgi apparatus and the directionality of movement of Xklp3 along microtubules (plus end–directed), we propose two mechanisms of action for Xklp3 along the secretory pathway. We know that traffic between the ER and the Golgi apparatus involves an anterograde and a retrograde transport of vesicles which requires dynein and probably plus end–directed motors like kinesin. Dynein carries transport intermediates from the ER exit sites towards the MTOC where they integrate into the Golgi apparatus (Presley et al., 1997). Although this has not been demonstrated, it is possible that a plus end–directed motor counteracts the activity of dynein during anterograde transport contributing to the steady-state organization of the Golgi network. Indeed, using an antisense approach, Feiguin et al. (1994) have demonstrated that conventional kinesin may be needed for organizing the Golgi network in astrocytes. Because expressing the Xklp3 mutant interferes

with the normal delivery of newly synthesized proteins to the Golgi, it is possible that Xklp3 participates in such a process.

On the other hand, two observations suggest that kinesin and/or KLP(s) are involved in the retrograde transport of vesicles from the Golgi apparatus to the ER. The inhibition of kinesin blocks the formation of tubular-vesicular structures between the Golgi and ER in the presence of BFA (Lippincott-Schwartz et al., 1995) and live studies have shown that the KDEL-receptor (important for the recycling of ER resident proteins) is associated with tubular-vesicular membrane structures forming at a velocity compatible with the involvement of a kinesin related protein (Sciaky et al., 1997). Therefore the steady-state size, location, and function of the Golgi apparatus may be strongly dependent on the relative rates of anterograde and retrograde transport. As a consequence, we can imagine that blocking a retrograde transport motor, here the Kinesin II, could also affect the anterograde pathway. An apparently surprising result is that even though inactivation of Xklp3 leads to a strong defect in ER to Golgi transport, it does not affect dramatically overall Golgi morphology, at least during the time course of our experiments. There are several possible explanations to this result. However, we favor the idea that blocking the retrograde transport results in freezing the flux of components throughout the secretory pathway, temporarily maintaining an apparently intact Golgi structure. It is also possible that by inhibiting Kinesin II, we block one specific part of the transport and that other motors keep other aspects of the secretory pathway active.

At this point, we emphasize that these two possibilities are only possible interpretations of our results, based both on our localization and functional data. More work will be required to establish exactly how the *Xenopus* Kinesin II is involved in the transport of components between the ER and the Golgi apparatus.

Kinesin II Functions

In various organisms, Kinesin II family members have been reported to associate with different types of membrane structures. In mouse neuronal cells, KIF3A/KIF3B/KAP3 is found on vesicles, distinct from synaptic vesicles, in the cell body and along the axon (Yamazaki et al., 1995). It has been recently reported that mouse KIF3C (able to heterodimerize with KIF3A but not with KIF3B [the Xklp3 mouse homologue]) colocalizes with Giantin, a Golgi membrane protein, in spinal cord neuronal cells (Yang and Goldstein, 1998). In sea urchin embryos, Kinesin II is associated with vesicles enriched at the poles, and at the spindle interzone (Henson et al., 1995). Antibody microinjection experiments have shown that, in sea urchin, KRP95/KRP85/KAP115 is necessary during embryogenesis for correct delivery of ciliary components (Morris and Scholey, 1997). In *Chlamydomonas*, the *fla10* gene product, the KRP85 homologue is associated to protein complexes beneath the flagellar membrane and mutant phenotypes suggest that FLA10 is involved in the intraflagellar transport (IFT) of protein complexes required for flagellar formation (Cole et al., 1998; Kozminski et al., 1995). In *Xenopus*, Kinesin II is present as well on purified melanosomes prepared from skin melanophores and a Xklp3 mutant protein equivalent to the Xklp3-ST used in this study inhibits the outward movement of melanosomes (Rogers et al., 1997; Tuma et al., 1998).

All these results indicate that Kinesin II associates with different organelles depending on the cell type and may play a role in a wide variety of processes. It is possible that this functional diversity is related to the heterotrimeric nature of the molecule and to the existence of isoforms or to the association of a core molecule with various proteins. Future work attempting to understand how it is targeted to a specific organelle and function should be of great interest.

It is worth noting that, in many cases, Kinesin II seems to antagonize the activity of dynein. In *Chlamydomonas*, cytoplasmic dynein is required to counteract FLA10 driven force and move the IFT particles back to the basal body of the flagella (Pazour et al., 1998). *Xenopus* melanosomes carry both the Kinesin II complex and cytoplasmic dynein. In this report we present evidence that *Xenopus* Kinesin II is involved in some step of the secretory pathway which also requires the activity of dynein.

This report is one of the first indications, together with the recent finding about the Rab6 interacting kinesin, and the mouse KIF1C, of the targeting of a KLP to a specific subcompartment of the secretory pathway and of a function for this KLP in this subcompartment. It is possible that different types of motors will be found targeted to specific domains of the secretory pathway, participating to the sorting process. The identification of such molecules may help to understand better the complexity of membrane traffic between the ER and the Golgi apparatus. The fact that the rabkinesin is targeted to the Golgi apparatus through a small G protein (Rab 6) raises the possibility that there is a whole pattern of localization of motors to specific membranes by adaptors like the G proteins. In this sense it is interesting to note that the human homologue of KAP3/KAP115, the third non motor subunit of Kinesin II, was cloned looking for proteins interacting with a small GTP-binding protein dissociation factor (Smg-GDS) (Shimizu et al., 1996). Smg GDS itself regulates the interaction of various small G proteins with membranes and might link the heterotrimeric kinesin to specific membrane cargoes via G protein interactions. Therefore, it will be of interest to examine whether different KLPs are involved in the transport of specific membrane compartments in the secretory pathway.

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