Kinfectin Anchors the Translation Elongation Factor-1\(\delta\) to the Endoplasmic Reticulum*

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Kinfectin has been proposed to be a membrane anchor for kinesin on intracellular organelles. A kinfectin isoform that lacks a major portion of the kinfectin-binding domain does not bind kinfectin but interacts with another resident of the endoplasmic reticulum, the translation elongation factor-1 delta (EF-1\(\delta\)). This was shown by yeast two-hybrid analysis and a number of in vitro and in vivo assays. EF-1\(\delta\) provides the guanine nucleotide exchange activities on EF-1\(\alpha\) during elongation step of protein synthesis. The minimal EF-1\(\delta\)-binding domain on kinfectin resides within a conserved region present in all the kinfectin isoforms. Overexpression of the kinfectin fragments in vitro disrupted the intracellular localization of EF-1\(\delta\) proteins. This report provides evidence of an alternative kinfectin function as the membrane anchor for EF-1\(\alpha\) on the endoplasmic reticulum and provides clues to the EF-1 complex assembly and anchorage on the endoplasmic reticulum.

Kinfectin has initially been proposed to be a membrane anchor for kinesin on intracellular organelles (1, 2). It is an integral membrane protein largely confined to the endoplasmic reticulum (ER)\(^1\) (1, 3). Kinfectin consists of a 120-kDa polypeptide and a 160-kDa polypeptide interacting through an \(\alpha\)-helical coiled-coil domain to form a heterodimer (4). The 120-kDa polypeptide is the truncated version of the 160-kDa polypeptide, lacking the first 232 amino acids, in the N terminus (4). The N terminus of the 160-kDa polypeptide consists of a transmembrane domain that anchors kinfectin to organelle membranes, potentially with the help of the 7 myristylation sites throughout the molecule (3, 4). The C terminus of kinectin consists of two functional domains. The kinectin-binding domain can interact with the cargo-binding site of the conventional kinesin and enhance the kinesin's microtubule-stimulated ATPase activity (5). A separate domain interacts with small G-proteins such as Rho A and Rac 1 (6, 7) and plays a key role in mediating the microtubule-dependent Rho G activity (8). The role of kinectin in organelle motility is further supported by antibody inhibition and kinectin-domain overexpression studies, in which the organelle motility is adversely affected both in vitro and in vivo (2, 5).

The role of kinectin as a universal membrane anchor for kinesin has been questioned when kinectin’s restricted intracellular and phagocytic distributions were discovered. Kinectin is not detected in axons of cultured neurons where kinesin is the major motor responsible for fast anterograde transport (9). Furthermore, the kinectin gene is not found in Caenorhabditis elegans or Drosophila genomes, where conserved conventional kinesin heavy chain gene is present (10). These findings suggest that additional or alternative membrane anchors for kinesin must exist for organelle motility. Recent yeast two-hybrid and biochemical studies have identified a few new kinesin-interacting partners on different organelles. For example, Sunday Driver interacts with the tricopeptide repeats of the kinesin light-chain subunit of kinesin-1 and mediates the axonal transport of post-Golgi vesicles (11). Kinesin-1 was proposed to link to a class of transport vesicles via the JIP-1 and JIP-2 scaffolding proteins that bind to members of the low density lipoprotein receptor family (12). Another scaffold protein, 14-3-3 proteins, may act as a membrane anchor for KIF1C (13). The transmembrane amyloid precursor protein is another potential membrane anchor for kinesin-1, which directly binds the tricopeptide repeats of the kinesin light chain (14). The AP-1 clathrin-associated adaptor complex, which mediates the transport of clathrin-coated vesicles from the trans-Golgi network to plasma membrane, binds KIF13A (15). There are studies revealing a potential interaction between KIF3 and fodrin (brain spectrin) on neuronal vesicles (16). The recently identified dendrite-specific kinesin KIF17 can also interact directly with the PDZ domain of mLin-10 (17). Therefore, a paradigm has emerged that motor proteins utilize different membrane anchors and the same motor protein, such as kinesin-1, can bind to different membrane anchors on different organelles (18). Since kinectin is primarily localized to the ER (1, 3), sparsely on the lysosome (8) and mitochondria\(^2\) but not Golgi apparatus, its primary functions may be restricted to these organelle compartments.

Different kinectin isoforms with combinations of variable domains (vd) have been reported in human, mouse and fox genomes (19–21).\(^2\) There are at least five small (23–33 amino acid residues) variable domains scattered throughout the C terminus of kinectin. Two variable domains (vd3: amino acid residues 1177–1200 and vdm4: amino acid residues 1229–1256) overlap the kinesin-binding domain on kinectin (5). This implies that the kinectin isoforms lacking either vdm3 or vdm4 can-

\(^1\) The abbreviations used are: ER, endoplasmic reticulum; EF-1, translation elongation factor-1; vdm, variable domain; BD, binding domain; AD, activation domain; H1S3, histidine; GST, glutathione S-transferase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SPR, surface plasmon resonance; RU, response unit; DIG, digoxigenin; GFP, green fluorescent protein.

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not serve as the membrane anchors for kinesin. Such isoforms without vd3 or vd4 have indeed been identified in cells (Fig. 1A) (20). We have investigated the function of one such kinecin isoform lacking vd4 in ER.

We have isolated from a human fetal brain cDNA library a naturally occurring kinecin isoform that contains variable domains vd1, vd2, vd3, vd5, and vd6 but lacks vd4 (Fig. 1A). This kinecin isoform did not interact with the conventional kinesin heavy chain in a yeast two-hybrid analysis (Fig. 1A). However, it interacted with another resident of the ER, the translation elongation factor-1a delta, in a series of in vitro and in vivo experiments.

EF-18 is a subunit of the protein complex that participates in the elongation step during the translation of mRNA. The EF-1 complex consists of four subunits, namely, α, β, δ, and γ. The GTP binding protein, EF-1α, is responsible for the transfer of aminoacyl-tRNA to ribosome with the hydrolysis of GTP. The EF-1β and EF-18 subunits are responsible for the nucleotide exchange activity, which converts the EF-1α-GTP to the active EF-1α-GTP (22–24). Both β and δ sequences appear unrelated other than the C-terminal domain, which is responsible for the nucleotide exchange activity (25). The exact function of EF-1γ is not yet known, although there is studies indicating that EF-1γ can enhance the guanine nucleotide exchange activity of EF-1β, and its association with tubulin suggests that EF-1γ may help to anchor EF-1β to cell structures like microtubules and membranes (26, 27).

We report here that kinecin interacts with EF-18 to anchor it to ER. Disruption of the kinecin-EF-18 interaction interferes with the EF-18 distribution in ER. The results provide us with a better understanding of the kinecin function in the ER.

EXPERIMENTAL PROCEDURES

The restriction enzymes used were from Promega (Madison, WI), and all of the other reagents were purchased from Sigma unless otherwise stated.

Construction of the Kinectin Bait D—A pair of oligonucleotides flanking 2539–4143 base pair (bp) of the human kinecin gene (GenBank™ accession number Z22551) was designed to isolate different kinecin isoforms. A standard polymerase chain reaction (PCR) (28) was carried out using the human fetal brain, fetal liver, adult brain, and prostate libraries (Clontech). The amplified PCR products were subcloned into pTOPO vector (Invitrogen) by TA cloning. The different kinecin isoforms were grouped by restriction mapping, and their sequences were assayed using an ABI PRISM™ 377 DNA sequencer, according to the manufacturer's instructions. The results were analyzed with the LASERGENE (DNASTAR) and the BLAST 2.0 (National Library of Medicine) software.

In Vitro GST Pull-down Assay—The glutathione S-transferase (GST) fusion constructs with the kinecin baits A, B, and D were made by subcloning the corresponding cDNA into the expression vector pGEX4T-1 (Amersham Biosciences). A His6-tagged fusion construct with EF-18 was made by subcloning the EF-18 cDNA into pRSET expression vector (Invitrogen). The fusion proteins were expressed in Escherichia coli strain BL21/pLyS8 (DE3) (generous gift from Niovit Santaamt, Cyprus Institute of Neurology and Genetics, University of Cyprus). Protein expression was induced by the addition of isopropyl-β-D-thiogalactoside. Intact bacterial cells containing the expressed fusion proteins were collected by centrifugation at 10,000 × g. Protein extracts were obtained by freezing and thawing the cell pellet and resuspend the pellet in GST purification buffer (1× phosphate-buffered saline (PBS), 50 mM Tris-HCl, pH 8, 0.5 mM MgCl2, 0.1% (v/v) Triton X-100) plus 5 mM dithiothreitol and a mixture of protease inhibitors (1 μg/ml each of aprotinin, leupeptin, pepstatin A and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of the GST fusion proteins with baits A, B, and D were immobilized on glutathione-agarose beads by end-over-end mixing in a 1.5-ml microcentrifuge tube for 2 h at 4 °C. The beads were washed three times with GST purification buffer to remove unbound proteins. The expressed His6-EF-18 (36 kDa) fusion protein extract was allowed to interact with the GST-bait fusion protein coupled beads for 2 h at 4 °C with end-over-end mixing. Any unbound proteins were removed through extensive washings. The proteins that remained bound to the immobilized GST-baits were released by boiling in SDS gel sample buffer, analyzed by SDS-polyacrylamide gel (12%) electrophoresis (PAGE), and immunoblotting with mouse RGS-His antibody (Qiagen). Antibody binding was detected with goat anti-mouse secondary antibody, coupled to horseradish peroxidase according to the manufacturer's instructions (Sigma).

Co-immunoprecipitation of Kinectin with EF-18—African green monkey kidney cells, COS7, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin. For transient transfection experiments, COS7 cells were transfected with the pGEX-GST tagged kinecin bait D (64 kDa) and pRSET-EF-18 (36 kDa) were generated by subcloning the corresponding cDNA into the pXJ-GST and pXJ-FLAG mammalian expression vectors, respectively (32). Both constructs were co-transfected into COS7 cells cultured in a 60-mm dish using TransFast™ transfection reagent (Promega). The transfected cells were washed with ice-cold 1× PBS at 48 h after transfection. The cells were isolated into 1 ml of the ice-cold GST purification buffer containing protease inhibitors (as described above). Cell lysate was prepared by passing the cells through 25-gauge needle for 10 times. Intact cells and debris were removed by centrifugation at 10,000 × g for 10 min at 4 °C. 60 μl of the equilibrated 50% glutathioneagarose bead suspension was added to the cell lysate and incubated for 1 h at 4 °C. Immunoprecipitation reactions were performed by adding a mixture of anti-GST (Santa Cruz Biotechnology) and anti-FLAG M2 monoclonal antibodies. Antibody binding was detected as described above.

Anti-EF-18 Polyclonal Antibody Preparation—The EF-18 recombinant protein was expressed in E. coli BL21 using pRSET vector (Qiagen). The fusion protein was purified using the nickel-nitriilotriacetic acid agarose bead affinity column (Qiagen). The purified EF-18 protein was injected into rabbit by standard protocol (33). The antiserum was affinity-purified using the first 100 amino acids of the EF-18 protein coupled to cytochrome oxidase-activated Sepharose 4B (Pharmacia).

In Vitro Phosphorylation of EF-18 by cdc2 Kinase—The HeLa cell lysate was prepared using SF buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1% sodium orthovanadate, 25 mM β-glycerophosphate) in the presence of protease inhibitors (as described above). 1 μg of the HeLa cell lysate was incubated with or without 10 units of cdc2 kinase in kinase reaction buffer (50 mM Tris-HCl, 10 mM MgCl2, 2 mM dithiothreitol, 1 mM EGTA, pH 7.4, 25 °C, 30 min). Reaction samples were separated by SDS-PAGE and the EF-18 protein was detected by immunoblotting with the purified anti-EF-18 polyclonal antibody. Antibody binding was detected with swine anti-rabbit secondary antibody (Dako), coupled to horseradish peroxidase according to the manufacturer's specifications.

In Vitro Binding of the Endogenous EF-18 (32 kDa) with Kinectin Baits—Bacterially-expressed kinecin baits A, B, and D were coupled onto glutathione-agarose beads as described above. HeLa cells were cultured in DMEM supplemented with 10% FCS, l-glutamine, penicillin, and streptomycin. The cells were harvested and re-suspended with

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RESULTS

The Translation Elongation Factor-1 Delta Interacts with Kinectin in Yeast Two-hybrid Screenings—To identify binding partners for kinectin isoforms lacking either vd3 or vd4 (kinesin-binding domain), we first isolated naturally occurring kinectin isoforms as baits. A PCR strategy was designed to amplify the region between 2539 and 4143 bp of the human kinectin gene using a pair of primers in the conserved sequences. The PCR reaction was carried out using human fetal brain, fetal liver, adult brain and prostate libraries. The amplified PCR bands were subcloned into pTOPO vector by TA cloning. A total of 7 unique naturally occurring kinectin isoforms were isolated and classified into two categories, namely either with or without vd4. One of the kinectin isoforms that contains vd1, 2, 3, 5, and 6 but lacks vd4 (Fig. 1A) was chosen for yeast two-hybrid screenings. The region between 3142 and 4139 bp (amino acid residues 1024–1536), which contains vd2, vd3, and vd5, was subcloned into the BD vector and renamed as bait D. Bait D does not contain the predicted leucine zipper motifs (amino acids 964–9621) in the core of a predicted α-helical coiled-coil. Baits A, B, and D showed no interaction with the conventional kinesin heavy chain in yeast two-hybrid analysis (Fig. 1A). This is consistent with our previous findings that the vd3 and vd4 of kinectin are required for kinesin binding (5).

In order to identify interacting partners of the kinectin isoforms that lack the kinesin-binding domain, we performed a yeast two-hybrid screening of the human fetal brain cDNA library. A total of 17 unique positive-clones were identified from screening ~2.35 × 10^7 transformants. Among them, one of the positive clones has sequence identical to the published human translation elongation factor-1 delta sequence (EF-18, GenBank™ accession number NM001960). The plasmid obtained has an insert of 1200 bp in length and encodes the full-length EF-18 protein.

GSTM-Kinectin Bait D Specifically Interacts with EF-18 in Vitro—To verify that kinectin bait D binds to the full-length EF-18 in vitro, we first performed a GST pull-down analysis. GST fusion constructs with the kinectin baits A, B, and D were made and expressed in E. coli. Equal amounts of the individual fusion protein extracts were immobilized onto glutathione-agarose beads. The beads were washed extensively to remove unbound proteins such that only the kinectin baits remained bound to the beads. The His-tagged EF-16 construct was expressed in E. coli, and the cell extract was allowed to interact with the immobilized GST-bait fusion proteins. The proteins remained bound to the immobilized GST-baits after extensive washings were analyzed by immunoblotting with anti-His antibody (Fig. 1B). Association of the EF-18 in vitro with the kinectin bait D, but not with baits A or B, is consistent with the interactions observed in the yeast two-hybrid analysis.

Kinectin Interacts with EF-18 in Mammalian Cells—To assess whether the interaction of EF-18 and kinectin occurs in mammalian cells, GST-tagged bait D and FLAG-tagged EF-18 were co-transfected into COS7 cells. Cell lysate was prepared after transfection and precipitated with glutathione-agarose beads. The protein complexes pulled down by the beads were analyzed by immunoblotting with anti-GST and anti-FLAG antibodies. The FLAG-tagged EF-16 indeed co-precipitated with the GST-bait D and not with GST-only (control) (Fig. 2), indicating that kinectin bait D interacts specifically with EF-16 in COS7 cells.

Interaction Analysis with Endogenous Proteins—To further
confirm their interaction, we investigated whether the ectopically expressed kinectin would interact with the endogenous EF-1\(\alpha\) in HeLa cells. Polyclonal anti-EF-1\(\alpha\) antibody was produced using purified His\(_6\)-tagged protein containing the first 100 amino acids of the human EF-1\(\alpha\) as antigen. The affinity-purified antibody recognizes two polypeptide bands (~32 kDa) on SDS-PAGE in HeLa cell lysate (Fig. 3A). The N terminus of the EF-1\(\alpha\) protein has shown no significant homology with other proteins (36). The purified antibody is specific for EF-1\(\alpha\) and has exhibited no cross-reactivity with EF-1\(\beta\) (25) (Fig. 3B). The two bands that the purified antibody recognizes are the un-phosphorylated and phosphorylated forms of EF-1\(\alpha\) (37, 38). When phosphorylated in vitro by cdc2 kinase, the un-phosphorylated EF-1\(\alpha\) undergoes a shift in electrophoretic mobility to the phosphorylated form (34) (Fig. 3C).

The HeLa cell lysate was incubated with the immobilized GST-bait fusion proteins. The endogenous proteins that remained bound to the baits were analyzed by immunoblotting with the purified anti-EF-1\(\alpha\) antibody. The association of the endogenous EF-1\(\alpha\) with the kinectin bait D, but not with baits A or B, supports the observation of specific kinectin-EF-1\(\alpha\) interaction (Fig. 3D) in vivo. Similarly, association of the endogenous kinectin with EF-1\(\beta\) was observed when the HeLa cell lysate was incubated with the immobilized GST-EF-1\(\beta\) proteins (Fig. 3E).

**Real-time Biomolecular Interaction Analysis**—To evaluate the kinectin-EF-1\(\alpha\) interaction quantitatively, we used a biosensor instrument, BIAcore\textsuperscript{TM}. BIAcore analysis is based on the surface plasmon resonance (SPR) measuring changes in refractive index upon binding of soluble analyte to an immobilized ligand (39). The purified recombinant proteins used for the analysis were shown in Fig. 4A. GST-only and GST-bait D were separately immobilized onto the sensor chip and 1 micro-molar of either His\(_6\)-EF-1\(\alpha\) or His\(_6\)-only recombinant proteins were injected over the sensor chip surface at a constant flow rate. The SPR signal is expressed as a sensorgram, in RU plotted as a function of time (40).

We found no association of both His\(_6\)-EF-1\(\alpha\) and His\(_6\)-EF-1\(\beta\) with the immobilized control GST-only ligand (Fig. 4B); however, His\(_6\)-EF-1\(\alpha\) showed a gradual association (association phase) with the immobilized GST-bait D (Fig. 4C). When His\(_6\)-EF-1\(\alpha\) was replaced with flow buffer, dissociation of the EF-1\(\alpha\) from GST-bait D was seen (dissociation phase). The association rate for the binding of EF-1\(\alpha\) was determined to be \(6.55 \times 10^4\) \(\text{m}^{-1}\text{s}^{-1}\) and the dissociation rate to be \(2.23 \times 10^4\) \(\text{s}^{-1}\). Kinetic analysis based on the sensorgram yielded the apparent dissociation constant of 34 nM. No significant binding was observed for His\(_6\) to the GST-bait D.
Characterization of the EF-1α Binding Domain on Kinectin—In order to identify the minimal sufficient domain on kinectin that interacts with EF-1α, we have constructed a series of 18 truncated fragments of kinectin by both N- and C-terminal deletions (Fig. 5). Their interactions with EF-1α were examined with the yeast two-hybrid analysis. Among the 18 clones, 12 were positive as indicated by the activation of both HIS3 and lacZ reporter genes. The proteins synthesized from clone D1, D4, D5, D6, D7, D8, D9, D11, D12, D14, D15, and D16 could interact with the EF-1α-bait, whereas D2, D3, D10, D13, D17, and D18 could not.

All the positive clones were confirmed experimentally for their interaction with EF-1α in the GST pull-down assay. The proteins synthesized from clone D1, D4, D5, D6, D7, D8, D9, D11, D12 but not D14, D15, and D16 could interact with EF-1α. The minimal sufficient domain for EF-1α-binding on kinectin is the clone D12 (amino acid residues 1116–1175), which encodes a 60-amino acid fragment in the conserved region of the kinectin sequence (Fig. 6). The binding affinity of D14, D15, and D16 to the EF-1α could be weak or transient and therefore was only detected in the yeast two-hybrid analysis but not in the GST pull-down assay. Therefore, D12 was used in further experiments to confirm its functional significance.

Intracellular Localization of Kinectin and EF-1α—For kinectin to interact with EF-1α in vivo, they need to encounter each other (i.e. co-localize in cells). We used confocal immunofluorescence to study the intracellular localization of kinectin and EF-1α proteins. Indirect double immunofluorescence staining was performed using monoclonal anti-kinectin antibody (41) and affinity-purified polyclonal anti-EF-1α antibody, on CV1 cells. Endogenous kinectin displayed an ER-like staining pattern, which overlapped significantly with that of the EF-1α (Fig. 7). Thus, the similar subcellular distributions of kinectin and EF-1α in the ER-like network suggest that the two proteins can interact with each other in vivo, with the possible function of kinectin anchoring EF-1α to the ER.

Kinectin Anchors EF-1α to the ER—To test the hypothesis that kinectin might anchor EF-1α to the ER, we examined the effect of the kinectin fragments (D and D12) overexpression on the EF-1α distribution in vivo. The kinectin baits D and D12 were cloned into a pEGFP-C vector and introduced into CV1 cells. 48 h post-transfection, the cells were treated with nocodazole for 2 h to perturb the microtubules and allowed the ER network (42) and EF-1α to retract toward the cell center.3 The EF-1α distribution in the cell was imaged by confocal immunofluorescence microscopy with anti-EF-1α antibody. The control cells (untransfected cells or cells transfected with pEGFP-C vector alone) exhibited the typical ER-like staining pattern in CV1 cells (Fig. 8). 89% of the pEGFP-C vector transfected control cells and all the untransfected control cells exhibited the ER-like EF-1α network (Table I). However, only 26 and 30% of cells transfected with baits D and D12, respectively exhibited the ER-like EF-1α network. It is possible that the overexpressed kinectin fragments disrupted the ER, or EF-1α anchors to ER. Nevertheless, kinectin seems to be important for maintaining the distinct EF-1α distribution in ER-like network manner.

We further investigated if the overexpression of the kinectin fragments disrupted the ER. The cells, transfected with baits D and D12, were stained with an antibody against calnexin, which is a marker protein for ER. All the transfected cells showed similar staining pattern as the untransfected control glutathione-agarose beads and allowed to interact with the HeLa cell lysate. Endogenous kinectin was pulled down by EF-1α. TP, total protein; FT, flow-through; W, wash; E, eluate.
FIG. 4. Analyzing EF-1α binding to kinectin bait D by surface plasmon resonance. A, Coomassie Brilliant Blue staining of the purified proteins (1 μg per lane) used for analysis. Either GST-only (B) or GST-bait D (C) protein was immobilized on CM5 sensor chip by amine coupling. 1 μM each of the His6-EF-1α and His6-only proteins was injected at 5 μl/min for 10 min. Their association and dissociation of the His6-tagged proteins were monitored for another 16 min. The response units (RU), which are relative indication of the protein-protein interaction, were plotted as a function of time in seconds.
The kinectin bait D was truncated by both N- and C-terminal deletions. The interaction of the truncated clones with EF-1 was assayed by the yeast two-hybrid analysis. The solid line indicates the cDNA clones whose gene products activated HIS3 and lacZ reporter genes in ≈90% of the transformants, whereas the dashed line indicates the clones that activated the reporter genes in <10% of the transformants.

**FIG. 6.** *In vitro interaction of EF-1 with its binding domain on kinectin.* Equal amount of the EF-1 protein extract was incubated with the beads-immobilized with 5 μg each of the GST-bait (D & D12) fusion proteins. After extensive washings, bound proteins were eluted and fractionated by SDS-PAGE, and analyzed in an immunoblot with anti-His antibody. EF-1 specifically bound the kinectin baits D and D12, but not baits A or B. TP, total protein; FT, flow through; W, wash; E, eluate.

Our previous work has implicated at least two categories of the kinectin isoforms. One category of kinectin contains both vd3 and vd4, which are essential for kinesin binding and important for organelle motility (5). The other category does not contain either vd3 or vd4 with unknown functions. In the present study, we have attempted to identify binding partners for kinectin at the region between 3142 and 4139 bp (amino acid residues 1024–1356), which contains vd2, vd3, and vd5 but lacks vd4 (i.e. a kinectin isoform lacking the kinesin-binding domain). A search of a human fetal brain library, using the two-hybrid screening, has identified EF-1 as a candidate binding partner of kinectin. The results from subsequent GST pull-down, co-immunoprecipitation, and BIAcore real-time binding analysis, have confirmed that EF-1 is indeed a binding partner of kinectin. The observed interaction of kinectin bait D with EF-1 is unlikely to be due to nonspecific interaction, commonly seen between coiled-coil domains spanning leucine zipper motifs as the kinectin bait D does not contain such motifs. Our circular dichroism analysis of the kinectin baits A, B, and D indicated that all three baits exhibit the typical α-helical structures as previously predicted (3, 4, 41), suggesting that the differences in the ability of the kinectin baits to interact with EF-1 are unlikely due to gross misfolding of the baits, even though we cannot rule out the possibility of minor misfolding.

We have constructed a series of deletion mutants of kinectin to characterize the minimal sufficient domain for the kinectin-EF-1 interaction. The EF-1-binding domain on kinectin resides near the C terminus and is in close proximity to the kinesin-binding domain (5). The resolution of the fine mapping of the EF-1-binding domain on kinectin was restricted by the qualitative nature of the yeast two-hybrid assay. Despite >90% of the D14, D15, and D16 transfected yeast cells can activate both HIS3 and lacZ reporter genes, a larger fragment D12 was deduced to be the minimal binding domain. D12 is the minimal domain on kinectin that can interact with the EF-1 protein in both yeast two-hybrid and GST pull-down assay. The minimal EF-1-binding domain on kinectin is located within the conserved region, between vd2 and vd3, indicating that EF-1 can interact with all kinectin isoforms. Unlike EF-1, the conventional kinesin heavy chain can only interact with the kinectin isoforms containing both vd3 and vd4 (5). Our findings suggest that all kinectin isoforms are capable of interacting with EF-1 and only a subset of isoforms, which contains vd3 and vd4, is likely to play an additional role in organelle motility (5). In preliminary *in vitro* binding studies, we have not observed an interference of the kinesin-kinectin interaction by the addition of excess recombinant EF-1. Further studies are needed to elucidate the relationship between the two adjacent binding domains on kinectin.

Both, transient expression of the full-length kinectin and immunolocalization of the endogenous kinectin in cells, display an ER-like staining pattern (3, 41). Previous studies have also demonstrated that EF-1 clearly co-localizes with the protein-disulfide isomerase, which is one of the most abundant ER-resident proteins (34). We have shown here that kinectin and EF-1 co-localize significantly in CV1 cells, and display an ER-like staining pattern. Such a co-localization of kinectin with EF-1 is consistent with the hypothesis that kinectin anchors EF-1 to the ER.

The hypothesis has been confirmed in the kinectin fragment overexpression study, where a disruption of the EF-1 network...
was observed. The overexpression of the kinectin fragments containing the EF-1α-binding domain but lacking the transmembrane N terminus competed with the EF-1α binding to the native kinectin on ER. ER network remained intact in this overexpression study suggesting that the observed disruption of the EF-1α network was likely due to the disruption of the EF-1α anchorage to the intact ER network via the kinectin residing on ER. The anchorage of EF-1α and EF-1 complex by kinectin on ER might be important for coupling protein synthesis and membrane translocation machineries on ER.

EF-1α plays an important role in the regulation of protein synthesis (43). EF-1α is a component of the EF-1βγδ complex responsible for GDP/GTP exchange on EF-1α (22, 24, 36, 44). The only known kinectin-interacting protein in the EF-1 complex is found to co-elute with the heterodimer EF-1αβδ in the complex (44, 46).

The precise mode of the interaction of the EF-1βγδ complex with ER is unknown (35). Our current findings suggest two possible models on how EF-1 complexes are anchored to the ER membrane. The first model suggests the anchorage of the EF-1βγδ complex to ER is via kinectin. In *Artemia*, EF-1α was found to co-elute with the heterodimer EF-1βγ during purification (45). It has also been reported that EF-1γ contains 2 internal repeats (VFGEXNXS) at amino acid residues 35–42 and 355–362. It is conceivable that the region of these two octapeptides of the EF-1γ could be part of binding motif for EF-1βδ and EF-1α, respectively, hence suggesting that EF-1γ subunit observed from one-to-one yeast two-hybrid analysis (3).

The exact mechanism of how kinectin may affect protein synthesis will need to be addressed in the future. The current findings indicate that kinectin seems to play an important role in supporting protein synthesis by anchoring the EF-1 complex via EF-1α.

The distribution of EF-1α proteins disrupted by kinectin overexpression. CV1 cells were transiently transfected with GFP-tagged kinectin baits. 48-h post-transfection, the cells were treated with 1 µg/ml of nocodazole for 2 h and cultured for another 3 h after removal of nocodazole. The distribution of EF-1α proteins was examined by staining with anti-EF-1α antibody (A and B). Transfected cells were identified by GFP expression (green channel in B, C, and inset A). Two phenotypes were observed: (A) typical cells that overexpressed GFP-tagged kinectin baits D and D12 displayed reduced, disrupted EF-1α network; or (B) typical cells that overexpressed pEGFP-C vector alone or untransfected cells displayed distinct ER-like EF-1α network. The cells overexpressing GFP-tagged kinectin baits D & D12 were also stained with anti-calnexin monoclonal antibody for ER localization (C). The ER networks were intact in the cells overexpressing kinectin baits D and D12. Bar, 10 µm.

**FIG. 7.** Co-localization of endogenous kinectin and EF-1α in CV1 cells. CV1 cells were double-stained with CT-1 monoclonal antibody (A, A′) and anti-EF-1α (B, B′) polyclonal antibody. Both proteins overlapped significantly and displayed an ER-like staining pattern (C, C′). Bar, 10 µm.

**FIG. 8.** Distribution of EF-1α proteins disrupted by kinectin overexpression. CV1 cells were transiently transfected with GFP-tagged kinectin baits. 48-h post-transfection, the cells were treated with 1 µg/ml of nocodazole for 2 h and cultured for another 3 h after removal of nocodazole. The distribution of EF-1α proteins was examined by staining with anti-EF-1α antibody (A and B). Transfected cells were identified by GFP expression (green channel in B, C, and insert A). Two phenotypes were observed: (A) typical cells that overexpressed GFP-tagged kinectin baits D and D12 displayed reduced, disrupted EF-1α network; or (B) typical cells that overexpressed pEGFP-C vector alone or untransfected cells displayed distinct ER-like EF-1α network. The cells overexpressing GFP-tagged kinectin baits D & D12 were also stained with anti-calnexin monoclonal antibody for ER localization (C). The ER networks were intact in the cells overexpressing kinectin baits D and D12. Bar, 10 µm.

**TABLE I**

**Effects of overexpressing kinectin on the distribution of EF-1α in CV1 cells.** Cells were transfected with GFP-tagged kinectin baits and were stained with anti-EF-1α antibody. 30 transfected cells were randomly selected from each experiment, and were scored either with distinct or disrupted EF-1α distribution. The numbers represent mean percentage (± S.E.) of cells in each category from three independent experiments.

| EF-1α phenotype | Untransfected | GFP | GFP-Bait D | GFP-Bait D12 |
|-----------------|---------------|-----|------------|--------------|
| %               | %             | %   | %          | %            |
| Distinct network| 100           | 89 ± 0.9 | 26 ± 5.2 | 30 ± 6.5 |
| Disrupted network| 0            | 11 ± 0.9 | 74 ± 5.2 | 70 ± 6.5 |

The only known kinectin-interacting protein in the EF-1 complex is EF-1δ. In addition, no direct interaction of kinectin with EF-1α, β, or γ subunit observed from one-to-one yeast two-hybrid analysis (3).

The effects of overexpressing kinectin on protein synthesis will need to be addressed in the future. The current findings indicate that kinectin seems to play an important role in supporting protein synthesis by anchoring the EF-1 complex via EF-1α.
Kinectin Anchors EF-1\textsuperscript{a} to ER

in protein synthesis (46). The restricted localization of kinectin to ER could be related to its primary role as the membrane anchor for the translation elongation factors. The concentration of the translation elongation factors to ER should allow efficient protein synthesis by preventing the intermittent diffusion of the translation elongation factors into the cytoplasm (47).

An alternative model suggests that kinectin anchors the EF-1\textsuperscript{a} onto specific regions of the ER membrane while the EF-1\textsuperscript{βγ} complex interacts with other regions of the ER membrane in kinectin- and EF-1\textsuperscript{βγ}-independent manners. EF-1\textsuperscript{a} was found to have specific affinity for membranes and tubulin (45, 48). EF-1\textsuperscript{βγ} was also found to be an actin-binding protein associating the protein synthesis apparatus with the cytoskeleton (49). This model can explain the presence of two different guanine-nucleotide exchange proteins in the EF-1 complex in cells. The C termini of both EF-1\textsuperscript{βγ} and EF-1\textsuperscript{βγ} contain domains that are responsible for the guanine nucleotide-exchange activity of EF-1\textsuperscript{a} but their N termini exhibit no significant sequence homology (25, 36). It is possible that the EF-1\textsuperscript{a} complex and the EF-1\textsuperscript{βγ} kinectin complex serve similar functions on different regions of the ER membrane. Even though the first model seems better supported by our and other reported findings, further investigations are needed to clarify the detailed mechanism of the anchorage of EF-1 complex to ER.

In conclusion, our findings reveal a new role of kinectin in cells in addition to its involvement in organelle motility. This report provides evidences suggesting that kinectin acts as a membrane anchor of EF-1\textsuperscript{a} onto ER.

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