Kinectin-dependent Assembly of Translation Elongation Factor-1 Complex on Endoplasmic Reticulum Regulates Protein Synthesis*

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Lee-Lee Ong†§, Pao-Chun Lin†§, Xin Zhang†, and Harry Yu†§**‡‡§§2

From the †National University Medical Institutes, ‡Department of Physiology, §Graduate Program in Bioengineering, ¶NUS Graduate School of Integrative Sciences and Engineering, and **NUSTEP, National University of Singapore Singapore 117597, Singapore, the ††Singapore-MIT Alliance, Singapore 117576, Singapore, and the ‡‡Institute of Bioengineering and Nanotechnology, Agency for Science, Technology, and Research, Singapore 138669, Singapore

Kinectin is an integral membrane protein with many isoforms primarily found on the endoplasmic reticulum. It has been found to bind kinesin, Rho GTPase, and translation elongation factor-1α. None of the existing models for the quaternary organization of the elongation factor-1 complex in higher eukaryotes involves kinectin. We have investigated here the assembly of the elongation factor-1 complex onto endoplasmic reticulum via kinectin using in vitro and in vivo assays. We established that the entire elongation factor-1 complex can be anchored to endoplasmic reticulum via kinectin, and the interacting partners are as follows. Kinectin binds EF-1α, which in turn binds EF-1γ but not EF-1β; EF-1γ binds EF-1α and EF-1β but not kinectin. In vivo splice blocking of the kinectin exons 36 and 37 produced kinectin lacking the EF-1α binding domain, which disrupted the membrane localization of EF-1α, EF-1γ, and EF-1β on endoplasmic reticulum, similar to the disruptions seen with the overexpression of kinectin fragments containing the EF-1α binding domain. The disruptions of the EF-1α/kinectin interaction inhibited expression of membrane proteins but enhanced synthesis of cytosolic proteins in vivo. These findings suggest that anchoring the elongation factor-1 complex onto endoplasmic reticulum via EF-1α/kinectin interaction is important for regulating protein synthesis in eukaryotic cells.

Kinectin 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, 3). The NH₂ terminus of the 160-kDa polypeptide consists of a trans-membrane domain that anchors kinectin to organelle membranes, potentially with the help of the seven myristoylation sites throughout the molecule (3, 4). The COOH terminus of kinectin consists of the kinesin-binding domain, which can interact with the cargo-binding site of the conventional kinesin and enhance the microtubule-stimulated ATPase activity of kinesin (5). Different kinectin isoforms with combinations of variable domains (vds) have been reported in human, mouse, and fox genomes (6–10). At least two alternatively spliced forms have been detected in chicken, whereas eight more multiple variants and a differential splicing pattern of kinectin are found in human hepatocellular carcinoma (11). Five partial kinectin splice variants with alternative COOH termini in spleen and testis were identified in mouse (7), and at least two isoforms have been observed in different fox tissues (8). Recently, 15 novel kinectin isoforms have been isolated from mouse nerve tissues, such as embryonic/adult hippocampus and cultured astrocytes (9). There are at least five small (23–33 amino acid residues) variable domains scattered throughout the COOH terminus of kinectin (7). Two variable domains (vd3 (amino acid residues 1177–1200) and vd4 (amino acid residues 1229–1256)) overlap the kinesin-binding domain on kinectin (5).

The role of kinectin as a universal membrane anchor for kinesin has been questioned when the restricted intracellular and phylogenetic distributions of kinectin were discovered. Kinectin is not detected in axons of cultured neurons, where kinesin is the major motor responsible for fast anterograde and retrograde transport (12). The kinectin gene is not found in Caenorhabditis elegans or Drosophila genomes, where conserved conventional kinesin heavy chain gene is present (13). Furthermore, kinectin isoforms without vd3 or vd4 have indeed been identified lacking either vd3 or vd4 (overlapping the kinesin-binding domain) cannot serve as the membrane anchors for kinesin (10). These findings suggest that kinectin should have additional or alternative functions other than interacting with kinesin for organelle motility.

In the search for interacting partners for kinectin isoforms lacking the kinesin-binding domain, translation elongation fac-

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† Graduate Research Scholar of the National University of Singapore.

‡ To whom correspondence should be addressed: Dept. of Physiology, National University of Singapore, Block MD11, 04-01A, 10 Medical Dr., Singapore 117597. Tel.: 65-6516-3466; Fax: 65-6872-7150; E-mail: phsyuh@nus.edu.sg.

§ The abbreviations used are: ER, endoplasmic reticulum; vδ, variable domain; EF, translation elongation factor; GST, glutathione S-transferase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; cLuc, cytosolic luciferase; mLuc, membranous luciferase; RT, reverse transcription; TRITC, tetramethylrhodamine isothiocyanate; AD, activation domain; BD, binding domain.
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We present here evidence from a series of in vitro and in vivo assays in support of this model and further characterize the functional roles of the EF-1 complex anchorage onto ER via EF-1δ/kinectin interaction. Overexpression of the kinectin fragments and in vivo splice-blocking of the exons containing the EF-1δ binding domain disrupted the membrane localization of EF-1δ, EF-1γ, and EF-1β on endoplasmic reticulum. In cells with disruption of the EF-1δ binding domain on kinectin, we observed inhibition in expression of membrane proteins but enhancement of cytosolic protein synthesis. These findings suggest that anchoring the elongation factor-1 complex onto ER via EF-1δ/kinectin interaction is important and potentially plays a role in regulating synthesis of the membrane/secretory and cytosolic proteins in eukaryotic cells, apart from the involvement of kinectin in organelle motility.

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 Kinectin Baits and EF-1 Subunits—Human kinectin (GenBank™ accession number Z22551) bait A (amino acids 46–444), bait B (amino acids 444–1049), and bait D (amino acids 1024–1356) were constructed by cloning in the whole-length bait A into pGEX4T-1 vector (Clontech). The sequence of each bait was verified by sequencing and subcloned in frame to both pGEM-T Easy vector (Promega) and pET32 expression vector (Novagen). The fusion proteins were expressed in E. coli strain BL21/pLysS (DE3) (a generous gift from Niogi,

Three of the models also include the valyl-tRNA synthetase, which in turn binds other subunits and anchors the EF-1 complex to ER together with ribosomes, which give rise to rough membranes. The transformants were assayed for their expression of the his-3 reporter genes by colony lift assay (30). False positives were further eliminated by testing whether the clones could activate both BD and AD vector alone.

In Vitro GST Pull-down Assay—The glutathione S-transferase (GST) fusion constructs with the kinectin bait A, B, and D and EF-1α, -β, -δ, and -γ were carried out using a yeast two-hybrid system (Clontech). Yeast two-hybrid analysis was performed according to the supplied protocol. The constructs were co-transformed into yeast strain Y190 (Clontech) using the lithium acetate method (28). The transformants were assayed for their expression of the histidine (HIS3) reporter gene by plating them onto the synthetic medium deficient in tryptophan, leucine, and histidine. A 25 mM concentration of 3-amino-1,2,4-triazole was included to limit the number of false HIS3 positives (29). Colonies that grew successfully in the selective medium were further screened for their expression of the lacZ reporter gene by colony lift assay (30). False positives were further eliminated by testing whether the clones could activate both HIS3 and lacZ reporter genes with controls (BD and AD vector alone).

We present here evidence from a series of in vitro and in vivo assays in support of this model and further characterize the functional roles of the EF-1 complex anchorage onto ER via EF-1δ/kinectin interaction. Overexpression of the kinectin fragments and in vivo splice-blocking of the exons containing the EF-1δ binding domain disrupted the membrane localization of EF-1δ, EF-1γ, and EF-1β on endoplasmic reticulum. In cells with disruption of the EF-1δ binding domain on kinectin, we observed inhibition in expression of membrane proteins but enhancement of cytosolic protein synthesis. These findings suggest that anchoring the elongation factor-1 complex onto ER via EF-1δ/kinectin interaction is important and potentially plays a role in regulating synthesis of the membrane/secretory and cytosolic proteins in eukaryotic cells, apart from the involvement of kinectin in organelle motility.

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Santama, 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 6000 × g for 10 min. Protein extracts were obtained by freezing and thawing the cell pellet and resuspending 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 bait D and EF-1γ were immobilized onto 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-bait D (58 kDa), His₆-EF-1 (52 kDa), and His₆-EF-1β (46 kDa) fusion protein extract was allowed to interact with the GST-tagged protein-coated 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-bait D or GST-EF-1γ were released by boiling in SDS-gel sample buffer, analyzed by SDS-15% polyacrylamide gel electrophoresis, and immunoblotting with mouse RGS-His antibody (Qiagen). Antigen binding was detected with goat anti-mouse secondary antibody, coupled to horseradish peroxidase according to the manufacturer’s instructions (Sigma).

Anti-EF-1β and EF-1γ Polyclonal Antibody Preparation—The first 90 amino acids of both EF-1β and EF-1γ were expressed in E. coli BL21 using pGEX-4T-1 vector (Amersham Biosciences). The specificities of the sequences were confirmed by a BLAST 2.0 search against National Center for Biotechnology Information data bases (31). The fusion proteins were purified using the glutathione-agarose bead affinity column. The purified proteins were injected into rabbits by standard protocols (32). The antisera were affinity-purified using His₆-tagged first 90 amino acids of the EF-1β coupled to cyanogen bromide-activated Sepharose 4B (14).

In Vitro Binding of the Endogenous Kinectin and EF-1βγ with EF-1β—Bacterially expressed GST-tagged EF-1β was coupled onto glutathione-agarose beads as described above. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1-glutamine, penicillin, and streptomycin. The cells were harvested and resuspended with 1 volume of the GST purification buffer in the presence of protease inhibitors. The cell lysate was prepared as described above and was added to the beads coated with GST-EF-1β and incubated with end-over-end mixing for 2 h at 4 °C. Proteins eluted from the beads were detected by immunoblotting with antikinectin CT-1 monoclonal antibody (generous gift from Prof. Martin Krönke, University of Cologne, Germany) and anti-EF-1β and anti-EF-1γ polyclonal antibodies. Antibody binding was detected as described above.

Immunostaining of Cultured Cells—Cells cultured on coverglasses were fixed with 3.7% paraformaldehyde in PBS for 10 min at 37 °C and permeabilized with 0.05% Triton X-100 in PBS for 10 min at room temperature. The fixed cells were blocked with 10% fetal calf serum in PBS for 1 h prior to incubation with appropriate primary antibodies, anti-EF-1δ, anti-EF-1β, anti-EF-1γ, anti-calnexin (StressGen Biotechnologies Corp.), anticalreticulin (StressGen Biotechnologies Corp.), or anti-β tubulin overnight. The cells were washed three times, followed by a 1-h incubation with appropriate secondary antibodies, TRITC-conjugated goat anti-rabbit/anti-mouse IgG. The coverglasses were washed thoroughly, mounted in FluorSave™ (Calbiochem), and imaged using a Carl Zeiss LSM510 Meta laser-scanning confocal microscope.

Overexpression Studies—The EF-1β binding domains on kinectin, baits D (amino acids 1024–1356) and D12 (amino acids 1116–1175), were subcloned into the pEGFP-C vector (Clontech) for optimal expression in mammalian cells. The pEGFP-C vector alone was used as a control. HeLa cells were seeded on a coverslip and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1-glutamine, penicillin, and streptomycin overnight. Both kinectin constructs were transfected into the HeLa cells using TransFast™ transfection reagent (Promega). 48 h post-transfection, cells were subsequently fixed and stained with appropriate antibodies as described above. Transfected cells were identified by green fluorescence protein expression and scored either with ER-like network or disrupted EF-1β and EF-1γ distributions. A total of 30 cells were observed for each clone during each experiment. The data tabulated were based on three independent experiments.

Morpholino Antisense Studies—Two 25-nucleotide morpholino oligonucleotides were synthesized by Gene Tools (Philomath, OR) targeting the exon-intron boundaries of exons 36 (positions 3415–3504) and 37 (positions 3505–3597) of the human kinectin gene. The morpholinos HS1 (5’T-ATCATTT CCTACTGTGTTCGCAAGG-3’), HS2 (5’T-TCTTGTGTA AATACCTGTITTAATAG-3’), and Std Ctrl (5’T-CCTCTTAC CTCAGTTACATAATATA-3’) were delivered into HeLa cells using EPEI special delivery solution according to the manufacturer’s protocol (Gene Tools). The delivery of morpholinos was repeated at 72 h to sustain the splice blocking for an additional 48 h. For RNA analysis, total RNA was isolated from cells at 24 and 48 h post-transfection with morpholinos using the RNeasy minikit (Qiagen). The RNAs were analyzed by semiquantitative reverse transcription (RT)-PCR using spliced primers (position 3244–3754), which flanked the splice-blocked regions (exons 36 and 37), for not more than 20 cycles. A set of control primers (positions 3244–3415), which amplify outside the spliced region, was used as control. For immunolocalization analysis, the cells were immunostained with antibodies against EF-1β, EF-1δ, EF-1γ, ER (calreticulin), and microtubules (β-tubulin) at 48 h postretransfection. The average staining signal was quantified using ImagePro™ Plus (MediaCybernetics, Silver Spring, MD) to determine the mean intensity per pixel for each cell. A total of 30 cells were observed for each staining in each experiment. The data tabulated were based on mean percentage intensity ± S.E. of cells in each category normalized against the untransfected cells from three independent experiments. The confocal microscope was calibrated using intensity calibration beads (Molecular Probes) during each experiment to ensure consistency in the microscope, laser, and detector settings. For total protein analysis, the cells were lysed with GST
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TABLE 1
Kinectin baits interaction with EF-1α, -β, -δ, and -γ subunits

|                | AD/ EF-1α | AD/ EF-1β | AD/ EF-1δ | AD/ EF-1γ |
|----------------|-----------|-----------|-----------|-----------|
| BD/ Bait A     | -         | -         | -         | -         |
| BD/ Bait B     | -         | -         | -         | -         |
| BD/ Bait D     | -         | -         | +         | +         |
| BD/ EF-1α      | -         | -         | -         | -         |
| BD/ EF-1β      | -         | -         | -         | +         |
| BD/ EF-1δ      | -         | -         | -         | +         |
| BD/ EF-1γ      | -         | +         | +         | +         |

Human kinectin bait A (amino acid residues 46–444), B (amino acid residues 444–1049), and D (amino acid residues 1024–1356) were cloned in frame into GAL4 DNA-BD vector. Full-length EF-1α, -β, -δ, and -γ subunits were cloned in frame into both GAL4 DNA-BD and GAL4 DNA-AD vectors. Pairwise interaction analysis of kinectin and EF-1α, -β, -δ, and -γ subunits was carried out using a yeast two-hybrid assay. − and +, no interaction and positive interaction, respectively, based on interaction of both HIS3 and lacZ reporter genes.

RESULTS

Interaction Analysis of Kinectin with EF-1 Subunits Using the Yeast Two-hybrid Method—To study the assembly of EF-1α, -β, -δ, and -γ subunits onto kinectin, we first isolated EF-1α, EF-1β, and EF-1γ cDNAs from the human fetal brain cDNA library by PCR. The amplified gene products of 1389, 678, 842, and 1313 bp for EF-1α, -β, -δ, and -γ, respectively, were subcloned in frame into both GAL4 DNA-binding domain and activation domain vectors. A thorough pairwise interaction analysis of kinectin baits A, B, and D as well as EF-1α, -β, -δ, and -γ were carried out using a yeast two-hybrid system as described under “Experimental Procedures.” The co-transformants were assayed for the activation of both HIS3 and lacZ. Kinectin bait D can only interact with EF-1δ (10) (Table 1). EF-1δ interacts with kinectin bait D and EF-1γ. EF-1γ interacts with EF-1β and EF-1γ.
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A proposed model of the assembly of EF-1γ/β subunits onto ER via EF-1Δ/kinectin based on the yeast two-hybrid analysis. Kinectin bait D and EF-1γ (as indicated by the arrows) were used as ligands for subsequent GST pull-down assay. Equal amounts of the His6-kinectin bait D, His6-EF-1Δ, and His6-EF-1β protein extracts were incubated with the glutathione beads immobilized with GST-bait D and GST-EF-1γ fusion proteins. Bound proteins were eluted and fractionated by SDS-PAGE and analyzed in an immunoblot with anti-His antibody. EF-1γ specifically binds to both EF-1Δ and EF-1β but not bait D; bait D binds to EF-1β but not EF-1β. TP, total protein; FT, flow-through; W, wash; E, eluate.

Figure 1. In vitro interaction of kinectin bait D with EF-1Δγβ subunits. A, proposed model of the assembly of EF-1Δγβ subunits onto ER via EF-1Δ/kinectin based on the yeast two-hybrid analysis. Kinectin bait D and EF-1γ (as indicated by the arrows) were used as ligands for subsequent GST pull-down assay. Equal amounts of the His6-kinectin bait D, His6-EF-1Δ, and His6-EF-1β protein extracts were incubated with the glutathione beads immobilized with GST-bait D and GST-EF-1γ fusion proteins. Bound proteins were eluted and fractionated by SDS-PAGE and analyzed in an immunoblot with anti-His antibody. EF-1γ specifically binds to both EF-1Δ and EF-1β but not bait D; bait D binds to EF-1β but not EF-1β. TP, total protein; FT, flow-through; W, wash; E, eluate.

with both EF-1Δ and EF-1β. EF-1β can interact only with EF-1γ. No interaction was detected for kinectin baits A, B, and EF-1α with any other kinectin baits and EF-1 subunits. These results are consistent with reports that EF-1γ interacts strongly with EF-1β (25, 27).

**Interactions Confirmed Using an In Vitro Binding Assay**—To corroborate the yeast two-hybrid data and our proposed model (Fig. 1A), we performed an in vitro binding study. GST fusion constructs with the kinectin bait D and EF-1γ were made, and equal amounts of the individual fusion protein extracts were immobilized onto glutathione-agarose beads. The beads were washed extensively to remove unbound proteins. Kinectin bait D, EF-1β, and EF-1γ genes were expressed with a His6 tag in E. coli, and the fusion protein cell extracts were allowed to interact with the immobilized GST-bait D and GST-EF-1γ. The proteins that remained bound to the immobilized GST-bait D and GST-EF-1γ were analyzed by immunoblotting with anti-His antibody. EF-1γ ligand binds both His6-EF-1Δ and His6-EF-1β but not kinectin bait D (Fig. 1B). Kinectin bait D binds His6-EF-1β but not EF-1β. The results are consistent with the yeast two-hybrid analysis and our model in which kinectin bait D binds EF-1Δ, EF-1β binds EF-1γ, and EF-1γ in turn binds EF-1β.

**EF-1γ Associates with Endogenous Kinectin, EF-1β, and EF-1γ**—To further confirm the interactions, we investigated whether ectopically expressed EF-1Δ would interact with the endogenous kinectin, EF-1β, and EF-1γ. Polyclonal anti-EF-1β and EF-1γ antibodies were produced using GST-tagged fusion proteins containing the first 90 amino acids of both human EF-1β and EF-1γ as antigens. The protein sequences were BLAST-searched at the National Center for Biotechnology Information. No significant homology was detected with other known proteins. The crude anti-EF-1β serum was affinity-purified using a His6-tagged protein containing the first 90 amino acids of EF-1β. The purified anti-EF-1β antibody recognizes a single band (~26 kDa) on SDS-PAGE of HeLa cell lysate (Fig. 2A). The crude anti-EF-1γ serum only recognizes a single band (Fig. 2B) consistent with a previously reported affinity-purified anti-EF-1γ antibody that detected a single protein of ~45 kDa in human VH25 primary fibroblast lysates (14). The specificity of the anti-EF-1β, -Δ, and -γ antibodies was further verified using His6-tagged recombinant proteins. The anti-His antibody detected the expression of the three recombinant proteins (Fig. 2C). The anti-EF-1β, anti-EF-1Δ, and anti-EF-1γ have been demonstrated to be specific and do not exhibit cross-reactivity with other EF-1 subunits.

The HeLa cell lysate was incubated with the bead-immobilized GST-EF-1Δ protein. The endogenous proteins that remained bound to the bead-immobilized GST-EF-1Δ protein were eluted and analyzed by immunoblotting with anti-Kinectin (CT-1), anti-EF-1γ, and anti-EF-1β antibodies. Indeed, endogenous kinectin, EF-1γ, and EF-1β were associated with ectopically expressed GST-tagged EF-1Δ but not with GST tag control (Fig. 2D).

**Kinectin Anchors the EF-1 Subunits to ER**—To test the hypothesis that kinectin anchors the entire EF-1 complex onto ER via EF-1Δ/kinectin interaction; we examined the effects of disrupting the EF-1Δ binding domain on kinectin (baits D and D12) to the distributions of EF-1β and EF-1γ proteins in vivo. For overexpression studies, HeLa cells were transfected with the GFP-tagged bait D and D12 constructs for 48 h, fixed, and immunostained with anti-EF-1β and anti-EF-1γ antibodies. Both anti-EF-1β and anti-EF-1γ antibodies stained ER-like structures (Fig. 3, A and C, respectively) in the control cells (untransfected cells or cells transfected with pEGFP-C vector alone), consistent with the known distributions of the EF-1β, EF-1γ, and EF-1δ subunits as ER-like...
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FIGURE 2. EF-1γ interaction with endogenous kinectin and EF-1γ/β subunits. The specificity of the affinity-purified rabbit anti-EF-1γ/β (4) and crude anti-EF-1γ (5) polyclonal antibody was verified by SDS-PAGE followed by immunoblotting. The cell lysate was stained with Coomasie Brilliant Blue (lane 1). Both anti-EF-1γ and anti-EF-1γ antibodies recognized specifically the 26-kDa EF-1γ and 45-kDa EF-1γ, respectively (lane 2). C, His6-EF-1γ (lane 1), His6-EF-1γ (lane 2), and His6-EF-1γ (lane 3) proteins were immunoblotted with anti-His, anti-EF-1γ, anti-EF-1γ, and anti-EF-1γ antibodies. The antibodies raised are specific. D, GST-EF-1γ was immobilized onto glutathione-agarose beads and allowed to interact with the HeLa cell lysate. Bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting with anti-kinectin, anti-EF-1γ, and anti-EF-1γ antibodies. Endogenous kinectin, EF-1γ, and EF-1γ subunits were pulled down by EF-1γ. TP, total protein; FT, flow-through; W, wash; E, eluate.

networks (14). $95 \pm 2.8\%$ of the control cells have their EF-1β distributed as an ER-like network (Fig. 3, A (a) and B). In cells overexpressing GFP-tagged baits D and D12, only $43.9 \pm 2.3$ and $51.1 \pm 9.2\%$ of the respectively transfected cells have their EF-1β distributed as an ER-like network (Fig. 3, A (b) and B). Similarly, $94.8 \pm 1.8\%$ of the control cells have their EF-1γ distributed as an ER-like network (Fig. 3, C (a) and D). Upon overexpressing kinectin baits D and D12, cells that have their EF-1γ distributed as an ER-like network were reduced to $40 \pm 5.5$ and $51.3 \pm 3.7\%$, respectively (Fig. 3, C (b) and D). The HeLa cells transfected with kinectin baits D and D12 were immunostained with antibodies against the ER marker protein, calnexin. All transfected cells showed a similar staining pattern as the untransfected control cells (Fig. 3A, c), indicating that the ER-membrane network remained intact. The overexpression of kinectin fragments containing the EF-1δ binding domain specifically disrupted the intracellular localization of EF-1δ (10), EF-1γ, and EF-1β on ER membrane networks, supporting our hypothesis that kinectin serves as a membrane anchor for all three EF-1 subunits onto ER.

Aberrant Splicing of EF-1γ Binding Domain on Kinectin mRNA Using Morpholinos—To corroborate the results from our overexpression studies, we induced splice blocking of the EF-1δ binding domain on kinectin in HeLa cells using morpholino antisense oligonucleotide “knockdown” technology (36). The minimal EF-1γ binding domain on kinectin, bait D12 (positions 3418–3595), spans exon 36 (positions 3415–3504) and exon 37 (positions 3505–3597) of the kinectin gene. Two 25-nucleotide morpholinos, HS1 and HS2, were designed to target the exon-intron boundaries of exons 36 and 37, respectively. The morpholinos block the access of the splicing machinery to the pre-mRNA, resulting in partial or complete deletion of the EF-1γ binding site in newly synthesized kinectin protein.

The efficiency of the morpholino uptake and splice blocking was characterized using semiquantitative RT-PCR. We used a set of control primers targeting a region (positions 3244–3415) outside the spliced domains to normalize the amount of cDNA. We observed a single 511-bp PCR product in control (untransfected and control morpholino-treated) cells when using spliced primers that flank the spliced region (positions 3244–3754). However, two PCR products of 511 and 421 bp were detected in cells as early as 24 h post-transfection (Fig. 3A). The 511-bp band indicates normal kinectin mRNA splicing, whereas the 421-bp band indicates that the 90-nucleotide exon 36 was splice-blocked and not incorporated into the kinectin mRNA. No splice blocking was detected in control morpholino-treated cells, confirming the target specificity of the morpholinos. Splice blocking was still observable at 48 h post-transfection with HS1 (Fig. 4A). The 511-bp band indicates normal kinectin mRNA splicing, whereas the 418 bp band indicates that the 93-nucleotide exon 37 was splice-blocked and not
incorporated into the kinectin mRNA (Fig. 4A). In order to delete the entire EF-1β binding domain on kinectin, both HS1 and HS2 were transfected simultaneously into HeLa cells. A single 511-bp PCR product was generated in control cells, whereas three PCR products were observed in HS1-plus HS2-transfected cells when using spliced primers. The
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Kinectin Anchoring EF-1 Complex to ER Is Confirmed by Morpholino Studies—To confirm that kinectin is the membrane anchor for the EF-1 complex onto ER, we examined the intracellular distributions of EF-1β, EF-1γ, and EF-1β proteins by confocal microscopy. Kinectin possesses a relatively long half-life (38) and might take some time to be replaced by the newly synthesized spliced kinectin variants. Therefore, retransfection with the same set of morpholinos was performed at 72 h post-transfection to sustain the splicing blocking. Significant reduction of EF-1-β staining in HS1, HS2, and HS1 plus HS2 morpholino-treated cells was observed at 48 h posttransfection (Fig. 4B, c–e). The EF-1-β staining signal is reduced in cells whose EF-1-β binding domain on kinectin has been splice-blocked by morpholinos when compared with the control cells (Fig. 4B, a and b). Quantitatively, the average signal per surface area is 100 and 93.3 ± 2.4% for untransfected and control morpholino-treated cells, respectively (Fig. 4C). The average signal per surface area was reduced to 50.7 ± 2.4, 58.7 ± 2.7, and 53.3 ± 1.9% when transfected with HS1, HS2, and HS1 plus HS2, respectively.

Similarly, there is also a significant reduction in the EF-1β and EF-1γ staining signals as ER-like networks in splice-blocking morpholino-treated cells (Fig. 4, D and F, c–e). Both proteins were mainly localized in the perinuclear region, and virtually no EF-1β and EF-1γ proteins were detected near the cell periphery. In contrast, the control cells showed extensive ER-like networks for both EF-1β and EF-1γ proteins (Fig. 4, D and F, a and b). The average EF-1β signal per surface area for untransfected and control morpholino-treated cells is 100 and 104 ± 3.0%, respectively, compared with HS1, HS2, and HS1 plus HS2 with 60.7 ± 2.2, 61.1 ± 2.6, and 61.1 ± 2.2%, respectively (Fig. 4E). Correspondingly, the average EF-1γ signal per surface area for untransfected and control morpholino-treated cells is 100 and 88.8 ± 3.0%, respectively, compared with HS1, HS2, and HS1 plus HS2 with 45 ± 2.0, 42.8 ± 2.8, and 44 ± 1.8%, respectively (Fig. 4G). The total EF-1 subunits remained unchanged upon morpholino treatment (supplemental Fig. 1). These results further confirm that EF-1β/γ subunits anchor onto ER via EF-1-β/kinectin interaction as we have proposed.

We investigated if the disruption of the distribution of EF-1 complex as ER-like networks in HS1, HS2, and HS1 plus HS2 morpholino-treated cells could be caused by the disruption of microtubule or ER-membrane network. The ER membrane network was examined by detecting calreticulin, which is an ER luminal resident protein (39). We did not observe any difference in the calreticulin distribution in either splice-blocking morpholino-treated cells or control cells (supplemental Fig. 2A). Similarly, the microtubules grow outwards from the microtubule-organizing center adjacent to the nucleus and extend throughout the cytoplasm toward the cell periphery in both the control and splice-blocking morpholino-treated cells (supplemental Fig. 2B). These results confirm that EF-1β/kinectin interaction but not microtubule or ER membrane is responsible for the disruption of the EF-1βγ δ distribution in splice-blocking morpholino-treated cells.

EF-1β/Kinectin Interaction Is Important for Protein Synthesis—Since the elongation factor-1 complex is important for protein synthesis, we investigated the functional consequence of disrupting the EF-1β/kinectin interaction on the protein synthesis of a model secretory protein, chromogranin B (40). 48 h posttransfection with morpholinos, the cells were microinjected with pEFGP-chromogranin B or pEFGP-only constructs for another 2 h. The protein expression levels were quantified by measuring the average fluorescent intensity per surface area. The expression level of chromogranin B was significantly reduced in cells treated with the splice-blocking morpholinos (Fig. 5A, c–e). The average signal per surface area for untransfected and control morpholino-treated cells is 100 and 87 ± 5.5% respectively, compared with 61.3 ± 3.4, 70 ± 5.0, and 61.8 ± 4.6% in cells transfected with HS1, HS2, and HS1 plus HS2, respectively (Fig. 5B). In contrast, the expression level of GFP-only protein was increased in cells transfected with splice-blocking morpholinos (Fig. 5C, c–e). The average signal per surface area for untransfected and control-morpholino treated cells is 100 and 107 ± 7.1%, respectively, compared with 145 ± 9.8, 143 ± 9.5, and 128 ± 8.8% in cells transfected with HS1, HS2, and HS1 plus HS2, respectively (Fig. 5D). These results lead us to hypothesize that kinectin potentially plays a role in regulating protein synthesis in eukaryotic cells.

In order to confirm our hypothesis, an alternative quantitative assay was designed using the firefly luciferase gene as the reporter gene. The mLuc was generated by cloning an endoplasmic reticulum targeting sequence of calreticulin (33) fused to the 5′ end of firefly luciferase cDNA and the endoplasmic reticulum retention sequence, KDEL, fused to the 3′ end (Fig. 6A) (34, 35). A cLuc was constructed with the firefly luciferase cDNA alone. To verify their intracellular distributions, the two GFP constructs were transiently expressed in HeLa cells stably expressing fusion protein con-
sisting of red fluorescent protein DsRed: the ER targeting sequence of calreticulin (33) fused to the 5’ end of DsRed and the ER retention sequence, KDEL (34, 35), fused to the 3’ end of DsRed. The GFP-only protein produced strong fluorescence, which is present in both nucleus and cytoplasm (Fig. 6B, a and a”). The cytosolic luciferase exhibited similar diffused distribution with GFP-only protein (Fig. 6B, b and b”). However, the fluorescence generated by membranous luciferase construct was entirely excluded from the nucleus and displayed a distinct reticular ER-like network distribution that overlapped significantly with the ER signal from the DsRed vector (Fig. 6B, c, c’, and c”). The endoplasmic reticulum-targeting sequence of calreticulin and the KDEL retention sequence localized the membranous luciferase to the ER. The observations agree with the sequence analysis of the two constructs using TargetP version 1.0, a computer program designed to predict the subcellular localization of eukaryotic proteins based on the presence of the NH2-terminal presequence (41). It was predicted that the membranous luciferase protein is likely to follow the secretory pathway, whereas cytosolic luciferase does not have a discrete subcellular localization.

We transfected morpholino-treated or control cells with both luciferase constructs (mLuc and cLuc) and measured the luciferase activity 7 h post-transfection, normalizing against the luciferase mRNA amounts by semi-quantitative RT-PCR. The efficiency of protein synthesis was expressed as luciferase activity per respective relative luciferase mRNA amount. The splice-blocking morpholinos reduce the protein synthesis of the membranous luciferase construct (Fig. 6C). The protein synthesis is reduced by 30.7 ± 4.1% in cells treated with HS1 plus HS2 as compared with the untransfected cells. On the other hand, the splice-blocking morpholino-treated cells showed an increase in cytosolic luciferase protein synthesis (Fig. 6D). The protein synthesis is increased by 51.3 ± 1.5% in cells treated with HS1 plus HS2 as compared with the untransfected cells. These observations support the hypothesis that kinectin anchors the EF-1 complex via EF-1/kinectin interaction, which is important for the efficient synthesis of membrane or secretory proteins.

**FIGURE 5.** Kinectin reduces expression of chromogranin B and increases expression of GFP protein. A, expression level of GFP-chromogranin B was significantly reduced in cells treated with HS1 (c), HS2 (d), and HS1 plus HS2 (e) compared with untransfected cells (a) and cells transfected with standard control morpholino (b). Bar, 5 μm. B, average fluorescent intensity per surface area quantification using ImagePro™ Plus software. The numbers represent mean percentage intensity (n = 30 ± S.E.) of cells in each category normalized against the untransfected cells from three independent experiments. C, expression level of GFP-only protein was significantly increased in cells transfected with HS1 (c), HS2 (d), and HS1 plus HS2 (e) compared with untransfected cells (a) and cells transfected with standard control morpholino (b). D, the numbers represent mean intensity ± S.E. of cells in each category from three independent experiments. *, p < 0.05, unpaired Student’s t test.
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FIGURE 6. **Kinectin affects synthesis of membranous and cytosolic luciferase.** A, cytosolic luciferase contains a 1.6-kb firefly luciferase gene; membranous luciferase consists of endoplasmic reticulum-targeting sequence of calreticulin and KDEL endoplasmic reticulum retention sequence fused to the 5’ and 3’ end of luciferase gene, respectively. B, transient expression of GFP (a), GFP-tagged cytosolic luciferase (b), and GFP-tagged membranous (c) fusion proteins in HeLa cells stably expressing DsRed-ER (a’, b’, and c’). GFP-only protein produced strong fluorescence in both nucleus and cytoplasm (a and a’); cytosolic luciferase exhibited similar diffused distribution (panels b and b’), whereas membranous luciferase protein localized entirely as distinct reticular ER-like distribution in the cytoplasm (c and c’). Splice-blocking morpholinos reduce membranous luciferase but increase cytosolic luciferase protein synthesis. HeLa cells transfected with membranous luciferase (C) or cytosolic luciferase (D) constructs for an additional 7 h postmorpholino treatment. The luciferase protein level was analyzed by measuring total luciferase activity of the cell lysate, and luciferase mRNA was analyzed by RT-PCR from the total RNA isolated. Densitometric measurement on the PCR products was performed using Molecular Analyst software from Bio-Rad. The numbers represent mean percentage luciferase activity per mRNA ± S.E. in each category normalized against the untransfected cells from triplicate samples. **, p < 0.01, unpaired Student’s t test.
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and potentially plays a regulatory role in the synthesis of cytosolic proteins as well.

**DISCUSSION**

We have demonstrated previously that kinectin interacts with EF-1α to anchor it to ER (10); this current work is to clarify the detailed mechanism of the anchorage of EF-1 complex to ER via kinectin. Five different models have previously been proposed for the quaternary organization of the EF-1 complex in various higher eukaryotes (18–22). These models share a number of similarities, but none of the models are equivalent, and none of them have taken kinectin into account. In this study, we have proposed a model of how EF-1 complex can anchor to the ER membrane. An extensive yeast two-hybrid analysis of kinectin and EF-1α, -β, -δ, and -γ subunits was carried out. The results from both yeast two-hybrid and GST pull-down analysis suggest that anchorage of the EF-1βγδ complex to ER is via kinectin. Kinectin bait D binds EF-1δ, followed by EF-1δ binding to EF-1γ, and EF-1γ in turn binds EF-1β. No interaction was detected for EF-1α with all of the kinectin baits and EF-1 subunits, which is consistent with the previous findings that kinectin and EF-1βγδ subunits are predominantly localized on ER (1, 3, 14), whereas EF-1α exhibits a diffused distribution throughout the cytoplasm and can appear in nucleus (14). We also have demonstrated that the endogenous EF-1βγ and kinectin can be precipitated by EF-1δ. Although EF-1γ has been postulated as a candidate to anchor the elongation complex to ER (20), our findings here suggest that kinectin is the major membrane anchor for the entire EF-1βδγ complex onto ER via EF-1δ/kinectin interaction.

To investigate if EF-1δ/kinectin interaction is the major anchor for the entire EF-1βδγ complex to ER in vivo, we determined the distribution of EF-1β and EF-1γ in cells overexpressing the kinectin fragment containing the EF-1δ binding domain. Both the EF-1β and EF-1γ intracellular networks were indeed disrupted in these cells where EF-1δ/kinectin interaction has been disrupted. It is likely that the overexpression of the kinectin fragments containing the EF-1δ-binding domain but lacking the trans-membrane amino terminus competed with EF-1δ binding to the native kinectin on the ER. Since EF-1δ localization on ER is disrupted, both EF-1δ and EF-1γ proteins were also displaced from the ER. Such phenomena will not occur if EF-1γ has direct affinity for membranes or tubulin as suggested previously (23, 25).

Our hypothesis has been further confirmed in the morpholino antisense studies. The highly specific morpholinos can reproducibly delete exons of the EF-1δ binding domain on kinectin by blocking access of the splicing machinery to the pre-mRNA. The newly synthesized kinectin will have the EF-1δ binding domain deleted, leaving the remaining kinectin molecule intact. With the excellent RNA binding affinity, the morpholinos have effectively blocked the splicing of targeted mRNA as early as 24 h post-transfection in RT-PCR analysis. We observed that the distributions of EF-1β, -γ, and -δ networks were disrupted 48 h posttransfection, confirming that kinectin is the major membrane anchor for the entire EF-1βδγ complex to the ER in vivo.

There is much evidence indicating that the translation system is highly organized spatially in vivo and not freely diffusible in the cellular fluid (42). Functional studies indicate that protein biosynthesis is a channeled pathway in which aminoacyl-tRNAs are directly transferred from the aminoacyl-tRNA synthetases to the elongation factor to the ribosomes without dissociation into the cellular fluid (43, 44). It is important to maintain the supramolecular spatial organization of the protein biosynthetic machinery, which consists of a large number of proteins and nucleic acid components. The spatial organization may increase the efficiency of protein synthesis by limiting diffusion of intermediates and by increasing the local concentrations of individual components. Evidence suggests that this spatial organization of the translation components is essential to ensure highly efficient protein synthesis within the cell (45, 46). It is likely that the relatively low rates of protein synthesis in cell-free systems are the result of disrupting the spatial organization of the translation components that normally exists within the cell (47, 48).

To investigate whether kinectin-dependent anchorage of the EF-1 complex on ER plays a role in regulating protein synthesis, we characterized the expression of chromogranin B in cells containing kinectin that lacks the EF-1δ binding domain. The traffic of chromogranin B fused with GFP along the secretory pathway in HeLa cells has been well studied (49, 50). The expression level of chromogranin B was significantly reduced in cells treated with kinectin morpholinos. On the other hand, the expression level of the cytosolic GFP protein was increased. This finding suggests that the kinectin/EF-1δ interaction is important in regulating the levels of membrane versus cytosolic protein synthesis in cells. To confirm the finding, cytosolic and membranous luciferase constructs were produced and tested in a luciferase assay. The elimination of the EF-1δ-binding domain in the newly synthesized kinectin leads to disruption of the entire EF-1βδγ complex on ER. This in turn reduces the efficiency of the membranous luciferase synthesis but increases the synthesis of cytosolic luciferase. With these findings, we suggest that kinectin-dependent anchorage of EF-1 on ER can regulate the efficiency of membrane and cytosolic protein synthesis.

In eukaryotic cells, it is generally accepted that protein synthesis is compartmentalized. The soluble proteins are synthesized on free ribosomes (51–53), whereas secretory (54, 55) and membrane proteins (56–58) are synthesized on ER-bound ribosomes. Such compartmentalization limits access to the membrane-associated or secretory pathways to those proteins bearing signal sequences or other topogenic domains (54, 59). In the classical paradigm of mRNA translation, the first portion of the precursor protein containing the signal peptide is synthesized on free ribosomes. The signal sequence then directs the ribosome-nascent polypeptide complex to the signal recognition particle-signal recognition particle receptor, allowing targeting nascent polypeptides to be associated with the ER membrane and subsequently be inserted into the ER lumen via the translocon (60–62). Recent large scale identification of secreted and membrane-associated gene products using DNA microarrays, tran-
scripts known to encode secreted or membrane proteins, were enriched in the membrane-bound fractions, whereas those known to encode cytoplasmic proteins were enriched in the fractions containing mRNAs associated with free and cytoplasmic ribosomes (63). The only exception is the recent finding that a subset of mRNAs encoding soluble proteins was found to be highly partitioned onto membrane-bound polysomes (64). Upon export from the nuclear pore complex, the mRNAs associate with ER membrane and undergo translation on membrane-bound ribosomes. Should they encode a protein that lacks a signal sequence, such mRNAs would dissociate from the ER to complete translation in the cytosol (64, 65).

The components of the translational machinery are also limited and distributed nonhomogenously in eukaryotes. It is seen in electron microscopic images of the sections of many eukaryotic cells that a major portion of ribosomes is attached to the endoplasmic reticulum membrane, and at the same time there are regions of the cytoplasm completely devoid of ribosomes (66). There is also evidence of limited and nonuniform distribution of elongation factors and aminoacyl-tRNA synthetases in the cytoplasm of various eukaryotic cells (46). In experiments on injection of exogenous mRNA in Xenopus oocytes (67), there is a competition in mRNA translation within the same compartment (either membrane-associated or cytosolic). When two mRNAs encoding two secretory proteins or two cytosolic proteins were injected together, the syntheses of both proteins were reduced. It has been suggested that some components of the protein synthetic apparatus are limiting; the redistribution of such proteins can regulate the rate of different reactions in protein biosynthesis (46, 67). The overall elongation rate can be regulated through changes in the concentration of amino-tRNAs or modification of ribosomes and elongation factors (68). These findings support our hypothesis that kinecin can potentially regulate the differential expression of cytosolic versus membrane proteins. In cells treated with kinecin morpholinos, there is a redistribution of EF-1α subunits into the cytosol. Such an increase in cytoplasmic EF-1α enhances and decreases the cytosolic and membranous protein synthesis, respectively. Therefore, efficiency of mRNA translation could be enhanced by the ability of kinecin to localize elongation factors, synthetases, and ribosomes into an aggregated structure. Such spatial organization may increase the protein synthesis and provide a vehicle for transport and localization of specific mRNAs within the cells (69). It can also play a potential role in regulating local protein synthesis that is attracting increasing attention (70).

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