DISTINCT REGIONS WITHIN THE ERLINS ARE REQUIRED FOR OLIGOMERIZATION AND ASSOCIATION WITH HIGH MOLECULAR WEIGHT COMPLEXES

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Running head: Erlin oligomerization and multimerization

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The group of stomatin/prohibitin/flotillin/HflK/C (SPFH) domain-containing proteins comprises members of diverse subcellular localization and function. Association with detergent-resistant membranes (DRMs) and the propensity to form oligomers are two common properties of SPFH-domain proteins and likely important for the function of these proteins. Our laboratory recently discovered two novel members of this protein group, which, based on their endoplasmic reticulum (ER) localization and association with DRMs, were named ER lipid raft associated protein (erlin)-1 and -2. Here we characterized erlin oligomerization and identified domains within the erlins responsible for oligomerization and DRM association. Using co-immunoprecipitation and sucrose density gradient centrifugation approaches on endogenous and ectopically expressed erlin proteins, we found that they formed homo- and hetero-oligomers and were part of large multimeric complexes. These properties were independent of their DRM association. By analyzing truncation and point mutants of erlin-2 we discovered that interaction between erlin monomers (oligomerization) and association with high molecular weight (MW) complexes require distinct regions within the protein. While oligomerization and DRM association were mediated by a region immediately downstream of the SPFH domain (residues 228-300), integration into high MW complexes was absolutely dependent on a phenylalanine residue C-terminal of this region (F305), which lies within a short stretch of hydrophobic residues. Our data demonstrate that lower order oligomerization and incorporation into multimeric complexes are two separate biochemical properties of the erlins, as they are mediated by distinct regions.

Proteins containing the stomatin/prohibitin/flotillin/HflK/C (SPFH)-domain comprise a large protein group with members in many different species from prokaryotes to eukaryotes (1). Mammalian members include the flotillins, the prohibitins (PHBs), podocin, stomatin and stomatin-like proteins (2,3). The SPFH domain, also known as PHB or HflK/C domain, was originally considered to be evolutionarily conserved, but it was recently proposed that it emerged independently in different proteins by convergent evolution (4). The role of this domain mostly remains elusive, although the SPFH domain of podocin was found to be involved in cholesterol binding (5). Members of the SPFH domain containing proteins are attached to membranes of different subcellular compartments, including mitochondria (prohibitin), trans-Golgi network (flotillins), endosomes and the plasma membrane (flotillin and stomatin) (2,3). In conjunction with this diverse targeting these proteins carry out diverse functions, like regulation of membrane channels, endocytosis and coupling of plasma membrane signaling complexes to the cytoskeleton (2). All mammalian SPFH-domain containing proteins associate with detergent-resistant membranes (DRMs), and thus appear to occupy lipid raft-like domains of different cellular membranes (2,3).

Using an antibody screen against isolated lipid raft proteins from human myelomonocytic cells, our laboratory recently identified two novel members of this protein group named erlin (Endoplasmic Reticulum lipid raft protein) -1 and -2 (previously known as KE04p/ SPFH1 and C8orf2/ SPFH2 respectively) (6). The initial study characterized them as components of lipid raft-like
domains in the endoplasmic reticulum (ER) membrane (6). Erlin-2 was independently identified as a component of endothelial caveolae (7) and as an interacting partner of activated inositol 1,4,5-trisphosphate (IP3) receptors (7,8). The function of the erlins is still relatively unexplored, but the latter study suggests that erlin-2 acts as a substrate recognition factor during ER-associated protein degradation (ERAD) of activated IP3-receptors and other substrates (8).

Both erlins are closely related as they share ~80% identity at the amino acid level, whereas the extreme N- and C-termini show the lowest conservation (2,9,10). The erlins are evolutionarily conserved with homologous proteins being found in Caenorhabditis elegans and Arabidopsis thaliana (8-10). The extreme N-terminus of erlin-1 and -2 contains a transmembrane domain, which anchors and targets these proteins to the ER membrane (6). Erlin-2 was shown to be a type II ER membrane protein, with only the first three N-terminal amino acids on the cytosolic side of the ER membrane, and most of the protein, including central and C-terminal regions, being found in the ER lumen (8). Erlin-1 likely has the same membrane topology, due to its high similarity to erlin-2.

In addition to DRM association, another common characteristic of SPFH domain-containing proteins is the propensity to form oligomers, although the size of these complexes varies somewhat between members. Stomatin and podocin both form large homo-oligomers of ~9-12 and ~20-50 monomers respectively (5,11), while prohibitin-1 and -2 form multimeric complexes of ~1.2 MDa, which were shown to have a ring-like structure (12). Flotillin-1 and -2 on the other hand form homo- and hetero- tetramers (13), which likely assemble to larger clusters with a diameter of ~100 nm (14,15). Oligomerization of all SPFH domain containing proteins investigated thus far depends on regions close to their C-terminus. Domains of predicted coiled-coil secondary structure are required for oligomerization of the prohibitins and the flotillins (12,13). While the oligomerization domains of most SPFH domain containing proteins have only been defined in relatively broad terms, the domain necessary for oligomerization and DRM association of stomatin was mapped to a nine amino acid stretch downstream of the SPFH domain (16).

Since oligomerization and DRM association are common features of SPFH-domain containing proteins (2), they are likely essential for their function. Domains or motifs, which mediate these properties, may be functionally important. In the first part of this study, we characterized complex formation of endogenous and ectopically expressed erlin proteins. Consequently our second aim was to identify domains within the erlins mediating oligomerization as well as DRM association, as these two properties seem to be mediated by the same general regions in other SPFH domain containing proteins. Here we report the identification of two distinct erlin regions, which mediated specific biochemical properties of these proteins. While one region was found to be required for lower order oligomerization and DRM association, another one was responsible for incorporation into multimeric complexes.

Experimental Procedures

Antibodies and Chemicals. The following commercially available antibodies were used: rat α-HA mAb (3F10, Roche), mouse α-actin (MAB1501, Chemicon), rabbit α-calnexin (SPA-860, Stressgen), mouse α-flotillin-1 (BD Transduction Labs.), mouse α-FLAG (M2, Sigma), rabbit α-DYDDDK (FLAG-) Tag (Cell Signaling), HRP-conjugated goat α-rabbit, goat α-mouse and goat α-rat IgGs (Santa Cruz) and HRP-conjugated mouse α-rabbit and goat α-rat light chain specific IgGs (Jackson Immuno Research Labs.). Mouse α-erlin-1 monoclonal antibody (7D3) has been previously described (6). Polyclonal antibodies against erlin-1, erlin-2 or both erlins (PAN-erlin Ab) were generated by immunizing rabbits with GST-fusion proteins to erlin-1(305-346) (6), erlin-2(321-339) or erlin-1(184-346) (6) respectively (see figure S1). All other reagents and chemicals were purchased from Sigma unless indicated otherwise.

Bioinformatics. Conserved domains in erlin-1 and -2 were determined as follows: The prohibitin (PHB) domain (smart00244) was identified by the Simple Modular Architecture Research Tool (SMART; http://smart.embl.de). The SPFH domain corresponds to the Band_7/SPFH domain (pfam01145), and the HflK/C domain corresponds to the conserved domains Band_7/HflK (cd0304) and Band_7/HflC
(cd0305), which were determined based on homologies to the bacterial membrane protease subunits HflK and HflC. All are available at the conserved domain database at NCBI.

**Plasmid constructs.** HA- and FLAG-tagged full-length (FL) erlin-1, FL erlin-2FLAG and HA-tagged erlin-2 truncation mutants were generated by PCR using erlin-1 and -2 cDNAs described in (6) as templates. FL erlin-2HA has been described previously (6). All HA-tagged constructs were cloned into the pLPCX and all FLAG-tagged constructs into the pLNCX2 retroviral vector (Clontech). In all cases constructs were inserted into vectors using XhoI and ClaI restriction sites. Erlin-2 (E2) F305A HA and E2 IPMFM→A HA were generated from the erlin-2HA/pLPCX plasmid using the QuickChange Site-Directed Mutagenesis kit (Stratagene) with minor modifications.

**Cell culture and generation of stable cell lines.** Hela, NIH-3T3 and Bosc23 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. NIH-3T3 cell lines stably expressing erlin constructs were generated by retroviral infection as described previously with some modifications (17). Briefly, retrovirus containing supernatants were produced by transiently transfecting Bosc23 cells with retroviral vectors described above using Fugene6 (Roche) according to manufacturer’s instructions. For infection, retroviral supernatant diluted 1:1 in growth media containing 8 µg/ml polybrene was added to cells. Cells infected with HA-tagged erlin constructs in pLPCX were selected for positive clones using 2 µg/ml puromycin. To generate NIH-3T3 cells co-expressing HA-tagged and FLAG-tagged erlin constructs, cell lines expressing erlin-HA constructs were re-infected with erlin-FLAG constructs in pLNCX2 and selected for positive clones by adding 2 mg/ml G418 (Invitrogen) to puromycin containing growth medium. Frozen pellets of two 150 mm tissue culture dishes of nearly confluent cells were used for Immunoprecipitations, Sucrose Density Gradient Centrifugations, DRM isolations and methyl-β-cyclodextrin (M-β-CD) treatment of membrane fractions. For harvesting, cells were washed briefly in phosphate buffered saline (PBS), detached with a cell scraper and pelleted by centrifugation at 1000 g for 5 mins. Cell pellets were stored at -80 °C until use.

**Immunoprecipitation.** For immunoprecipitations frozen cell pellets were lysed in lysis buffer (1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, 10% glycerol, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) containing protease inhibitors (1 mM PMSF, 10 µg/ml each of aprotinin and leupeptin) and homogenized with 20 strokes in Dounce homogenizer. Lysates were cleared by centrifugation at 16,000 g for 10 mins at 4 °C, and 0.5 to 1 mg protein of the cell lysate was used per immunoprecipitation reaction. After preclearing lysates by incubation with protein G sepharose for 45 mins, samples were incubated with either α-HA, mouse α-FLAG, normal IgG, erlin antiserum or pre-immuniserum (PI) for 1 hr, followed by protein G sepharose for an additional hour. Immunoprecipitates were washed in lysis buffer, resuspended in Laemmli sample buffer, and analyzed by Western blotting. Rabbit α-FLAG was used as primary antibody for all α-FLAG Western blots of immunoprecipitation samples. For all other Western blots, the identical antibodies used for immunoprecipitations were also employed as primary antibodies in combination with light chain specific secondary antibodies.

**siRNA transfection.** Predesigned siRNA duplexes specific for human erlin mRNAs were purchased from Qiagen. We obtained two erlin-1 specific siRNAs with catalogue numbers SI00731402 (Hs_SPFH1_2) and SI00731409 (Hs_SPFH1_3), which we here refer to as siRNAs E1_1 and E1_2 respectively. Catalogue numbers of the two erlin-2 specific siRNAs purchased are SI00731437 (Hs_SPFH2_3) and SI03167234 (Hs_SPFH2_6), here referred to as E2_1 and E2_2. “AllStars negative control siRNA” (Qiagen) was used as negative control. siRNA transfection of HeLa cells was performed using HiPerFect Transfection Reagent (Qiagen). Cells were transfected in 6-well plates (2.3*10⁶ cells/well) according to the manufacturer’s protocol using 10 nM siRNA and 12 µl HiPerfect for each well. 72 hrs after transfection cells were lysed in RIPA buffer and erlin protein levels were analyzed by Western blot.

**Sucrose Density Gradient Centrifugation.** Sucrose gradient centrifugation was performed as described previously (18) with minor modifications. Briefly, frozen cell pellets were lysed in 1% Triton-X100 in morpholino-
ethanesulfonic acid (MES) buffered saline (MBS; 25 mM MES, 150 mM NaCl, pH 6.5 and protease inhibitors) and homogenized with 25 strokes in Dounce homogenizer. Lysates were cleared by centrifugation as described above, and 1 ml of lysate was layered on top of an 11 ml 40-5% continuous sucrose gradient (containing 1% Triton X-100). Gradients were spun at 170,000 g for 17 hrs at 4 ºC in an SW41 rotor (Beckman). Gel Filtration molecular weight (MW) standards (Amersham) were resuspended in 1% Triton/MBS and ran in parallel. Twelve 1 ml fractions were collected from each gradient, and equal volumes of each fraction were mixed with Laemmli sample buffer. Protein samples were resolved by SDS-PAGE and analyzed by Coomassie staining using GelCode Blue Stain (Pierce) or Western blotting.

Methyl-β-cyclodextrin (M-β-CD) treatment. Treatment of crude membrane fractions with 50 mM M-β-CD was performed as previously reported (6). Subsequently, DRM isolation, immunoprecipitation and sucrose gradient centrifugation was performed with M-β-CD treated membranes and controls, which were treated with PBS only.

Isolation of detergent resistant membrane (DRM) fractions. DRM fractions were prepared as described previously (17). Briefly, frozen cell pellets were lysed in 1% Triton X-100/MBS with protease inhibitors, and lysate was adjusted to 40% sucrose in a volume of 2 ml. Lysate was overlayed with a 10 ml continuous 30-5% sucrose gradient and spun at 170,000 g for 17 hrs in SW41 rotor. After centrifugation the top ~3.5 ml of the gradient were discarded and the next 3.5 to 4 ml containing the opaque band of detergent resistant material were diluted with 8 ml MBS and subjected to another ultracentrifuge spin of 1 hr to pellet the DRM fraction. The soluble fraction in the bottom 2 ml of the gradient was TCA precipitated, both DRM and soluble fraction were resuspended in a 1:1 mixture of 2x Laemmli sample buffer and 1 M Tris (pH 11), and equal parts of each fraction were analyzed by Western blotting. When DRM isolation was performed on M-β-CD treated membranes the gradient was fractionated into six 2 ml fractions and further subfractionated into insoluble and soluble material as described previously (6). Equal portions of insoluble material from all fractions (1-6) and soluble material from fraction 6 were analyzed on Western blots.

Microscopy. For immunofluorescent staining, NIH-3T3 cells were seeded onto acid-washed coverslips in DMEM containing 10% charcoal-stripped FBS one day prior to fixation. Transient transfection with E2 N300 HA was performed on cells seeded onto coverslips using Fugene6 (Roche) according to manufacturer’s instructions and cells were fixed 24 hrs post transfection. Cells were fixed with 4% paraformaldehyde in PBS for 10 mins at RT and permeabilized for 5 mins with 0.2% Triton X-100 in PBS. Primary and secondary antibodies were diluted in 1% BSA in PBS, and cells were incubated with each antibody for 1 hr at RT. After fixation and each antibody incubation cells were washed trice in PBS. Endogenous erlins in 3T3 pLPCX cells were stained with PAN-erlin antibody and donkey α-rabbit Alexa Fluor 488. HA-tagged erlin-2 constructs were stained with 3F10 antibody, cells were co-stained with rabbit α-calnexin as ER marker and goat α-rat Alexa Fluor 488 and donkey α-rabbit Alexa Fluor 568 were used as secondary antibodies (all fluorescently labelled secondary antibodies from Molecular Probes). Coverslips were mounted using Fluorescence Mounting Medium (Dako). Images were acquired as Z-stacks using an LSM 510 Meta confocal on an Axiovert 200M microscope with a 63x/1.4 Plan Apochromat objective (all from Carl Zeiss) and are presented as projections of three focal planes, which were generated using LSM Image Browser (Carl Zeiss). For presentation purposes, levels, contrast and brightness were adjusted across the entire image using Adobe Photoshop.

RESULTS

Erlin-1 and -2 form Homo- and Hetero-Oligomers. We previously reported that, in addition to the 43 kDa band corresponding to the monomeric form of ectopically expressed HA-tagged erlin-2, a ~80 kDa band, which might represent a dimeric version of the protein, can be detected in DRM fractions isolated from NIH-3T3 murine fibroblasts (6). Similar higher MW bands were also found on erlin-1 Western blots (Data not shown). This finding and the fact that oligomerization appears to be a common feature of SPFH domain-containing proteins (2), lead us to
investigate, whether the erlins form homo- and hetero-oligomers.

We used co-immunoprecipitation experiments to test whether erlin monomers could interact with each other, which is the prerequisite for oligomerization. To examine homo-oligomerization, we stably co-expressed HA- and FLAG-tagged versions of either human erlin-1 or erlin-2 and performed immunoprecipitations with HA- and FLAG-specific antibodies (Fig. 1A). We found that erlin-1HA precipitated with erlin-1FLAG, and vice versa, and the same was observed for HA- and FLAG-tagged erlin-2 (Fig. 1A). This showed that erlin-1 and -2 monomers have the ability to homo-oligomerize. Next, we examined interactions between erlin-1 and erlin-2 by immunoprecipitations on NIH-3T3 cells co-expressing HA-tagged erlin-1 and FLAG-tagged erlin-2, or vice versa, and found an interaction between ectopically expressed erlin-1 and erlin-2 (Fig. 1B). To confirm this finding in an endogenous setting, immunoprecipitations on endogenous erlin proteins were performed in HeLa cells, a human cervical carcinoma cell line (Fig. 1C). We used polyclonal antibodies specific for either erlin-1 or -2, which were raised against the extreme N-terminus of erlin-1 or erlin-2 respectively, a region with very little conservation between erlin-1 and -2 (Fig. S1, see also Fig. 5A). These antibodies were specific for human erlin proteins as they recognized endogenous protein in HeLa cells but not in vector control murine NIH-3T3 cells (Fig. S1). Consistent with our data on overexpressed proteins, we observed an interaction of endogenous erlin-1 and -2 (Fig. 1C). The interaction between erlin monomers appears to be very stable, since all immunoprecipitation experiments described here (Fig. 1A-C) were carried out in a lysis buffer, containing substantial amounts of various detergents (see Experimental procedures). Based on these data we concluded that erlin-1 and -2 form homo- and hetero-oligomers.

Protein levels of erlin-1 and -2 are independent from each other. Earlier studies on the prohibitins and the flotillins suggest that hetero-oligomerization is required for protein stability (2,12,13,19). PHB-1 and -2 are both dependent on each other for protein stability, since loss of one PHB protein leads to the loss of the partner protein (12,19). siRNA mediated knockdown of flotillin-2 causes proteosomal degradation of flotillin-1, while protein levels of flotillin-2 are not dependent on its partner protein (13). We therefore investigated if protein levels of erlin-1 and -2 are also dependent on each other. To this end, HeLa cells were transfected with siRNA duplexes against either erlin-1 or erlin-2 (Fig. 1D). For targeting of each erlin mRNA, two siRNA sequences were used in separation, to control for off-target effects of siRNAs. While siRNAs against erlin-1 and -2 dramatically reduced protein levels of erlin-1 and -2 respectively, they did not have an obvious effect on levels of the partner protein (Fig. 1D). Lysates of siRNA transfected HeLa cells were probed with a previously described erlin-1 antibody (7D3) (6), the newly generated polyclonal antibodies described above, which were specific for either erlin-1 or erlin-2, and a polyclonal antibody, which detected both erlin proteins (PAN-erlin antibody) (Fig. 1D). The PAN-erlin antibody was raised against the C-terminal half of erlin-1, which for the most part is conserved between erlin-1 and -2 (Fig. S1). The antibody detected a double band on Western blots, whereas the lower band corresponded to erlin-1 and the upper band to erlin-2, which was corroborated by the siRNA experiment (Fig. 1D and S1).

In contrast to the erlins, siRNA mediated knockdown of PHB1 or PHB2 reduces protein levels of PHB1 and PHB2 by the same degree, while only reducing PHB1 or PHB2 respectively at the mRNA level (19). A similarly dramatic effect on reduction of flotillin-1 levels after siRNA mediated knockdown of flotillin-2 has also been reported (13). However our data suggested that protein levels of erlin-1 and -2 are not dependent on each other.

The erlins associate with high molecular weight (MW) complexes. Since our initial experiments showed that the erlins have the ability to form homo- and hetero-oligomers, we wanted to characterize these oligomeric complexes in more detail. Total lysates of HeLa cells or NIH-3T3 cells, expressing empty vector (pLPCX), erlin-1HA or erlin-2HA, were subjected to sucrose density gradient centrifugation and gradient fractions were analyzed by SDS-PAGE and Western blotting (Fig. 2). Stained SDS-PAGE gels of gradient fractions demonstrated that this technique resulted in efficient and reproducible
separation of cellular proteins, with the bulk of the proteins being concentrated in the low density fractions 2 to 6. Thus most proteins appeared to be part of low MW protein complexes. HeLa and vector control NIH-3T3 cells showed a similar distribution of cellular proteins across sucrose gradient fractions (Fig. 2A). MW standards were also run on the gradient to confirm separation of size markers (Fig. 2B, bottom). Interestingly, both erlin-1 and -2 were enriched in high density fractions 7 to 12, with endogenous proteins in HeLa and NIH-3T3 cells and ectopically expressed HA-tagged proteins all showing similar distributions (Fig. 2B). Based on the migration pattern of MW standards within the gradient, we estimated that most of the erlin protein was associated with high MW complexes of ≥600 kDa. In NIH-3T3 cells stably expressing erlin-2HA, a smaller pool of ectopically expressed erlin-2HA was also found in lower density fractions 3 to 5, which might represent monomeric and dimeric versions of erlin-2. To confirm that enrichment in high density fractions is not just a general feature of ER membrane proteins, we also examined the distribution of the ER-membrane associated chaperone calnexin across density gradient fractions. In contrast to the erlins, calnexin, an ~80 kDa protein, was almost exclusively found in low density fractions of both HeLa and NIH-3T3 cells (Fig. 2B), indicating that it primarily exists as monomer, which is consistent with previous reports (11).

**Interactions between monomers, incorporation into high MW complexes and lipid raft association of erlin-2 depend on regions between the SPFH domain and the non-conserved C-terminus.** Oligomerization and DRM association of the erlins are properties shared with other SPFH domain-containing proteins and are likely required for their function. Consequently, domains or motifs within the erlins mediating these properties could be functionally important. The next objective of this study was therefore to identify erlin regions responsible for interactions between erlin monomers and association with high MW complexes and DRMs. We focused on erlin-2 for this part of the study, since erlin-1 and -2 are very similar at the amino acid level (see Fig. 5A) and functional domains are likely conserved between the two proteins. Because oligomerization and DRM association domains of other SPFH domain-containing proteins are localized to regions close to their C-terminus (2), we assumed a similar scenario for the erlins. For the initial characterization we generated three different C-terminal erlin-2 truncations (Fig. 4A). The shortest truncation mutant encompassed amino acids 1-188, therefore called E2 N188, and consisted of the N-terminus with the transmembrane domain and the complete PHB domain (Fig. 4A). The PHB domain is essentially the same as the SPFH domain, but different domain recognition algorithms were used to define the domains, resulting in slightly different boundaries (2). The PHB domain is therefore 23 residues shorter than the SPFH domain (Fig. 4A). The next erlin-2 truncation mutant was named E2 N227 and was comprised of the N-terminus with the transmembrane domain and the complete PHB domain (Fig. 4A). 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To examine whether these erlin-2 truncation mutants are able to interact with FL proteins, we stably co-expressed HA-tagged versions of the mutants together with a FLAG-tagged version of FL erlin-2 in NIH-3T3 cells and carried out HA- and FLAG-specific immunoprecipitations with these cell lines (Fig. 4B). While E2 N305HA, like FL erlin-2HA, precipitated with FL erlin-2FLAG, this was not the case for E2 N188 and N227 (Fig. 4B). This showed that E2 N188 and N227 lacked the domain required for interaction between erlin monomers, which is on the other hand included in the N305 mutant. Next we performed sucrose gradient centrifugation on NIH-3T3 cells stably expressing HA-tagged erlin-2 truncations alone, to test their association with high MW complexes (Fig. 4C). Consistent with co-immunoprecipitation results, only E2 N305 exhibited the same distribution across gradient fractions as FL erlin-2, with most of the protein being enriched in high density fractions 7 to 12, and a smaller pool in low density fractions 2 to 5 (Fig. 4C, also compare to fig. 2B). In contrast, E2 N188 and N227 accumulated in low density fractions, demonstrating that they do not contain the erlin-2 region necessary for incorporation into high MW complexes (Fig. 4C). Lastly DRM association of these erlin-2 truncations was examined. To assess DRM association equal parts of soluble and DRM (raft) fractions isolated from NIH-3T3 cells expressing HA-tagged erlin-2 truncations were analyzed by Western blotting. While the FL and the E2 N305 constructs were found in both soluble and raft fraction, E2 N188 and N227 were detected in the soluble fraction only (Fig. 4D). Taken together, these data showed that the region of erlin-2 located downstream of the SPFH domain and upstream of the non-conserved C-terminus (residues 228-305) was required for interaction between monomers, high MW complex formation and lipid raft association of erlin-2.

To ensure that the phenotypes of erlin-2 truncation mutants were not just caused by incorrect subcellular targeting, we confirmed their ER localization by immunofluorescence (IF) microscopy (Fig. S2). We did not expect C-terminal mutations to interfere with ER-localization, since the N-terminal transmembrane domain has been shown to be sufficient for correct ER targeting (6). Endogenous erlins in vector control NIH-3T3 cells (Fig. S2A) and ectopically expressed erlin-2HA (Fig. S2B, first row) revealed a reticular cytoplasmic and perinuclear staining pattern, indicative of ER localization, which is consistent with previous reports (6-8). The erlin-2 truncation mutants also showed ER staining, which greatly overlapped with staining for the ER protein calnexin (Fig. S2B).

Erlin-2 residue F305 is essential for association with high MW complexes, but not for interaction between erlin monomers or DRM association. Our next goal was to further define the erlin-2 region essential for monomer interaction and association with high MW complexes and DRMs. A recently published study on stomatin identified a short, largely hydrophobic stretch of nine amino acids close to the C-terminus, which is necessary for oligomerization as well as DRM association of stomatin. This stretch contains a phenylalanine, which is absolutely crucial for both oligomerization and DRM association of stomatin (16). E2 N305 was the only truncation mutant showing oligomerization and DRM and multimeric complex association. Interestingly, residue 305 is a phenylalanine and three out of four residues upstream and one residue downstream of F305 are hydrophobic amino acids between, which was conserved in both erlin proteins (Fig. 5A). We speculated that the short hydrophobic stretch around erlin residue F305 was the equivalent to the hydrophobic DRM association and oligomerization domain of stomatin (16). To test this, three additional erlin-2 mutants were generated. We made another erlin-2 truncation mutant E2 N300, which lacks the complete hydrophobic region; a mutant in which all hydrophobic residues between positions 301-306 were replaced by alanines, called E2 IPMFM→A; and an alanine substitution mutant of residue F305 only, named E2 F305A (Fig. 5A).

However, when these new erlin-2 mutants were tested in co-immunoprecipitation and DRM association experiments described above, no impairment of their ability to interact with FL erlin-2 or to associate with DRMs could be detected (Fig. 5B and C). Based on these results it appeared that the hydrophobic stretch between residues 301 and 306 was not involved in DRM association and oligomerization. Finally, we examined high MW complex formation of these
erlin-2 mutants by sucrose gradient centrifugation. Strikingly, none of the mutant proteins were enriched in high density fractions as observed for the FL or N305 construct of erlin-2 (Fig. 5D, also compare to Fig. 4C). Instead, they accumulated in low density fractions, suggesting, that despite their ability to interact with FL erlin-2, they were unable to associate with high MW complexes (Fig. 5D). E2 F305A exhibited the same phenotype as mutants lacking the entire hydrophobic region (E2 N300 and IPMFM→A), demonstrating that mutation of a single residue, E2 F305, was sufficient to prevent erlin-2 from incorporating into high MW complexes. The three new erlin-2 mutants were concentrated in the same low density fractions (fraction 2 to 5/6) as E2 N188 and N227 (Fig. 4C and D). While the new mutants were still able to interact with FL erlin-2, and thus likely formed at least dimers, E2 N188 and N227 were unable to oligomerize and probably exist as monomers only. It therefore appears that erlin monomers and dimers could not be efficiently separated by sucrose gradient centrifugation due to the limited resolution of the technique. These data also demonstrated that the ability to interact with other erlin monomers and to associate with high MW complexes are two separate properties of the erlin and are mediated by distinct regions within the protein.

The new erlin-2 mutants were appropriately targeted to the ER, as determined by immunofluorescence. However, distribution of the E2 N300 mutant differed from the rest of the mutants. Although it showed ER staining, large cytoplasmic aggregates and vesicle like structures were also observed (Fig. S2C 1st row and S2D). These structures were only detected when E2 N300 was expressed at high levels, while they were not present in cells expressing low levels of E2 N300 (Fig. S2D). These structures appeared to be derived from ER, since most of them co-stained for ER chaperone calnexin, and potentially represented aggregates of misfolded proteins (Fig. S2C 1st row and S2D). Thus E2 N300 might be misfolded, because it is lacking C-terminal residues required for proper folding of this part of the protein. Five additional amino acids seemed to be sufficient for correct folding, since the E2 N305 mutant did not form these aggregate-like structures. The phenotype of E2 N300 was not caused by its inability to associate with high MW complexes, because it was not observed in the E2 F305A and IPMFM→A (Fig. S2C), which otherwise exhibited the same biochemical properties as E2 N300. Nevertheless, protein stability of E2 N300 did not seem to be severely compromised, since it could be stably expressed at high levels in NIH-3T3 cells, which also did not show any obvious defects in cell viability (data not shown).

**DISCUSSION**

The goal of the present study was to characterize oligomer formation of the erlins and to identify erlin regions that mediate oligomerization as well as DRM association. We showed here, that erlin-1 and -2 have the ability to interact with themselves and with each other, which suggests they form homo- and hetero-oligomers. Furthermore, we demonstrated that the erlins are part of high MW complexes. While interaction between erlin monomers, here referred to as oligomerization, and DRM association required a region of erlin-2, which lies downstream of the SPFH domain and upstream of the non-conserved C-terminus (residues 228-300), incorporation into multimeric complexes was absolutely dependent on residue F305. Oligomerization and high MW complex association therefore appear to be two distinct properties of the erlins, as they are mediated by different regions.

Residue F305 of erlin-2 lies within a short stretch of hydrophobic amino acids and replacing it by an alanine was sufficient to prevent erlin-2 from associating with high MW complexes. Mutation of this residue might prevent incorporation into multimeric complexes by reducing the hydrophobicity of this stretch or altering its secondary structure, or both. Since domains involved in protein-protein interactions are often hydrophobic (20), it is reasonable to assume that F305 promotes erlin multimerization due to its hydrophobic character and that the surrounding hydrophobic amino acids, are also involved in this process. Similarly, oligomerization of stomatin, another SPFH domain containing protein, depends on a short hydrophobic stretch close to its C-terminus (16). However, the hydrophobic residues at positions 301-306 in erlin-2 are not entirely equivalent to the hydrophobic domain in stomatin. The nine residue hydrophobic
domain of stomatin is not only required for oligomerization, but also for DRM association (16), whereas erlin DRM association and lower order oligomerization are mediated by a separate region of erlin-2. Because 81% of residues between positions 228-300 are identical between erlin-1 and -2, and the hydrophobic stretch between residues 301 and 306 is also conserved in erlin-1, these regions are likely also required for DRM association, oligomerization and high MW complex association of erlin-1.

The function of the SPFH domain still remains elusive, but it has been previously proposed to facilitate oligomerization and DRM association, since these are characteristics shared by most SPFH domain containing proteins (3,21). Our study showed that the erlin SPFH domain alone is not sufficient to induce these biochemical properties. Likewise, oligomerization of the flotillins and stomatin requires domains outside of their SPFH domain, which are unique for the each SPFH domain protein (13,16,22). Nevertheless the SPFH domain might contribute to DRM association by binding to cholesterol, which has been demonstrated for the SPFH domain of podocin and its C. elegans homologue MEC-2 (5), and potentially other membrane lipids.

Based on our results, one could envision the formation of multimeric erlin complexes as a two-step process. The first step would be the formation of lower order oligomers, potentially dimers, which is mediated by the region between erlin residues 228 and 300. Bands of ~80 kDa observed on erlin Western blots likely represent erlin dimers (6), indicating that dimers are relatively stable intermediates in the process of higher MW complex formation. Oligomeric complexes of only one or both types of erlins are conceivable, since we have found homo- and hetero-typic interactions between erlin-1 and -2 and erlin protein levels did not seem to be dependent on the partner protein. This is in contrast to PHB-1 and -2, which both depend on their partner for protein stability and can therefore only form hetero-oligomers (12,19). Erlin-2 mutants which contain residues 1-300, but not F305 (E2 N300, F305A and IPMFM→A) would only undergo the first step of multimeric complexes formation. In a second step, lower order erlin oligomers would assemble into high MW complexes of ≥600 kDa. These complexes might either consist of erlin proteins only, or include other ER membrane proteins. Such a two-step process has been demonstrated for the formation of ring-shaped multimeric complexes by yeast PHBs in the inner mitochondrial membrane (12). Upon import into the mitochondria, PHB-1 and -2 form hetero-oligomeric intermediates of ~120 kDa, which then aggregate into ring-like structures of 1.2 MDa. Similar to the erlins, each step requires distinct regions within the PHBs (12). Hence erlin multimerization at the ER membrane might proceed in a similar fashion, especially since the erlins have a membrane topology similar to PHB-1 and, of all SPFH domain containing proteins, the prohibitins and the erlins are most similar to each other (6).

In this study we also explored the relationship between DRM association of the erlins and their oligo- and multimerization. We found that disrupting erlin DRM association by removing cholesterol from cellular membranes after hypotonic lysis affected neither the interactions between erlin-1 and -2 nor their association with high MW complexes. DRM association therefore does not seem to be required for stabilization of existing erlin oligomers and high MW complexes. Due to their intracellular localization to ER membranes, DRM association of the erlins cannot be disrupted in live cells (6). This prevented us from examining whether DRM association is needed for initial assembly of erlin oligomers and multimers in live cells. However multimerization of podocin and its C. elegans homologue MEC-2 (5), suggesting that DRM association is generally not required for multimerization of SPFH domain containing proteins. This is in contrast to multimers of the caveolins, which require cholesterol for their stability (23).

We observed DRM association of erlin-2 mutants, which were unable to associate with high MW complexes, indicating that multimerization is not a prerequisite for lipid raft association of the erlins. As has been shown for podocin and MEC-2 (5), the erlins might be able to bind cholesterol and potentially other ER membrane lipids and thereby nucleate specific lipid molecules around them. Binding of specific lipids could be sufficient to induce detergent insolubility in the erlins. Erlin monomers and lower order oligomers, each bound to a certain number of cholesterol/lipid molecules...
might assemble into multimeric complexes, and thus form lipid raft-like microdomains in the ER membrane. According to this model, the erlins would provide a protein scaffold for the formation of specialized microdomains in the ER membrane, where they create a lipid microenvironment distinct from the remainder of the ER membrane. The flotillins have been proposed to form such “scaffolding microdomains” at the PM, which could provide platforms for flotillin dependent processes, which include certain PM receptor signaling pathways (21) and a novel type of endocytosis (24). Similarly, “erlin microdomains” in the ER membrane might facilitate certain ER associated processes by clustering proteins involved. ERAD of several protein substrates has been shown to require erlin-2 (8), and could therefore take place, at least partially, within the proposed “erlin microdomains”, but other ER associated processes might also be facilitated by the erlins.

Our data suggest that protein levels of erlin-1 and -2 are not dependent on each other, and it is therefore physically possible for each erlin protein to function in isolation from its partner. The strong conservation between the two proteins could hint towards redundant functions. However, siRNA mediated knockdown of erlin-2 only is sufficient to inhibit ERAD of activated IP3 receptors and other substrates, which shows that erlin-1 cannot functionally substitute for erlin-2 (8). Instead these data suggest two other possibilities: (1) Hetero-oligomers of both erlins might be required for their function or (2) the function of erlin-1 is at least partially different from erlin-2 suggesting that homo-oligomers of either erlin-1 or -2 play different roles in cellular processes.

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FOOTNOTES

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Abbreviations used are: Stomatin/ Prohibitin/ Flotillin/ HflK/C-domain, SPFH-domain; detergent resistant membranes, DRMs; Endoplasmic Reticulum, ER; ER lipid raft-associated protein, erlin; Molecular weight, MW; Methyl-β-cyclodextrin, M-β-CD; full length, FL; ERAD, ER-associated protein degradation; Inositol 1,4,5-Trisphosphate Receptor, IP3-receptor; prohibitin, PHB;

FIGURE LEGENDS

Fig. 1: The erlins form homo- and hetero-oligomers, but their protein levels are not dependent on each other. (A) Co-immunoprecipitation of FLAG-tagged with HA-tagged erlin-1 (left panel) or erlin-2 (right panel) stably co-expressed in NIH-3T3 murine fibroblasts indicates homo-oligomerization. Immunoprecipitations were performed with HA- and FLAG-tag specific antibodies and normal rat and mouse (ms) IgGs respectively were used as negative controls. Lysate: 10% of input. (B) Co-immunoprecipitation of HA-tagged erlin-1 with FLAG-tagged erlin-2 (left panel) or vice versa (right panel) indicates hetero-oligomerization of ectopically expressed erlin-1 and -2. Lysate: 10% of input. (C) Co-immunoprecipitation of endogenous erlin-1 and -2 was performed in HeLa cells using rabbit polyclonal antibodies against either erlin-1 (α-E1) or erlin-2 (α-E2). Pre-immunesera (PI) from corresponding rabbits were used as controls. Lysate: 15% of input. (D) HeLa cells were transfected with siRNA duplexes against either erlin-1 (E1_1, E1_2) or erlin-2 (E2_1, E2_2), or with a control siRNA duplex. Knockdown of erlin proteins was assessed by Western blotting using a panel of erlin antibodies.

Fig. 2: Sucrose density gradient centrifugation shows association of erlins with high MW complexes. Sucrose density gradient centrifugation was performed on total lysates from HeLa cells or 3T3 cells stably expressing pLPCX vector only, E1HA or E2HA. Cells were lysed in 1% Triton X-100 and fractionated by 40-5% sucrose gradient centrifugation. (A) Gels with gradient fractions from HeLa and 3T3 pLPCX cells were stained to visualize distribution of cellular proteins within the gradient. (B) Western Blot analyses of fractions: Distribution of endogenous erlin proteins within gradient was determined by probing Western blots with antibodies specific for either erlin-1 or erlin-2 (HeLa) or with PAN-erlin Ab, which detects both erlins (3T3). Ectopically expressed HA-tagged erlin-1 and -2 were detected with an HA-specific antibody. Western blots were also probed for calnexin, which localizes to the ER, but is not enriched in high density fractions. Distribution of MW standards is indicated at the bottom of the figure. 440 and 669 kDa standards were not separated by this procedure and therefore concentrated in identical fractions.
Fig. 3: Disrupting lipid raft association of the erlins does neither affect their ability to interact with each other nor their association with high MW complexes. Crude membrane fractions from HeLa cells were treated with PBS only (0 mM M-β-CD) or 50 mM M-β-CD in PBS for 30 mins at 37 °C and subsequently subjected to lipid raft isolation (A), Immunoprecipitation (B) and sucrose gradient centrifugation (C). (A) Pretreated membrane fractions were lysed in 1% T-X100, lysate was adjusted to 40% sucrose and overlayed with a 30-5% continuous sucrose gradient. After ultracentrifugation six fractions were collected from gradient, which were further separated into soluble and insoluble subfractions. Equal parts of insoluble fractions 1-6 and soluble protein from fraction 6 were analyzed by Western blotting using the PAN-erlin antibody and a flotillin-1 specific antibody as lipid raft marker. (B) Immunoprecipitation of endogenous erlins was performed as in figure 1D. Lysate: 20% of input. (C) Sucrose gradient centrifugation was performed as in Figure 2, and Western blot was probed with PAN-erlin antibody.

Fig. 4: A C-terminal region of Erlin-2 between the SPFH domain and the non-conserved C-terminus is required for its oligomerization and association with high MW complexes and DRMs. (A) Schematic of full length and truncated erlin-2 constructs used for initial characterization in this study: Transmembrane (TM) domain is shown in grey, prohibitin (PHB) domain is shown in red, red and orange boxes combined represent the SPFH domain and red, orange and yellow boxes combined represent HflK/C domain. The blue box indicates a C-terminal region, which shows very little conservation between erlin-1 and -2. HA- and FLAG-tags (green) were added to the C-terminus of the erlins. Erlin-2 truncations lack various parts of the C-terminus and were named N188, N227 and N305, because they consist of the N-terminal 188, 227 and 305 amino acids respectively. (B) Full length (FL) FLAG-tagged erlin-2 and HA-tagged truncated (or FL) erlin-2 were stably co-expressed in NIH-3T3 cells and immunoprecipitations with HA- and FLAG-specific antibodies were performed. Western blots on the left show total lysate, the middle and right panels show immunoprecipitations HA- and FLAG-specific antibodies respectively. Lanes in middle and right panel marked with “+” show IPs with HA- or FLAG-specific antibodies, while lanes marked with “−” show control IPs with normal rat or mouse IgGs respectively. Lysate: 20% of input. (C) Sucrose gradient centrifugation was performed on 3T3 cells stably expressing truncated or FL erlin-2HA, as described for figure 3. (D) Lipid raft isolation was performed on 3T3 cells stably expressing truncated or FL erlin-2HA. Equal portions of soluble (s) and raft (r) fractions were analyzed by Western blotting to determine lipid raft association of erlin-2 truncation mutants. Flotillin-1 was used as a marker for lipid raft fractions.

Fig. 5: Residue F305 within a short hydrophobic stretch is dispensable for oligomerization and lipid raft association, but is required for high MW complex association of erlin-2. (A) CLUSTALW alignment of amino acid sequences of erlin-1 and -2 (modified from reference 6); Small grey box highlights residues 301 – 310, which are magnified in large grey box. Hydrophobic residues between 301 and 306 are marked as bold letters and underlined residues are included in erlin-2 N305 truncation mutant. Black box contains a list of additional erlin-2 mutants. (B) Testing new erlin-2HA mutants for oligomerization using the immunoprecipitation approach described for figure 4B. On α-HA Western Blot showing total lysate (top left), 10x the amount of protein was loaded onto gel for IPMFM→A compared to N300 and F305A. Longer exposure times are shown for IP blots of IPMFM→A mutant, due to low expression levels of this mutant in that specific cell line. Lysate: 10% of input. (C) Testing lipid raft association of new erlin-2HA mutants using same approach as in figure 4D. (D) Association of new erlin-2HA mutants was examined using sucrose gradient centrifugation, as described for figure 4C. Western blot of FL E2HA gradient fractions was included for comparison.

Fig. S1: Characterization of polyclonal erlin antibodies. Rabbit polyclonal antibodies (pAbs) generated against erlin proteins were tested on Western blots with total lysates from HeLa cells and NIH-3T3 cells stably expressing either empty vector (pLPCX), erlin-1HA (E1HA) or erlin-2HA (E2HA). On the right are schematics of GST-erlin constructs used for immunization of rabbits. Blue boxes indicate regions, which
show little conservation between erlin-1 and -2. All other regions are highly conserved between the two proteins.

**Fig. S2**: ER localization of erlin-2 mutants. NIH-3T3 were fixed with 4% PFA and permeabilized with 0.2% Triton X-100. Images were acquired with a confocal microscope, and pictures shown here represent projections of 3 focal planes. (A) Endogenous erlins were stained in 3T3 pLPCX cells using PAN-erlin antibody (left panel). Right panel shows a cell stained with preimmune serum (PI). (B,C) NIH-3T3 cells stably expressing erlin-2HA mutants or pLPCX only were stained with α-HA antibody to visualize erlin constructs (left column, green) and with calnexin antibody (middle column, red) as an ER marker. (D) 3T3 pLPCX cells were transiently transfected with erlin-2 N300 HA and fixed and stained with α-HA and calnexin antibodies 24 hrs post transfection. The upper row shows a cell with low expression levels of E2 N300 HA and the lower row shows cells with high expression levels. Scale bar = 20µm.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure S1
Figure S2
Distinct regions within the erlins are required for oligomerization and association with high molecular weight complexes
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