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Clustered hydrophobic amino acids in amphipathic helices mediate erlin1/2 complex assembly

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ABSTRACT

Erlin1 and erlin2 are highly homologous, ~40 kDa, endoplasmic reticulum membrane proteins that assemble into a ring-shaped complex with a mass of ~2 MDa. How this complex is formed is not understood, but appears to involve multiple interactions, including a coiled-coil region that mediates lower-order erlin assembly, and a short hydrophobic region, termed the "assembly domain", that mediates higher-order assembly into ~2 MDa complexes. Here we have used molecular modeling, mutagenesis and cross-linking to examine the role of the assembly domain in higher-order assembly. We find (i) that the assembly domains of erlin1 and erlin2 are amphipathic helices, (ii) that erlin1 alone and erlin2 alone can assemble into ~2 MDa complexes, (iii) that higher-order assembly is strongly inhibited by point mutations to the assembly domain, (iv) that three interacting hydrophobic residues in the assembly domain and aromaticity are essential for higher-order assembly, and (iv) that while erlins1 and 2 are equally capable of forming lower-order homo- and hetero-oligomers, hetero-oligomers are the most prevalent form when erlin1 and erlin2 are co-expressed. Overall, we conclude that the ~2 MDa erlin1/2 complex is composed of an assemblage of lower-order hetero-oligomers, probably heterotrimers, linked together by assembly domain hydrophobic residues.

1. Introduction

Erlin1 (E1) and erlin2 (E2), which are also known as SPFH1 and SPFH2, are integral endoplasmic reticulum (ER) membrane proteins that belong to a family of ~100 mammalian proteins that contain an "SPFH" domain [1]. This domain is an ~200 amino acid long motif named because of minor sequence similarities in the proteins Stomatin, Prohibitin, Flotillin, and HflC/K [1–3]. SPFH domain-containing proteins share several structural and biochemical properties, including a size of 30–50 kDa, localization to membranes, and a propensity to assemble into very large oligomeric complexes [1,3], but have diverse functions, ranging from regulation of plasma membrane ion channels (stomatins) [4] to control of inner mitochondrial membrane functional integrity (prohibitins) [5].

E1 (348 amino acids) and E2 (340 amino acids) assemble into a complex that has been estimated to be ~2 MDa in size and is composed of ~40 subunits in an ~1:1 ratio [6]. The complex is found in the ER membrane and is ring-shaped [6]. It plays a role in ER-associated degradation [6,7], the pathway by which aberrant or misfolded proteins, or unassembled subunits of multimeric ER proteins, are degraded [8]. Specifically, the E1/2 complex has a clearly defined role in mediating the ER-associated degradation of activated inositol 1,4,5-trisphosphate receptors [6,7,9,10], contributes to the degradation of some model ER-associated degradation substrates [9], and may play a role in 3-hydroxy-3-methyl-glutaryl-CoA reductase degradation [11]. Assembly of the E1/2 complex appears to be mediated at two levels; lower-order oligomers are formed primarily via interactions between coiled-coil regions (residues 179–276 in E1 and 177–274 in E2), and "assembly domains" (residues 301–311 in E1 and 299–309 in E2), that are enriched in hydrophobic residues (Fig. 1A) [6,12], play a key role in higher-order assembly. Indeed, mutation of an individual hydrophobic residue (F^{305}) within the assembly domain of E2, blocks higher-order assembly, without affecting lower-order oligomerization [12]. Interestingly, a similar situation appears to exist for stomatin, for which the assembly of ~0.3 MDa complexes [13] is mediated by a short hydrophobic domain located near the C-terminus [14]. Prohibitins 1 and 2 and flotillins 1 and 2 also form large oligomeric complexes, but in these cases no clear role for a hydrophobic domain in assembly has been defined [15–17].

A critical unresolved question for SPFH domain-containing proteins concerns how lower-order oligomers are assembled into higher-order complexes. Here we have used molecular modeling,

Abbreviations: E1, erlin1; E2, erlin2; ER, endoplasmic reticulum; HA, hemagglutinin; DSS, disuccinimidyl suberate.

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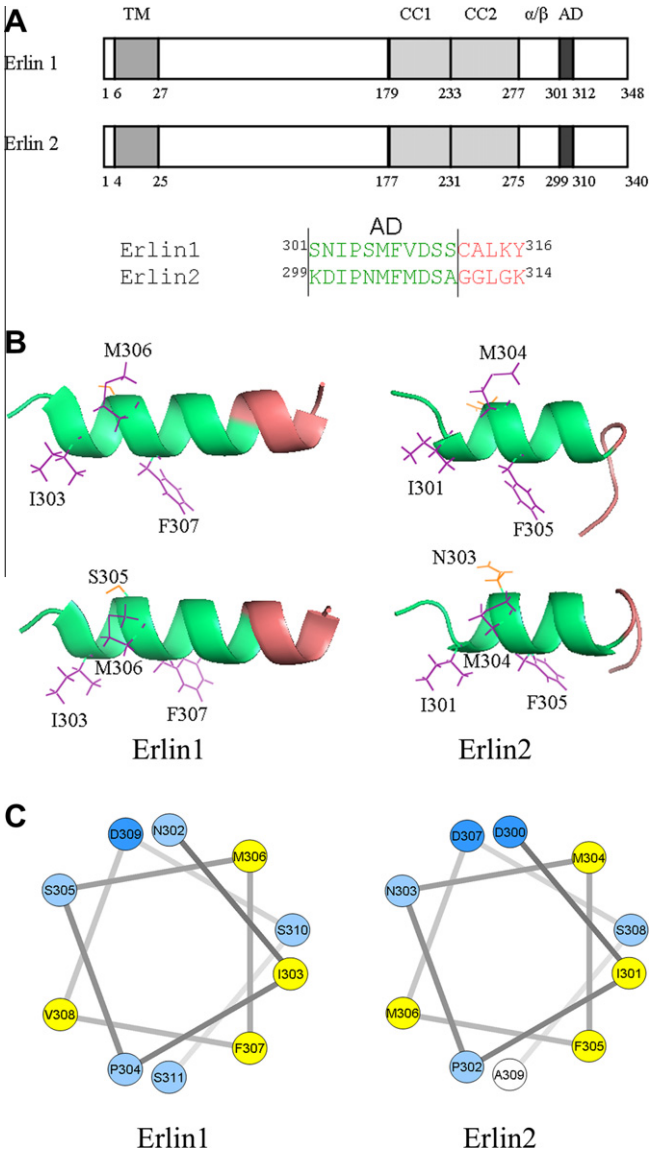

Fig. 1. Modeling of assembly domain structure (A). Domain organization of E1 and E2, with numbering according to mouse proteins, and indicating the first amino acid of each domain. TM, transmembrane domain; CC1 and CC2, coiled-coil regions 1 and 2; α/β, α/β domain; AD, assembly domain (B). Predicted structures with assembly domain amino acids shaded green and the side chains of selected residues highlighted. For E1 and E2, two images are shown, rotated differentially along the helical axis to best illustrate side chain orientation (C). Helical wheel projections of helical parts of the assembly domains with amino acids color-coded; yellow = more hydrophobic than alanine, light blue = less hydrophobic than alanine, dark blue = - less hydrophobic than alanine and charged [19]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutagenesis and cross-linking to examine this question for E1 and E2.

2. Materials and methods

2.1. Molecular modeling

The assembly domains of E1 and E2 were modeled with the server I-Tasser (zhanglab.ccmb.med.umich.edu/I-TASSER/). For the assembly domain amino acid sequences submitted, the program retrieves template proteins with homologous folds from the Protein Data Bank library. The fragments obtained are then re-assembled into full-length models by replica-exchange Monte Carlo simulations. The decoys generated during the simulation are clustered and the top five cluster centroids are used to generate full length models. The models are ranked based on the cluster density, with higher cluster density signifying that the structure occurs more often in the simulation trajectory and therefore is the most likely structure. Helical wheels were obtained using rzlab.ucr. edu/scripts/wheel/wheel.cgi.

2.2. Constructs, transfection, sample preparation and PAGE

cDNAs encoding mouse E1HA, E2HA and deletion mutants, E1flag and E2flag have been described previously [6]. Point mutations in E1HA and E2HA were created by mutagenic PCR. Briefly, a 50 μl mix containing 50 ng of template DNA, PfuUltraII buffer, dNTPs, primers (10 μM), and PfuUltraII enzyme, was cycled (initial denaturation step of 95 °C for 2 min, followed by 23 cycles of 95 °C for 30 s, 66 °C for 1 min, 68 °C for 6.5 min, and a final extension step of 72 °C for 10 min). The reaction products were then digested with *DpnI* to remove template DNA and the presence of mutations was confirmed by DNA sequencing.

HeLa cells (~6×10^5/well of a 6-well plate) were transiently transfected using Lipofectamine 2000 (10 μl plus 4.8 μg total cDNA), and ~24 h later cells were detached with 155 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4, were centrifuged (1000*g* for 2 min), were disrupted for 30 min at 4 °C with lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1% CHAPS, 10 μM pepstatin, 0.2 mM PMSF, 0.2 μM soybean trypsin inhibitor, 1 mM dithiothreitol, pH 8.0), were centrifuged (16,000*g* for 10 min at 4 °C), and supernatant samples were subjected to native or SDS–PAGE and immunoblotting as described [18]. Antibodies used for immunodetection were mouse monoclonal anti-hemagglutinin (HA) epitope clone HA11 (Covance), anti-flag epitope clone M2 (Sigma), anti-E1 clone 7D3 [12], and rabbit polyclonal anti-E1 or anti-E2 [6].

2.3. Cross-linking

Transfected HeLa cells in 6-well plates were rinsed once with PBS and were then incubated for 1 min at 22 °C with 1 mM disuccinimidyl suberate (DSS, Thermo Scientific) in PBS. Cells were then detached and incubated for 30 min at 4 °C with 1% CHAPS lysis buffer supplemented with 10 mM lysine and 25 mM glycine, were centrifuged (16,000*g* for 10 min at 4 °C), and supernatant samples were subjected to SDS–PAGE. Immunoprecipitated αT3-1 cell E1/2 complex, prepared using anti-E2 [6], was incubated for 2 min at 22 °C with 1 mM DSS in PBS, reactions were stopped by adding gel-loading buffer supplemented with 10 mM lysine and 25 mM glycine, and samples were subjected to SDS–PAGE.

3. Results

3.1. Molecular modeling of the assembly domain

The domain structures of E1 and E2 are shown in Fig. 1A, together with the amino acid sequences of the assembly domain regions. Previous studies have indicated that E1/2 complex assembly is mediated by two distinct interactions; the primary interaction (lower-order oligomerization) being mediated by the two coiled-coil motifs and the α/β domain, and the secondary interaction (the linkage of lower-order oligomers into higher-order ~2 MDa complexes) being mediated by the assembly domain [6,12]. Evidence for the existence of the assembly domain came from experiments showing that deletion of amino acids 299–309 of E2 blocked ~2 MDa complex formation [6], but not lower-order

assembly, and also that mutation of hydrophobic amino acids within the E2 assembly domain to alanine, or mutation of just F^{305} to alanine had the same effect [12]. To further explore assembly mechanisms we derived structures of the assembly domain regions of E1 and E2 (Fig. 1B). Helical regions that encompass the assembly domains are predicted, with a preponderance of hydrophobic residues aligned towards one face of the helices (amino acids I^{303}, M^{306} and F^{307} in E1, and I^{301}, M^{304} and F^{305} in E2). Helical wheel projections (Fig. 1C) confirm this distribution and indicate that the helices are amphipathic.

3.2. Mutation of the assembly domain

The extent of E1 or E2 assembly was assessed by native-PAGE, which allows for the separation of proteins under native conditions, and preserves protein complexes [20]. When expressed in HeLa cells, E1HA and E2HA predominantly migrated as a smear at ~2 MDa, (Fig. 2A, lanes 4 and 5), similarly to endogenous E1 and E2 (lanes 1 and 2), indicating that each exogenous erlin alone is capable of oligomerizing into higher-order complexes. There was a tendency, however, for some E2HA to migrate in the ~0.3–1 MDa range (lane 5). Interestingly, when E1HA and E2HA were co-expressed, much less immunoreactivity was detected in the ~0.3–1 MDa range, suggesting that higher-order complexes are most stable when composed of both erlins. These findings were recapitulated when E1flag and E2flag were expressed (lanes 10–12). Importantly, there was little or no comingling of endogenous and exogenous erlins, as immunoprecipitation of exogenous

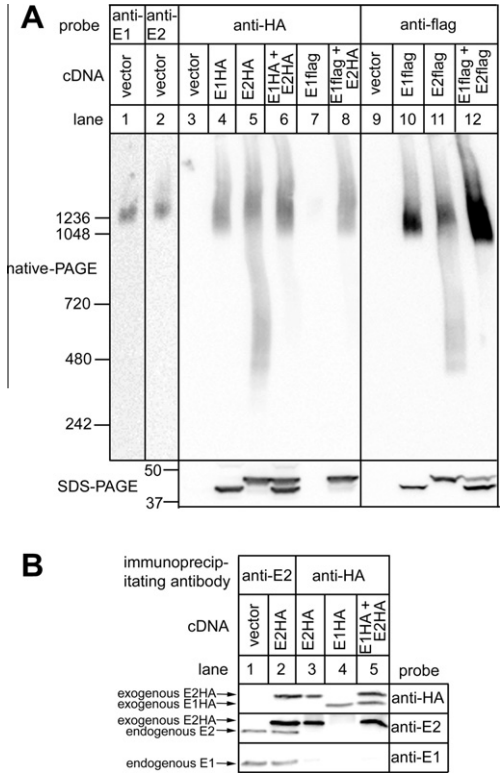

Fig. 2. Exogenous erlins form higher-order complexes Hela cells were transfected with vectors encoding epitope-tagged erlins, or empty vector, and cell lysates were prepared. In (A) lysates were subjected to either native-PAGE or SDS–PAGE and were probed as indicated, with the migration positions of molecular mass markers indicated (masses in kDa). In (B) lysates were incubated with anti-E2 or rabbit polyclonal anti-HA [10] and immunoprecipitates were subjected to SDS–PAGE and were probed as indicated. Note that mouse monoclonal anti-E1 is human specific [9,12] and recognizes endogenous E1, but not exogenous E1HA. E1, E2, E1HA and E2HA migrated, respectively, at 41,43, 42 and 44 kDa.

E1HA or E2HA with anti-HA did not co-immunoprecipitate endogenous E1 or E2 (Fig. 2B, lanes 3–5), while both endogenous E1 and E2 were readily immunoprecipitatable from vector-transfected, or E2HA-expressing cells with anti-E2 (lanes 1 and 2). Thus, the signals seen after native-PAGE (e.g. Fig. 2A) reflect the properties of exogenous proteins only.

Systematic point mutation of each residue within the E2 assembly domain to alanine, which has a relatively small hydrophobic side chain and does not disrupt helical motifs [21], showed that all mutants, with the exception of N303AE2HA, failed to migrate predominantly at ~2 MDa (Fig. 3A, upper panel, lanes 2–10), indicating that assembly is extremely sensitive to perturbation of the assembly domain. Consistent with these results, Δ310–340E2HA migrated at ~2 MDa (lane 12), while Δ299–340E2HA, which lacks the assembly domain, did not (lane 11). Interestingly, E1HA co-expression reversed the effects of several of the point mutations (lower panel, lanes 2–10), but the migration of three E2HA mutants, I301A (lane 3), M304A (lane 6) and F305A (lane 7) was not substantially altered. The ability of E1HA to facilitate the assembly of some of the E2HA mutants suggests that it combines with E2 in such a way to buffer the effects of mutations, whereas the resistance of I301AE2HA, M304AE2HA and F305AE2HA to E1HA indicates that I^{301}, M^{304} and F^{305} are indispensable for assembly. Each of these residues are hydrophobic and are clustered on one face of the assembly domain helix (Fig. 1B and C). The same appears to be true for E1, in which an identical cluster is found, and F307AE1HA fails to migrate at ~2 MDa, in the absence or presence of E2HA (data not shown).

Additional information was obtained from co-expression of the different types of E2HA mutant with E1flag, since this allowed for effects of E2 mutants on E1 assembly to be observed directly (Fig. 3B). Notably, the ability of E1flag to form ~2 MDa complexes was disrupted by resistant mutants like F305AE2HA, but not by reversible mutants like P302AE2HA (lower panel, lanes 3–6). Thus, the influence of E1 and E2 on each other is bi-directional, indicative of an intimate interaction between the assembly domains of the two proteins.

Other mutations were made to further probe the assembly domain. To examine why N303AE2HA appears to migrate more predominantly in the ~2 MDa region than wild-type E2HA (Fig. 3A, upper panel, compare lanes 1 and 5; Fig. 3B, upper panel, compare lanes 1 and 7), we replaced N^{303} (relatively large, polar, uncharged) with serine (relatively small, polar, uncharged), or with isoleucine (relatively large, hydrophobic). When expressed alone, N303SE2HA migrated very similarly to N303AE2HA, while N303IE2HA failed to form an ~2 MDa complex (Fig. 3B, upper panel, lanes 7, 9 and 11). This suggests that there are steric constraints at position 303 of E2, with small amino acid side chains (those of alanine and serine) enhancing assembly, and large side chains (that of isoleucine) inhibiting assembly. Interestingly, a serine (S^{305}) is found in E1 in the position equivalent to N^{303} of E2 (Fig. 1), which may explain why E1 alone migrates more predominantly at ~2 MDa than E2 alone (Fig. 2A).

To examine whether the aromaticity of phenylalanine at position 305 in E2 is critical to assembly, we replaced F^{305} (large hydrophobic, aromatic) with leucine (large hydrophobic, non-aromatic) and found that F305LE2HA does not assemble into ~2 MDa complexes in the absence or presence of E1flag, suggesting that aromaticity, rather than just hydrophobicity at position 305, is required for assembly (Fig. 3B, lanes 13 and 14). These data also show that the inability of I301AE2HA, M304AE2HA and F305AE2HA to assemble is not simply because the number of residues in the hydrophobic cluster is reduced from 3 to 2, since F305LE2HA possesses 3 clustered hydrophobic residues.

Overall, these data indicate that the formation of higher-order erlin complexes is extremely sensitive to perturbation of the

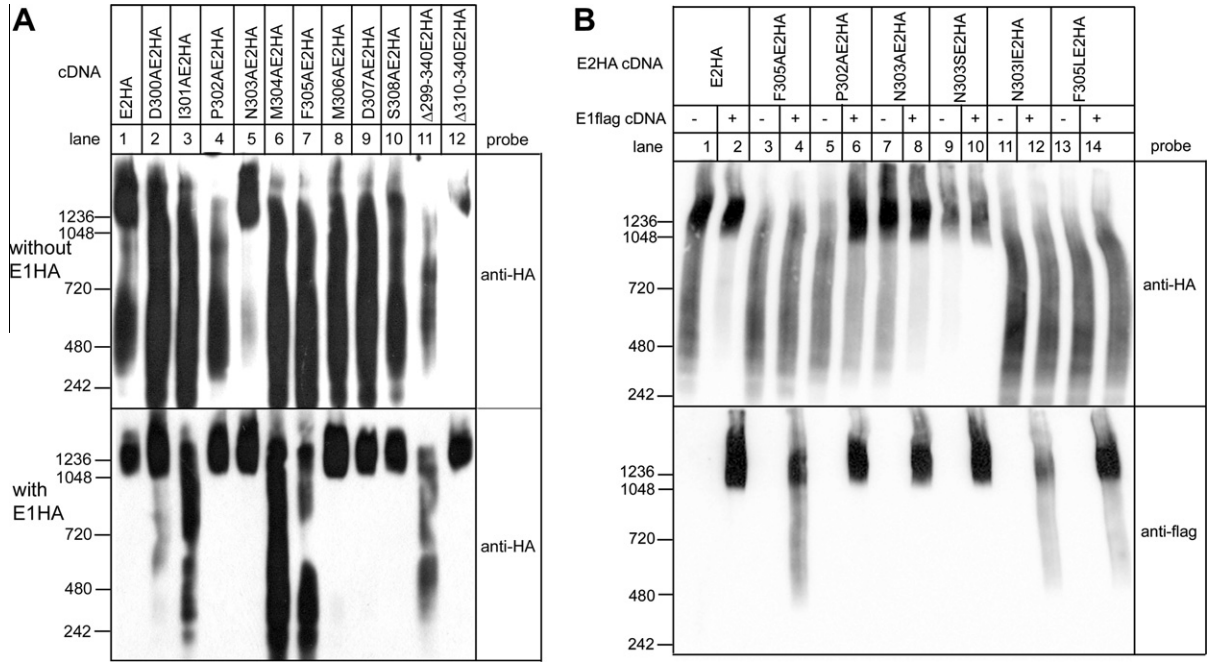

Fig. 3. Effects of mutations to the E2 assembly domain on higher-order complex formation Hela cells were transfected with vectors encoding E2HA and its mutants, (A) either without E1HA or with E1HA, or (B) either without or with E1flag. Cell lysates were prepared and were subjected to native-PAGE and were probed with anti-HA or anti-flag as indicated. The migration positions of molecular mass markers are indicated (masses in kDa).

assembly domain, that three hydrophobic residues oriented to one side of the assembly domain form a hydrophobic patch that drives assembly, that aromaticity of F^{305} contributes to assembly, and that even residues on the non-hydrophobic side of the assembly domain (e.g. at position 303 in E2) influence assembly through steric effects.

3.3. Cross-linking of the E1/2 complex

To examine whether there is a lower-order assemblage or "basic unit" from which the E1/2 complex is constructed, we employed chemical cross-linking. Previous immunoprecipitation studies have shown that E1 and E2 can form homo-oligomers when expressed individually, can form hetero-oligomers when co-expressed, and that the formation of these oligomers does not require ~2 MDa complex assembly [6,12]. Brief exposure of cells expressing exogenous E1HA or E2HA to DSS, a homobifunctional primary amine-specific cross-linker that couples adjacent lysine residues, yielded species indicative of partial cross-linking (Fig. 4A, lanes 1–12). For E1HA, clear bands at ~112 kDa and ~87 kDa were seen, corresponding in size to dimers and trimers, respectively, with the ~112 kDa band predominating (lane 2). For E2HA, bands of similar size were seen, but with the ~87 kDa band predominating (lane 3). Thus, erlins appear capable of forming homodimers and homotrimers when expressed alone. To examine whether there is a preference for the formation of homomers or heteromers when both erlins are expressed, E1HA and E2HA were co-expressed with Δ310–340E2HA, which contains the AD and forms ~2 MDa complexes (Fig. 3A, lane 12), but which is substantially smaller than E1HA and E2HA, and which when expressed alone is cross-linked into an ~72 kDa band, corresponding in size to dimers (Fig. 4A, lanes 4, 7 and 10). When Δ310–340E2HA was

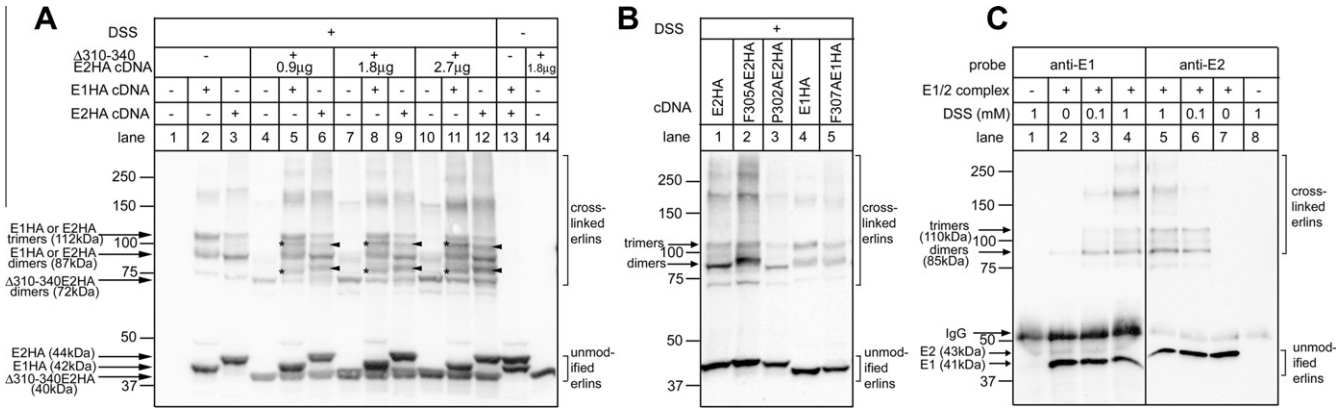

Fig. 4. Cross-linking of erlins (A) and (B). Hela cells were transfected with the vectors indicated, were exposed to 1 mM DSS for 1 min, and cell lysates were subjected to SDS–PAGE and were probed with anti-HA (C). E1/E2 complex immunopurified from αT3 cells (lanes 2–7) and control material lacking E1/E2 complex (lanes 1 and 8) were exposed to DSS for 2 min as indicated and subjected to SDS–PAGE and probed with anti-E2 and rabbit polyclonal anti-E1 [6]. The migration positions of molecular mass markers are indicated (masses in kDa) as are the positions of unmodified and cross-linked erlins and IgG heavy chain.

co-expressed with E1HA, new bands were formed at ~75 and 100 kDa (lanes 5, 8 and 11, marked with asterisks), indicative of the formation of heterodimers and heterotrimers between E1HA and the E2HA mutant. Interestingly, similarly sized bands were formed with the same efficiency when Δ310–340E2HA was co-expressed with E2HA (lanes 6, 9 and 12, marked with arrowheads), indicating that Δ310–340E2HA interacts equally well with E1HA and E2HA. These data indicate that there is no preference for homomeric versus heteromeric interactions in the formation in lower-order erlin oligomers, and that when co-expressed, E1 and E2 associate in an unbiased manner. That these lower-order interactions were independent of higher-order assembly is demonstrated by the observations that E1HA and E2HA mutants incapable of forming ~2 MDa complexes (F305AE2HA, P302AE2HA and F307AE1HA) were cross-linked into dimers and trimers similarly to wild-type E2HA and E1HA (Fig. 4B).

Lower-order assembly was also examined using endogenous E1/2 complex immunopurified from αT3-1 cells (Fig. 4C), that is composed of an ~1:1 mixture of E1 and E2 [6]. While E1 and E2 migrate at 41 and 43 kDa, respectively, brief exposure to DSS caused the formation of anti-E1 and anti-E2 immunoreactive bands at ~85 and 110 kDa, corresponding in size to dimers and trimers. The co-migration of anti-E1 and anti-E2 immunoreactivity suggests that these bands correspond to heterodimers and heterotrimers, and again support the notion that there is no preference for homomeric lower-order assemblages. Thus, it is reasonable to conclude that the basic unit from which the ~2 MDa complex is assembled is a lower order hetero-oligomer, either a heterodimer or a heterotrimer.

4. Discussion

Our studies have provided insight into both the nature of the assembly domain that is critical for erlin1/2 complex assembly and the basic unit from which the complex is assembled. A fundamental conclusion is that the assembly of E1 and E2 appears to be an unbiased process. This stems from the findings that E1 alone and E2 alone can form ~2 MDa complexes, and that Δ310–340E2HA cross-links equally well with E1HA and E2HA. Further support for this conclusion comes from data showing that exogenous E1 and E2 can form homo-oligomers equally as well as hetero-oligomers [12], and that essentially any ratio of E1:E2 will assemble into ~2 MDa complexes [6]. Thus, it appears that any E1 and E2 present in the ER membrane will randomly associate. This lack of bias is most probably a reflection of the great similarity between E1 and E2; the amino acid sequences of mouse E1 and E2 are 76% identical, and the region from the transmembrane domain to the end of the assembly domain (amino acids 27–311 in E1 and 25–309 in E2), that includes all of the determinants for assembly, are 85% identical [6]. Finally, these conclusions are consistent with the observation that E1 is not dependent upon E2 for stability, and vice versa [6,12]. Interestingly, this contrasts with the situation for prohibitins 1 and 2 and flotillins 1 and 2, that are destabilized in the absence of their counterparts [5,22]. This may be because, for these proteins, there are constraints to how oligomers form; for prohibitins, at least, it appears that hetero-oligomers are the only viable lower-order assemblage [5].

Our cross-linking data also provide evidence for the existence of lower-order E1/E2 assemblages that may represent the basic unit that is multimerized into higher-order complexes. For both wild-type erlins, and mutant erlins incapable of assembling into ~2 MDa complexes, DSS caused the formation of species corresponding in size to dimers and trimers. Thus, these lower-order oligomers exist independently of higher-order assembly and are likely held together by the coiled-coil and α/β domains of E1 and E2 (Fig. 1 A), deletion of which inhibits interaction between E1 and E2 [6]. While there are currently no structural data for E1 or E2, the crystal structure of a prokaryotic stomatin has been solved, revealing a trimeric assembly [23]. Given the similarities between the erlins and stomatin [1], it is quite plausible that erlins too are trimeric. Thus, the basic unit may well be erlin trimers, and as E1 and E2 are co-expressed in all cell types so far examined [6,9,12], heterotrimers (E1 + E1 + E2 or E2 + E2 + E1) will be the most likely assemblage.

It appears that these basic units are linked into ~2 MDa complexes by the assembly domains of E1 and E2. Molecular modeling and mutagenesis indicate that assembly domains are amphipathic helices and that a cluster of 3 interacting hydrophobic residues are essential to this process. Mutation of any one of these 3 residues to alanine may reduce the mutual attraction between the hydrophobic/aromatic faces of the assembly domains of adjacent erlins, such that linkage into higher-order complexes becomes impossible. The importance of the hydrophobic cluster is consistent with the notion of "hot spots" in protein interfaces – amino acids or small regions whose mutation leads to a significant drop in binding free energy [24]. The requirement for phenylalanine for ~2 MDa complex assembly raises the possibility that phenylalanine aromaticity in adjacent assembly domains provides binding free energy, perhaps by stacking, as has been seen in other proteins [25]. Interestingly, computational analysis of protein structures shows that clusters of aromatic residues commonly participate in protein–protein interactions; for example, a cluster of 20 interacting aromatic residues interact in epoxide hydrolase oligomers [26]. The same ideas may apply to stomatin, in which mutation of any of the amino acids in a small hydrophobic stretch near the C-terminus (STIVFPLPI) inhibits higher-order oligomerization [14].

As the E1/E2 complex is ring-shaped and is composed of ~40 E1 and E2 subunits [6], a model that accommodates our new data would be that ~14 E1/E2 heterotrimers are linked into a ring by the three critical hydrophobic residues on the hydrophobic faces of assembly domains. Such a model would be consistent with the data showing that lower-order assembly is independent of higher-order assembly (Fig. 4B, [6,12]), that higher-order assembly is mediated by the 3 hydrophobic residues on one face of the assembly domain helices (Figs. 1 and 3), and that the non-hydrophobic side of the helices can also influence higher-order assembly (Fig. 3). Finally, the importance of the assembly domain to erlin function appears to be exemplified by a recently described autosomal recessive disease that is caused by a frameshift mutation in E2 that truncates the protein at residue 272 [27]. This mutant should be able to assemble into lower-order oligomers [6], but lacks the assembly domain (Fig. 1A), and thus will be incapable of forming ~2 MDa complexes.

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