Loss of Extended Synaptotagmins ESyt2 and ESyt3 does not affect mouse development or viability, but in vitro cell migration and survival under stress are affected

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Keywords: Extended-Synaptotagmin, Esyt1, Esyt2, Esyt3, expression analysis, genetic deletion, phenotypic analysis, cell migration defects, cell survival defects, signal transduction

The Extended Synaptotagmins (Esyts) are a family of multi-C2 domain membrane proteins with orthologs in organisms from yeast to human. Three Esyt genes exist in mouse and human and these have most recently been implicated in the formation of junctions between endoplasmic reticulum and plasma membrane, as well as the Ca²⁺ dependent replenishment of membrane phospholipids. The data are consistent with a function in extracellular signal transduction and cell adhesion, and indeed Esyt2 was previously implicated in both these functions in Xenopus. Despite this, little is known of the function of the Esyts in vivo. We have generated mouse lines carrying homozygous deletions in one or both of the genes encoding the highly homologous Esyt2 and Esyt3 proteins. Surprisingly, esyt2⁻/⁻/esyt3⁻/⁻ mice develop normally and are both viable and fertile. In contrast, esyt2⁻/⁻/esyt3⁻/⁻ mouse embryonic fibroblasts display a reduced ability to migrate in standard in vitro assays, and are less resistant to stringent culture conditions and to oxidative stress than equivalent wild type fibroblasts.

Introduction

The Extended Synaptotagmins (Esyts) are multiple C2 domain containing membrane proteins. The first member of this family of proteins was isolated from preparations of plasma membranes and high density microsome fractions of rat adipocytes.¹ However, the Esyts were not further considered until 2007, when the primary structures of the 3 human Esyts1 to 3 were determined and their membrane associations investigated.² Human Esyt1 was shown to contain 5 C2 domain homologies, while human Esyts 2 and 3 each contain 3. The C2 domains are preceded by a ~300aa N-terminal region containing one or 2 putative membrane spanning domains and a predicted SMP domain ³,⁴,⁵ (Fig. 1A). Solution studies of the C2 domains of Esyt2 have confirmed their structural identity and shown that when linked they exhibit calcium dependent multimerization, while the domains display different abilities to coordinate Ca²⁺.⁶,⁷,⁸ The C2C domains of Esyt2 and 3 interact with phospholipids driving the recruitment of these Esyts to phospholipids within the plasma membrane.²,⁹ In previous studies, we identified Xenopus ESyt2 as an endocytic adapter that determines the timing of ERK activation in blastula embryos by binding both Fibroblast Growth Factor Receptor (FGFR) and Adaptin 2 (AP-2) to catalyze rapid receptor endocytosis via the Clathrin pathway.⁹ We further showed that ESyt2 recruits the cytoskeleton regulator p21-Activated-Kinase-1 (PAK1) to modulate local actin polymerization,¹⁰ a function required during endocytosis.¹¹ Most recently it was shown that the ESyts and the related yeast Tricalbins are in fact found inserted into the endoplasmic reticulum (ER) at sites of contact with the Plasma Membrane (ER-PM junctions).¹²,¹³,¹⁴ This has given rise to the model of the ESyts as 2-pass ER membrane proteins that link the ER to the PM via a C2C-domain-PtdIns(4,5)P₂ interaction. Most recently, Esyt1 was shown to stimulate the formation of ER-PM junctions in a Ca²⁺-dependent manner, and in this way to promote recruitment of the phosphatidylinositol transfer protein (PTTP) Nir2 and phospholipid incorporation into the PM.¹⁴ To date the demonstration that Esyt2 is required for mesoderm formation in early Xenopus embryos remains the only
Figure 1. For figure legend, see page 2618.
Table 1. Genotype analysis of the progeny born A) from esyt2+/−/esyts2+/− (alleles CA0077 and AN0678) and esyt3+/−/esyts3+/− crosses, and B) from esyt2+/−/esyts3+/− crosses as compared with the expected Mendelian frequencies.

| Gene      | Number of pups | WT/WT | ++/− | −/− |
|-----------|----------------|-------|------|-----|
| ESYt2 (CA0077) | 171         | 41 (24%) | 86 (50%) | 44 (26%) |
| ESYt2 (AN0678) | 180         | 53 (29%) | 87 (48%) | 40 (22%) |
| ESYt3      | 143          | 18 (23%) | 43 (55%) | 17 (22%) |
| % Expected |              | 25%    | 50%  | 25% |

B) ESyt2/ESyt3

| Number of pups | ++/− ++/− | ++/− −/− | −/− ++/− | −/− −/− |
|----------------|-----------|----------|----------|----------|
| 143            | 10 (7.0%) | 17 (11.9%) | 9 (6.3%) | 10 (7.0%) |
| % Expected     | 6.25%     | 12.50%   | 6.25%    | 12.50%   |

Results

Targeted disruption of the mouse ESYt2 and ESYt3 genes

ES cells carrying insertions in the esyt2 gene (#CA0077 and AN0678, International Gene Trap Consortium (IGTC)) (Fig. 1B–D), and “Knockout First” ES cells carrying a potentially conditional insertion in esyt3 (EPD0458_5_A10, European Conditional Mouse Mutagenesis Program (EUCOMM)) (Fig. 1E–G) were used to generate chimeric mice. Southern blotting and targeted PCR analysis showed that transmission of the mutant alleles was obtained in each case.

ESyt2, −3 and 2/3 null mice are viable

We found that not only were the esyt2+/− and esyt3+/− mice viable, but the frequency of wild-type, heterozygous and homozygous null genotypes followed a Mendelian pattern of inheritance (Table 1A). Moreover, we found that the esyt2+/− and esyt3+/− mice were fertile, produced litters of normal size and did not show any overt morphological defects compared to their heterozygous and wild-type littermates. When esyt2+/− and esyt3+/− mice were crossed they also generated viable esyt2+/−/esyts3+/− offspring at near Mendelian ratios (Table 1B). The ratios did however show some skewing toward esyt2+/−/esyts3+/− double heterozygotes at the expense of esyt2+/−/esyts3+/− and esyt2−/+/esyts3−/−, suggesting minor effects on viability during development. As expected, the esyt2−/+/esyts3−/− mice expressed no detectable level of the corresponding mRNAs, but continued to express ESYt1 mRNA at wildtype levels (Fig. 1). ESYt2−/−, esyt3−/−, and esyt2−/+/esyts3−/− mice also displayed a normal life span, several being kept for 18 months with no premature signs of senescence. Thus, the esyt2 and esyt3 genes are not essential for mouse development, viability, survival or reproduction.

Expression pattern of the ESYts in mouse adult tissues

Expression of the esyt2 and particularly of esyt3 genes were found to be highly tissue specific in adults. ESYt2 mRNA was predominantly detected in lung, spleen, testis and stomach, and at much lower levels in all the other tissues tested (Fig. 1). The same tissue specific expression pattern was reflected for ESYt1 mRNA with the sole exception of testis, which showed low levels of ESYt1 mRNA. In contrast, ESYt3 mRNA was only expressed strongly in lung and testis, and was present at low levels only in stomach and possibly brain. The strongly overlapping expression profiles may provide some explanation for the lack of an ESyt2/3-null phenotype if ESYt1 can functionally replace the other 2 ESyts.

Expression of ESYt2 and 3 in mouse embryos

It was possible that the lack of a developmental phenotype simply correlated with a lack of expression of esyt2 and/or −3. However, using hetero- and homozygous embryos expressing β-galactosidase from the respective endogenous gene promoters we found that the esyt2 gene was expressed throughout the 10.5 to 12.5 dpc embryo with little regional specificity. Expression was, however, highest in the neural tube and later in the dorsal root ganglia (Fig. 3). In complete contrast, at 10.5 dpc the esyt3 gene was expressed only at the midbrain-hindbrain border.
Esyt2 and Esyt3 deficiency does not impair organ development

It was possible that the Esyt2/3 null mice harbored minor organ defects that did not affect their viability. Hence, we studied the structure of a range of organs from adult mice. However, we failed to detect anything unusual in the histology of lung, testis or spleen, in which Esyt2 and 3 are strongly expressed, or kidney, in which Esyt1 and 2 are expressed only weakly and Esyt3 was not detected (Fig. 4). Similarly, cursory inspection of brain and muscle histology detected no abnormalities (data not shown).

Esyt2 loss does not affect FGF activation of ERK in MEFs

Given that previous data had implicated Xenopus Esyt2 in FGF signaling in early Xenopus embryos, we generated embryonic fibroblasts from both Esyt2 and Esyt2/3 null mice and studied their response to FGF and other stimuli. As shown in Fig. 2, MEFs do not contain Esyt3 mRNA, hence we first determined whether or not activation of signaling pathways were affected in esyt2−/− MEFs. After overnight serum withdrawal, FGF, EGF and serum (FBS) induced robust and similar levels of activation of ERK and AKT in both wt and esyt2−/− MEFs (Fig. 5).

Esyt2/3 loss does affect migration of MEFs and their viability under stress

Despite the lack of effect on signal transduction, “scratch-test” assays to determine the ability of cells to migrate when stimulated by FGF revealed that both esyt2−/− esyt3−/− MEFs (Fig. 6A) and the esyt2−/− MEFs (not shown) tended to migrate in a far less coordinated fashion and maintained little cell-cell contact during their migration as compared to wt (esyt2+/+ esyt3+/+) MEFs. The esyt2−/− and esyt2−/− esyt3−/− MEFs also migrated far less rapidly (Fig. 6A & B). As would be expected given the lack of esyt3 expression in MEFs (Fig. 2), esyt2−/− esyt3−/− MEFs displayed the same migration defect as the esyt2−/− MEFs (Fig. 6B).

The esyt2−/− esyt3−/− MEFs were also significantly less resistant to serum withdrawal or oxidative stress as compared to the wt ones. Withdrawal of serum over 4 d of incubation caused a 75% reduction in viability in wt MEFs, while less than 3% of esyt2−/− esyt3−/− MEFs survived this treatment (Fig. 6C). Despite this, FGF afforded a similar level of protection in both cell types, consistent with its ability to activate signaling pathways in both. The esyt2−/− esyt3−/− MEFs were also extremely sensitive to oxidative damage as compared to the wt, and again here FGF provided some degree of protection in both cases. These data show that inactivation of the esyt2−/− and esyt2−/− esyt3−/− genes does indeed affect aspects of cell migration and viability. These defects must, however, be compensated for in the in vivo context of the mouse.

Discussion

Given the apparent importance of Esyt2 during Xenopus development and the recent demonstrations of the role of the Esyt in ER-PM junction formation and phospholipid generation, the lack of phenotypic effects due to the loss of Esyt2 and 3 in mice was fully unexpected. It is, however, not without precedent. Yeast contains 3 Tricalbin (Tcb) proteins that are structurally closely related to the mammalian Esyts. Deletion studies in yeast of the Tricalbin family show that they are highly functionally redundant and in concert with other membrane tethering proteins they promote ER-PM junction formation. Indeed, deletion of all 3 Tcb s was not in itself sufficient to eliminate ER-PM tethering and this required deletion of 3 other proteins, Ist2 (a TMEM16 ion channel family member) and the vesicle-associated membrane protein-associated protein (VAP) orthologs Scs2 and Scs22. We previously demonstrated a requirement for Xenopus Esyt2 in FGF signal transduction, receptor

Figure 2. Expression of Esyt1, −2 and −3 mRNA in adult mouse tissues and MEFs. RT-PCR analyzes are shown for tissues from both wild type esyt2+/+ esyt3+/+ and esyt2−/− esyt3−/− mice as compared with GAPDH.
Figure 3. Expression pattern of the esyt2 and esyt3 genes in early mouse embryos. Expression was determined by conversion of X-Gal (blue-green) by β-galactosidase produced from the gene inserted into the esyt2 and esyt3 gene loci. Enlarged panels on the right show a limb-bud and the hindbrain region of esyt3+/βGal embryos. "aer" apical ectodermal ridge, "mhb" midbrain-hindbrain boundary, "fb" forelimb bud, "hb" hindlimb bud, "url" and "lrl" upper and lower rhombomere lips, "r2-6" rhombomeres, "ov" otic vesicle, "nt" neural tube, "drg" dorsal root ganglion.
endocytosis and mesoderm induction.\textsuperscript{9,10} Why very early Xenopus development was sensitive to Esyt2 depletion, while mouse is clearly not, is still unclear. This said, the expression profiles of the Xenopus Esys suggest that only Esyt2 mRNA is present maternally (NCBI Unigene EST_Profiler, Xenbase).\textsuperscript{22} Thus, Esyt2 may be the only family member present during early cleavage divisions.

Despite the apparent lack of a requirement for Esyt2 and -3 in mouse, MEFs carrying homozygous deletion of one or both genes display clear migration deficits in scratch test assays and are significantly more susceptible to stringent culture conditions and to oxidative stress than otherwise isogenic wt MEFs. Given the connection with the PAK1 function, it is tempting to suggest that this is due to defects in cytoskeletal dynamics. We note that mRNA levels of Esyt1, the only remaining Esyt in the \texttt{esyt2\textsuperscript{-/-}} and \texttt{esyt2\textsuperscript{-/-} esyt3\textsuperscript{-/-}} MEFs, are low. Possibly then this level of Esyt1 is insufficient to compensate. Clearly, these issues will only be resolved by the generation of Esyt1-null and possibly Esyt1/2/3 null mice.

**Materials and Methods**

Genotype analysis of targeted ES cells and mice

\texttt{esyt2\textsuperscript{+/+} esyt3\textsuperscript{+/+}}, \texttt{esyt2\textsuperscript{-/-} esyt3\textsuperscript{+/+}} (gene trapped clones Esyt2\textsuperscript{Gr} (AN0678)/Wtsi and Esyt3\textsuperscript{Gr} (CA0077)/Wtsi) and \texttt{esyt3\textsuperscript{+/+}} (targeted clone Esyt3\textsuperscript{tn4a(EUCOMM)Wtsi}) embryonic stem (ES) cells were generated respectively by SIGTR and EUCOMM from Wellcome Trust Sanger Institute with the targeting vectors shown in Fig. 1. These clones were each used to generate 2 independent mouse lines. Southern blot analysis was used to determine the genotype of single \texttt{esyt2} or \texttt{esyt3} mutant ES cell lines and mice. For the Esyt2 clone AN0678, genomic DNA was
and D amplified a fragment of 446 bp from the β-gal allele. The mice were housed and manipulated according to the guidelines of the Canadian Council on Animal Care and experiments were approved by the institutional animal care committee.

**Gene expression analysis by RT-PCR**

Total RNA was extracted from mouse tissues using Trizol (Invitrogen) and quantified by absorbance at 260 nm. Two μg of total RNA was reverse transcribed using random primers (GE Healthcare) and mMLV reverse transcriptase (Invitrogen). PCR was performed using the primers designed with Primer3 (Untergasser et al. 2007) and the number of PCR cycles was optimized to be within the linear range of amplification. The primers used were: mESyt1.FOR (5'-TGGGATCCCTGTATCTCAGC), mESyt1.REV (5'-CTGGAGATCAGTCCATT), mESyt2.FOR (5'-CGAATCAGGTCCTTCTTG), mESyt2.REV (5'-GCTCTGGAAGATTTGGTT), mESyt3.FOR (5'-CAAGCGCTTCTACTGAGCTG), mESyt3.REV (5'-AGCAATGGAGCTCGGATCAC), mGAPDH.FOR (5'-AACTTGCGATTGTGAAGGAG), mGAPDH.REV (5'-ACACATTGGGGAAGAACATA). Amplicons were of the expected sizes of 296 bp for ESyt1, 192 bp for ESyt2, 246 bp for ESyt3 and 223 bp for GAPDH. Products were sub-cloned and sequenced to confirm their specificity.

**X-gal staining**

Mouse embryos were isolated at E10.5 to E12.5 and fixed for 30 minutes in 1% Formaldehyde, 0.2% Gluteraldehyde, 0.02% NP-40 in 1 x PBS, washed 3 times 20 min. each in Wash Solution (2 mM MgCl2, 0.02% NP40, 1 x PBS). Embryos were protected from light and incubated overnight at R/T in the Staining buffer solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal in Wash Solution). Embryos were rinsed 3 times, 20 min. each, in 1 x PBS. Clarification was performed with “Scale” solution as described previously.15

**Histopathology**

Organs were dissected from 11-month old adult mice and fixed for more than 24 hours in 4% paraformaldehyde in PBS. Samples were progressively dehydrated and embedded in paraffin. Cross sections of 5 to 20 microns were cut and stained with hematoxylin and eosin.

**Cell culture and migration assay**

Primary mouse embryo fibroblasts (MEFs) from E14.5 embryos were prepared as described16,17 and cultured in Dulbecco’s modified Eagle medium (DMEM) – high glucose (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Wisent) and Penicillin/Streptomycin/Antimycotic (Anti-Anti, Invitrogen). The effects of ESyt2 and ESyt3 loss on MEF’s migration were determined in a wound-healing assay (Scratch Test).18 Cells were seeded in a multi-6 well plate, 12 h later serum was withdrawn and cells incubated for a further 16 h
Figure 6. For figure legend, see page 2624.
Signal transduction assays

Serum was withdrawn from cultures of Eṣyt2+/+ and −/− MEFs for 16 h prior to stimulation with bFGF (20 ng/ml)/heparin (5 μg/ml), EGF (100 ng/ml) or FBS (10%). Whole cell extracts were prepared using Triton lysis buffer (50 mM Tris [pH 7.5], 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of aprotinin, leupeptin and pepstatin), and cleared by centrifugation (20 min., 20,000 g, 4deg.C). Activation of ERK and AKT was examined by Western Blotting using 20 μg of protein extract and the antibodies to phospho-ERK1/2 (Sigma), phospho-AKT (Cell Signaling) and ERK2 (J. Grose). Immune complexes were detected using HRP-conjugated secondary antibodies and ECL+ (GE HealthCare).

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