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Unconventional secretion of tubby and tubby-like protein 1

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ABSTRACT

Tubby-like proteins (Tulps) with no signal peptide have been characterized as cytoplasmic proteins with various intracellular functions, including binding to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2]. PI(4,5)P_2 has been implicated in unconventional secretion of fibroblast growth factor-2 without a signal peptide. Here, we show that all Tulps are expressed intracellularly and extracellularly. Tubby secretion is partially dependent on its PI(4,5)P_2-binding activity with an essential secretory signal in the N-terminus. Pathogenic mutation in *Tubby* mice has no impact on tubby extracellular trafficking. Moreover, unconventional secretion of tubby and Tulp1 is independent of endoplasmic reticulum–Golgi pathway. These data implicate that Tulps may function extracellularly as well.

1. Introduction

Obesity has emerged as one of the most important public health issues. *Tubby* mice with a spontaneous mutation in *tubby* gene develop adult-onset obesity, progressive retinal and cochlear degeneration with unknown mechanism [1,2]. Tubby and tubby-like proteins are encoded by a gene family with a unique sequence similarity. Four mammalian tubby family members (tubby, tubby-like protein 1 (Tulp1), tubby-like protein 2 (Tulp2) and tubby-like protein 3 (Tulp3), or Tulps) have been identified [1,2], sharing a highly conserved C-terminal "tubby domain" of ~260 amino acids. The N-terminal half of the proteins is less well conserved and may define their functional specificity and distinct disease profile. Unlike *Tubby* mice, autosomal recessive mutations of human Tulp1 associate only with retinitis pigmentosa [3,4]. Similarly, Tulp1$^{-/-}$ mice develop early onset retinal degeneration [5,6].

Tubby is expressed in several major brain areas, including the hypothalamus controlling feeding behavior and in the spiral ganglion of the inner ear [7]. Tubby, Tulp1 and Tulp3 are expressed in the retina [8]. High level of Tulp1 expression has been demonstrated in the inner segment of the photoreceptors [8,9]. Due to lack of a signal peptide, Tulps are presumably expressed only as intracellular proteins [1,2]. In Neuro-2a cells, intracellular tubby associates with the inner leaflet of the plasma membrane by binding to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] through the "tubby domain" [10]. Receptor-mediated activation of G protein α_q releases tubby from the plasma membrane and triggers its translocation into the nucleus [10], implicating that tubby may be a membrane-bound transcription factors. However, the target genes transcriptionally regulated by the protein are yet to be identified. Other characterized intracellular functions include tubby involvement in an endocytic pathway for regulation of fat storage [11], and Tulp1 in vesicle transportation, rhodopsin localization in the photoreceptors [12], and interaction with F-actin and neuronal-specific GTPase dynamin-1 [13,14]. However, the pathological relevance of their intracellular functions is yet to be established.

Previous studies have demonstrated that a heterogeneous group of proteins with no signal peptide are secreted to the extracellular space through unconventional secretory pathways with poorly-defined mechanisms [15,16]. In light of a recent report that the well-documented unconventional secretion of fibroblast growth factor-2 (FGF-2) is mediated by its binding to PI(4,5)P_2 [17,18], we hypothesize that tubby with the similar binding

Abbreviations: Ab, antibody; ER, endoplasmic reticulum; FGF-2, fibroblast growth factor-2; FBS, fetal bovine serum; Gal-3, galectin-3; GFP, green fluorescence protein; LDH, lactate dehydrogenase; mAb, monoclonal antibody; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; tubby-2M, tubby with double mutation at Lys330Ala and Arg332Ala; tubby-ΔC44, mutant tubby with C-terminal 44 amino acid deleted; Tulp1, tubby-like protein 1; Tulp2, tubby-like protein 2; Tulp3, tubby-like protein 3

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activity may be secretory as well [10]. Here we demonstrate that all Tulps are expressed both intracellularly and extracellularly. Tubby extracellular trafficking is partially dependent on its C-terminal binding to $PI(4,5)P_2$. An essential signal sequence is located in tubby N-terminal region between Asn51 and Arg100. Unconventional secretion of tubby and Tulp1 is independent of classical endoplasmic reticulum (ER)–Golgi pathway. However, the pathogenic mutation in *Tubby* mice is irrelevant to its extracellular expression. These findings provide justification to extend the investigation of the pathological mechanisms of tubby and Tulp1 from the intracellular to the extracellular compartment.

2. Materials and methods

2.1. Cell culture condition

PC12 cells were cultured in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum (FBS), 2 mM L-glutamine, 1.5 g/l NaHCO3, 10 mM HEPES, 1 mM sodium pyruvate. Neuro-2a cells were cultured as previously described [19]. Alternatively, PC12 and plasmid-transfected Neuro-2a cells were cultured in serum-free 293SFM II medium (Invitrogen) supplemented with L-glutamine.

2.2. Plasmids

Full-length cDNA coding sequences for Tulps were generated from mouse retina by reverse transcription PCR. N-terminal FLAG-tagged Tulps were further generated by PCR using overlapping primers and cloned into pcDNA3 (Invitrogen). FLAG-tubby with double mutations at Lys330Ala and Arg332Ala (tubby-2M) was generated by PCR using overlapping primers with mutations embedded in primers appropriately and cloned into pcDNA3. FLAG-tagged tubby mutants with N- or C-terminal deletions or with C-terminal 44 amino acids (aa) replaced by 22-aa polypeptide derived from Intron 11 (Tubby-Δ44) were generated by PCR and constructed in a similar manner. C-terminal FLAG-tagged galectin-3 (Gal-3) was constructed in a similar manner. Control green fluorescent protein (GFP) with C-terminal FLAG tag was generated by PCR in pEGFP-N1 plasmid (Clontech) and validated by GFP expression. C-terminal FLAG-tagged Dickkopf homolog 3 (DKK3-FLAG) was a gift from Dr. Cristof Niehrs (Heidelberg, Germany). All plasmids were verified by DNA sequencing and Western blot using anti-FLAG M2 monoclonal antibody (mAb) (Sigma).

2.3. Extracellular expression of Tulps

Neuro-2a or HEK 293 cells were transfected with plasmids expressing Tulps or pcDNA3 by calcium phosphate method. Transfected Neuro-2a cells were switched to 293SFM II medium at 24 h post-transfection. The conditioned medium were collected every 12 h between 24 and 64 h, concentrated by filter concentrator units (Pierce, 9 kDa MW cutoff), and analyzed by Western blot using anti-FLAG M2 mAb. Alternatively, FLAG-Tulps collected in the complete media in the presence or absence of brefeldin A, monensin, nocodazole or methylamine were pulled down with anti-FLAG mAb affinity beads (Sigma) and analyzed by Western blot using anti-FLAG-mAb. The transfected Neuro-2a cells at 48 h post-transfection were also collected, washed, lysed and analyzed by Western blot for intracellular FLAG-Tulps. The bound primary antibody (Ab) was detected by horseradish peroxidase-conjugated secondary Ab, revealed by chemiluminescence, and recorded by ChemiDoc system (Bio-Rad). If necessary, the signals were quantified by Quantity-One software (Bio-Rad).

PC12 cells were cultured in 293SFM II medium. The conditioned medium was collected every 24 h, concentrated and analyzed by Western blot using anti-tubby Ab (Santa Cruz Biotechnology). The same membrane was stripped with a stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris–HCl, pH 6.8) at 50 °C for 30 min and re-probed with anti-lactate dehydrogenase (LDH) mAb (Santa Cruz Biotechnology).

2.4. LDH assay

LDH in conditioned media was detected by Cytotoxicity Detection Kit^{plus} (Roche) according to the manufacturer's protocol. Briefly, cell-free conditioned medium (100 μl) was mixed with the reaction mixture in 96-well plates, incubated at room temperature for 10–30 min, stopped by adding 50 μl of the stop solution, and quantified at 490 nm by a microplate reader. To verify the presence of LDH in the viable cells, we treated Neuro-2a with low level of UV-irradiation (254 nm, 20 mJ/cm^2). The cell-free medium was collected 24 h after the UV-irradiation and analyzed for LDH release. Alternatively, PC12 cells were treated with staurosporine (1 μg/ml) for 2 h, washed, cultured in 293SFM II medium or serum-containing medium for 16 h. The conditioned medium was analyzed for LDH.

2.5. Data analysis

All experiments were repeated independently for three times. All data are expressed as the means ± S.D. Student's *t*-test is used to analyze the statistical significance.

3. Results

3.1. Extracellular expression of Tulps in transfected cells

To analyze possible extracellular expression of Tulps, we expressed FLAG-tagged Tulps in Neuro-2a cells by plasmid transfection. Transfected cells were cultured in the defined serum-free 293 SFM II medium. The intracellular and extracellular FLAG-Tulps were analyzed by Western blot using anti-FLAG mAb (Fig. 1A). The results showed that all Tulps were expressed intracellularly and extracellularly. As a negative control, GFP-FLAG with no signal peptide was expressed exclusively as an intracellular protein under the same experimental conditions (Fig. 1A). It is noteworthy that GFP-FLAG had only about half of the molecular mass of FLAG-Tulps. If passive leakage across the plasma membrane was responsible for the extracellular FLAG-Tulps, GFP-FLAG would be detected in the culture medium as well. In this regard, the absence of GFP-FLAG in the conditioned medium suggested that extracellular FLAG-Tulps were resulted from selective secretion, rather than from passive release. Moreover, possible release of LDH from cytoplasm was analyzed. LDH is a stable enzyme normally found in the cytoplasm of all cells, but rapidly releases into the extracellular space upon damage of the plasma membrane. Because of its sensitivity, LDH has been widely used as a surrogate biomarker to detect possible release of cytoplasmic proteins from apoptotic cells [20]. LDH assay verified that the minimal amount of cytoplasmic proteins was passively released under the experimental conditions (Fig. 1B). On the other hand, low level of UV-irradiation rapidly induced LDH release, suggesting that the transfected cells were healthy and responsive (Fig. 1B).

3.2. Extracellular expression of endogenous tubby in PC12 cells

Constitutive expression of tubby has been previously reported in PC12 cells [21]. This offers an opportunity to analyze the secre-

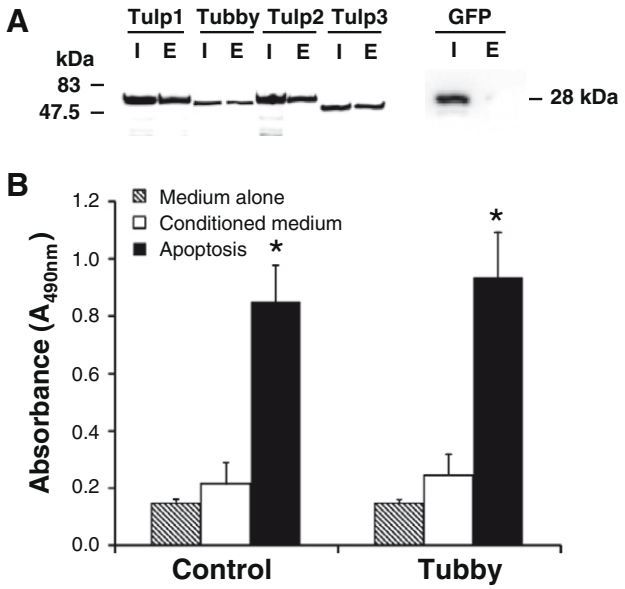

Fig. 1. Extracellular expression of recombinant Tulps. (A) Neuro-2a cells were transfected with plasmids expressing indicated FLAG-tagged Tulps. The conditioned serum-free medium of transfected cells was collected and concentrated, as described in Section 2. The intracellular (I) and extracellular (E) FLAG-Tulps were analyzed by Western blot using anti-FLAG mAb. I: lysate of $\sim 4 \times 10^4$ cells per sample; E: medium collected from $\sim 2 \times 10^5$ cells for 24 h. GFP-FLAG was a negative control. (B) LDH level in the conditioned medium was quantified in tubby-transfected cells. For the positive controls, cell apoptosis was induced by UV light (±S.D., $n = 3$, $^*P < 0.005$, vs. conditioned medium).

tion of endogenous tubby, rather than the overexpressed exogenous Tulps. To avoid possible interference of FBS, PC12 cells were cultured in the defined serum-free medium. Western blot analysis revealed that tubby was expressed intracellularly and extracellularly, whereas extracellular LDH was not detected (Fig. 2A). The level of LDH in the conditioned medium of PC12 cells was comparable to the fresh medium (Fig. 2B), suggesting negligible release of cytoplasmic proteins under the culture condition. The viability of the PC12 cells cultured in the serum-free medium was analyzed by trypan blue staining and was comparable to the cells cultured in the serum-containing complete medium (Fig. 2C). The cell responsiveness was confirmed by staurosporine-induced LDH release (Fig. 2B). These data suggested that endogenous tubby is expressed both intracellularly and extracellularly, and that extracellular tubby is secreted from living cells, but not from apoptotic cells.

3.3. Unconventional secretion is independent of the classical ER–Golgi secretion pathway

To define the mechanism of tubby and Tulp1 unconventional secretion, different pharmacological agents, including brefeldin A, monensin and nocodazole, were used to disrupt classical protein secretion pathway involving vesicular trafficking through ER–Golgi components [22,23]. Our results showed that the conventional secretion of DKK3 mediated by a signal peptide [24] was blocked by all the agents (Fig. 3). On the other hand, the well-characterized unconventional secretion of Gal-3 without a classical signal sequence [22] was minimally influenced by the inhibitors (Fig. 3). Similarly, the extracellular expression of tubby and Tulp1 was not affected by the pharmacological treatments, suggesting that their secretion is not mediated by the classical ER–Golgi pathway. It is noteworthy that methylamine, which inhibits endo- and exocytosis in some system [25], partially reduced Gal-3 secretion as previously described [22], completely abolished DKK3 export, but

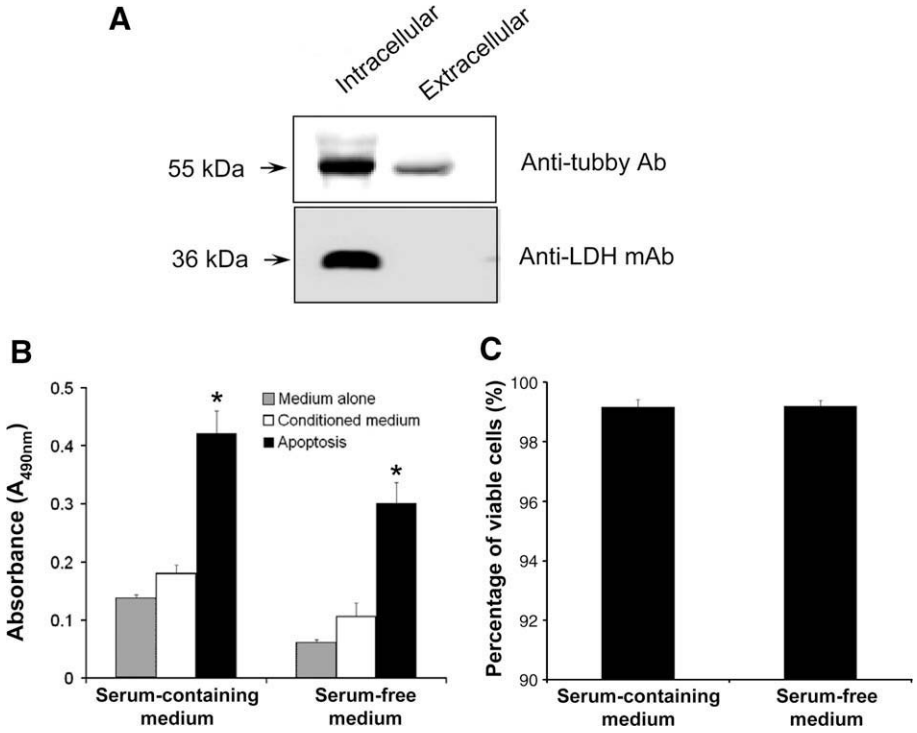

Fig. 2. Extracellular expression of endogenous tubby in PC12 cells. (A) PC12 cells were cultured in the serum-free medium. The conditioned medium was collected, concentrated and analyzed for extracellular tubby by Western blot using anti-tubby Ab. The same membrane was stripped and re-probed with anti-LDH mAb. Intracellular: lysates of $\sim 4 \times 10^4$ cells; extracellular: medium collected from $\sim 2 \times 10^5$ cells for 24 h. (B) LDH level in the culture medium was quantified in both healthy and staurosporine-induced apoptotic PC12 cells (±S.D., $n = 3$, $^*P < 0.01$, vs. conditioned medium). (C) The viability of PC12 cells cultured in the complete medium or serum-free medium was analyzed by trypan blue staining. At least 300 cells per group were analyzed.

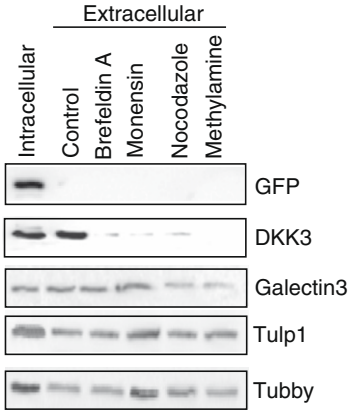

Fig. 3. Tubby and Tulp1 are not secreted through conventional ER–Golgi pathway. GFP-FLAG, DKK3-FLAG, Gal-3-FLAG, FLAG-Tulp1 and FLAG-tubby were expressed in Neuro-2a cells in the presence or absence of brefeldin A (18 μM), monensin (5 μM), nocodazole (33 μM) or methylamine (30 μM). FLAG-tagged proteins in the complete media were pulled down by anti-FLAG mAb beads and analyzed by Western blot using anti-FLAG mAb. The secretion of tubby and Tulp1 was not blocked by all the inhibitors. The signal peptide-mediated DKK3 secretion through ER–Golgi pathway was blocked. Unconventional secretion of Gal-3 was not inhibited. GFP was a cytoplasmic protein control. Intracellular, $\sim 4 \times 10^4$ cells; extracellular, conditioned medium from $\sim 2 \times 10^5$ cells for 24 h.

had minimal effect on the unconventional secretion of tubby and Tulp1 (Fig. 3). Rather than significantly enhancing Gal-3 extracellular trafficking [22], monensin only slightly promoted Gal-3 unconventional secretion in Fig. 3. The discrepancy could be the different cell lines used in these studies, because previous data showed that methylamine reduced Gal-3 export in baby hamster kidney (BHK) cells, but had not effect on MDCK cells [22,25].

3.4. Tubby secretion is partially dependent of its binding to PI(4,5)P_2

Previous study showed that Asn310, Lys330, Arg332 and Arg363 in tubby are essential residues for its binding to PI(4,5)P_2 [10]. Tubby-2M with double mutations at Lys330Ala and Arg332Ala abolishes its binding activity to PI(4,5)P_2 [10]. To investigate the importance of the binding activity in tubby extracellular trafficking, we analyzed the unconventional secretion of tubby-2M. The results showed that the level of extracellular expression of tubby-2M was reduced by ~41% ($P < 0.05$) (Fig. 4A and B), suggesting that PI(4,5)P_2-binding activity is partially responsible for tubby unconventional secretion.

Tubby mice bear a spontaneous mutation of IVS11 + 1G → T in *tubby* gene, which leads to mRNA alternative splicing and replacement of the C-terminal 44-aa chain with a 22-aa polypeptide derived from Intron 11 [26,27]. This mutation (Tubby-ΔC44) leads to adult-onset obesity, retinal and cochlear degeneration with undefined mechanisms. An interesting question is whether the mutation will affect its extracellular expression. Our results indicated that tubby secretion was not reduced by the mutation (Fig. 4A and B), suggesting that the C-terminal 44 aa is not necessary for its extracellular trafficking. Thus, tubby unconventional secretion should not be affected in *Tubby* mice.

3.5. Signal motif for tubby extracellular secretion

To define the signal sequence, we characterized tubby N-terminal (Tubby-N, Met1-Pro242) and C-terminal (Tubby-C, Val243-Glu505) domains, and demonstrated that both domains were expressed intracellularly without secretion in HEK293 cells (Fig. 5A). These results suggest that other than the C-terminal PI(4,5)P_2-binding site, an additional secretory signal is located in

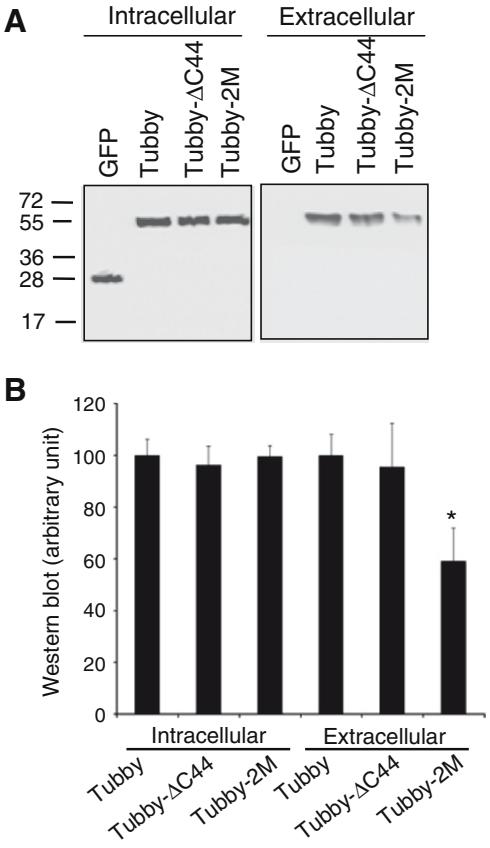

Fig. 4. Extracellular expression of mutant tubby. (A) FLAG-tagged wild-type tubby, tubby-ΔC44 and tubby-2M were expressed in Neuro-2a cells in the serum-free medium and analyzed for their extracellular expression as in Fig. 3. (B) Chemiluminescence signals in panel A were quantified (±S.D., $n = 3$, $^*P < 0.05$, vs. extracellular tubby).

Tubby-N. Further analyses revealed that the deletion of tubby N-terminal 50 aa (Tubby-ΔN50) had minimal effect, whereas the deletion of the N-terminal 100 aa (Tubby-ΔN100) completely abolished its extracellular expression in HEK293 cells (Fig. 5B). These data suggest that an essential secretory signal is located between Asn51 and Arg100.

Moreover, to characterize tubby signal motif and possible extracellular degradation, we analyzed extracellular expression of tubby mutants with various deletions in HEK293 cells because extracellular tubby derived from these cells is particularly susceptible to degradation. Unlike the stable extracellular expression by Neuro-2a cells (Fig. 4A), tubby secreted from HEK293 cells was partially degraded in the serum-free medium (Fig. 5), suggesting that protease(s) is secreted from HEK293 cells to degrade extracellular tubby. Given the susceptibility of extracellular tubby to possible protease degradation, the level and stability of tubby expression in the extracellular environments, including the blood, are yet to be characterized.

4. Discussion

As a protein family with no signal peptide, Tulps have been demonstrated to be expressed in the cytoplasm and nucleus [9,10,28]. Consequently, previous investigations focused almost exclusively on their intracellular functions [10,12–14,28], while their pathological mechanisms remain elusive. Perhaps the biggest conceptual barrier to recognize possible extracellular roles of Tulps is the absence of a typical signal peptide for secretion and no con-

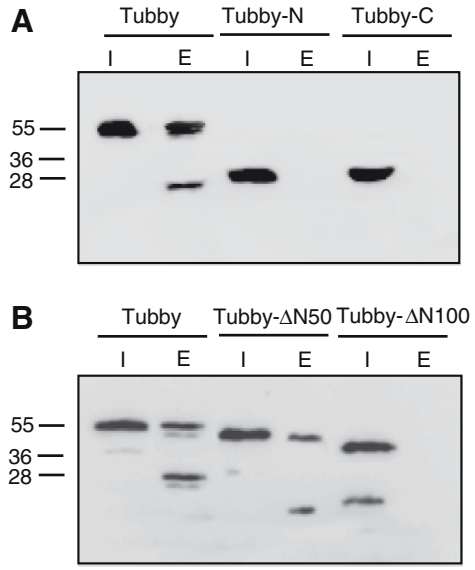

Fig. 5. Tubby signal sequence for extracellular expression. (A) Both N- and C-terminal regions are important for tubby secretion. Full-length tubby, Tubby-N (Met1-P242) and Tubby-C (V243-E505) were expressed in HEK293 cells in 293 SFM II medium and analyzed for their intracellular and extracellular expression as in Fig. 3. (B) Tubby N-terminal domain between Asn51 and Arg100 is essential for its secretion. Full-length tubby, Tubby-ΔN50 and Tubby-Δ100 were expressed in HEK293 cells and analyzed for their intracellular and extracellular expression as in Fig. 3. I, intracellular; E, extracellular.

firmed cell-surface receptor. Most secreted proteins have an N-terminal signal peptide, which facilitates the protein translocation across the membrane of the ER [29]. Once localized to the lumen of the ER, secretory proteins are packaged into transport vesicles, delivered to the Golgi apparatus, fused with the plasma membrane and released into the extracellular space. This secretion pathway can be blocked by several well-characterized inhibitors, such as brefeldin A, monensin and nocodazole [22,23]. However, lack of a signal peptide should not be the reason to exclude Tulps from possible extracellular trafficking. A heterogeneous group of proteins with no signal peptide has been exported to the extracellular compartment through alternative secretory pathways [15,16]. Some of these proteins have significant biological relevance, including FGF-2, galectins, interleukin-1b (IL-1b), macrophage migration inhibitory factor (MIF), heat shock proteins (HSPs), metallothionein, thymosin β-4 and myocilin [15,22,30–33].

Molecular mechanism of unconventional secretion is poorly defined. With the current knowledge, four possible paradigms for the unconventional trafficking have been proposed [15]: direct translocation across the plasma membrane, lysosomal secretion, secretion by exosomes derived from multivesicular bodies, and secretion by plasma membrane blebbing and vesicle shedding. Among all the secretory proteins with no signal peptide, FGF-2 is by far the best-characterized with different proposed modes [16–18]. Temmerman et al. demonstrated that binding to $PI(4,5)P_2$ is necessary for FGF-2 unconventional secretion [18]. Mutation analyses implicated that FGF-2 binding to both $PI(4,5)P_2$ and heparin sulfate proteoglycans (HSPGs) are essential for the translocation [18]. In this model, cytoplasmic FGF-2 binds $PI(4,5)P_2$ on the inner leaflet of the plasma membrane, translocates across the plasma membrane through an unknown mechanism, and associates with cell surface HSPGs [16]. HSPG-mediated trapping ensures net transport into the extracellular space. Despite a number of unsolved details in the new model, the well-established fact is that FGF-2 is a $PI(4,5)P_2$-binding protein and that the blockade of this binding reduced 50–60% of FGF-2 translocation [18].

Our findings in this study showed that Tulps are secretory proteins expressed both intracellularly and extracellularly. However, an intuitive explanation for our finding of extracellular Tulps is that they might be simply released to the extracellular space from the damaged cells due to possible membrane leakage. The negative controls of GFP-FLAG and LDH suggested otherwise. Similar to FGF-2, tubby mutant lacking $PI(4,5)P_2$-binding activity showed ~41% reduction in its extracellular expression, suggesting that $PI(4,5)P_2$-binding is partially responsible for tubby secretion. This is consistent with the previous data that several FGF-2 mutants (K127Q, R128Q or K127Q/R128Q) with minimal $PI(4,5)P_2$-binding activity retained 30–40% level of extracellular expression [18]. Moreover, an additional signal motif is located in tubby N-terminus between Asn51 and Arg100, essential for tubby secretion. However, its extracellular trafficking is not affected in *Tubby* mice with tubby mutation.

Our data showed that the unconventional secretion of tubby and Tulp1 is not mediated by the classical ER–Golgi pathway. Regardless of the molecular mechanism of unconventional secretion of Tulps, the secretion of tubby implicates that the protein may have additional extracellular functions. This provides a justification to expand the investigation of their pathological mechanisms from the intracellular to the extracellular space. For example, we have recently characterized tubby and Tulp1 as eat-me signals for retinal pigment epithelium (RPE) cell phagocytosis through a well-characterized phagocytic receptor (Caberoy et al., unpublished data). Tubby and Tulp1 activate the receptor by inducing receptor autophosphorylation. Therefore, the extracellular expression of tubby and its family members reported in this study will instigate the investigation of their pathological mechanisms with different approaches and in different cellular compartments. A thorough understanding of the extracellular trafficking of Tulps and their interaction with other extracellular or cell surface proteins may illuminate new opportunities for therapeutic management of obesity and retinal degeneration via the manipulation of this pool of extracellular Tulps and their receptors.

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