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Localization of EFA6 (exchange factor for ARF6) isoform D in steroidogenic testicular Leydig cells of adult mice

Surang Chomphoo[a], Sawetree Pakkarato[b], Tarinee Sawatpanich[a], Hiroyuki Sakagami[c], Hisatake Kondo[a], Wiphawi Hipkaeo[a,*]

[a] Electron Microscopy Unit, Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[b] Department of Social Sciences, Faculty of Sciences and Liberal Arts, Rajamangala University of Technology Isan, Sura Narai Rd, Nai-muang, Muang, Nakhon Ratchasima 30000, Thailand
[c] Department of Anatomy, School of Medicine, Kitasato University, Tokyo, Japan

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ABSTRACT

EFA6 (exchange factor for ARF6) activates Arf6 (ADP ribosylation factor 6) by exchanging ADP to ATP and the resulting activated form of Arf6 is involved in the membrane trafficking and actin remodeling of cells. Our previous study has shown the selective expression/localization of EFA6D in steroidogenic adrenocortical cells *in situ* of adult mice. In view of the previous finding, the present study was undertaken to examine its localization in mouse Leydig cells representing another steroidogenic cell species in order to further support the possible involvement of the EFA6/Arf6 cascade via membrane trafficking in the regulation of steroidogenesis and/or secretion. A distinct band for EFA6D with the same size as that of the brain was detected in the testis of adult mice. In immuno-light microscopy, immunoreactivity for EFA6D was seen throughout the cytoplasm in most Leydig cells without any distinct accumulation along the plasmalemma. Lack of immunoreactivity for EFA6D was seen in the seminiferous tubular epithelium. In immuno-electron microscopy, the immune-labeling was seen in sporadic/focal patterns on plasma membranes and some vesicles and vacuoles subjacent to the plasma membranes. More constant and rather predominant is the labeling on numerous mitochondria. No immuno-labeling was seen in lipid droplets. The present study suggests that EFA6D is somehow involved in regulation of the synthesis and/or secretion of testosterone through the membrane-traffic by activation of Arf6. In addition, EFA6D is suggested to play in mitochondria some yet unidentified roles rather independent of Arf6-activation, which remains to be elucidated.

1. Introduction

Control of the membrane trafficking including exo- and endocytosis is essential in the cell activity dynamics. The well-known molecules involved in the control are represented by ADP-ribosylation factors (Arfs), a family of Ras-related guanine nucleotide-binding (G) proteins with low molecular weights. Among six isoforms of Arfs identified in mammals, Arf6 is the most divergent and is considered to work on the plasma membrane and endosomes, while Arf1 to work in Golgi apparatus. Arf6 and other Arfs are activated by change from an inactive GDP-bound state to an active GTP-bound state, and this activation is mediated by guanine nucleotide exchange proteins (GEPs). Several GEPs for Arf6 have been identified such as EFA6 (Exchange factor for Arf6), ARNO (Arf nucleotide binding opener)/cytohesin 2 and ArfGEP10, all of which are individually composed of multiple isoforms and share a conserved SEC7 domain that is required for the GEP activity (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011; Sakagami 2008). Arf6 has been known to be ubiquitously expressed in eukaryotes, with its developmental timing differing in a tissue-specific manner as shown by *in situ* hybridization histochemistry (Akiyama et al., 2010). Therefore, the presence of such multiple species of GEPs suggests that Arf6 is delicately regulated in cell-specific and cellular function-specific manners by individually different GEP species. It is thus important to clarify what GEP species is involved in activation of Arf6 in a given cell for more understanding of the functional significance of the plasma membrane trafficking.

From this viewpoint, together with the general principle that information on the localization of a given molecule in cells *in situ*, presents important clues on its function beyond that in cells *in vitro*, we have studied the localization of EFA6 family, which is composed of isoforms A, B, C and D, in various tissue cells such as neurons, salivary gland cells, testicular spermatogenic cells and adrenal cortical cells

* Corresponding author.
E-mail address: wiphawi@kku.ac.th (W. Hipkaeo).

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(Chomphoo et al., 2016; Matsuya et al., 2005; Sakagami, 2008; Suzuki et al., 2009; Tachow et al., 2017). The reason why EFA6 family has been focused on as targets in those studies is that EFA6 was the first Arf GEF identified as working specifically to Arf6 (Franco et al., 1999) and some of the present authors (HS and HK) clarified the complete primary structure of mouse EFA6D (Sakagami et al., 2006; Sakagami, 2008). The present study was thus attempted, as one of a series of studies on the localization of EFA6 family, to examine the localization of EFA6D in testicular Leydig cells of steroidogenic activity. It was because the present authors had previously clarified the selective localization of EFA6D in the adrenocortical cell, another representative steroidogenic cell (Chomphoo et al., 2016), and because the comparison between the two steroidogenic cells in terms of its localization was regarded as valuable for understanding the possible involvement of the EFA6-Arf6 cascade in the steroidogenic cell function.

2. Materials and methods

2.1. Animals

Ten male mice of postnatal 8 weeks were purchased from National Laboratory Animal Center (NLAC), Bangkok, Thailand and grown under standard laboratory conditions with free access to food and water. All procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals at Khon Kaen University. The study was reviewed and approved by the ethics board with the ethics number AEKKU 44/2016.

2.2. Immunoblotting analysis

After testes as well as whole brains were extirpated from five mice under pentobarbital sodium (Cat# 76744, Merck; KgaA, Germany; 50 mg/kg BW) anesthesia, the tunica albugineae of testes were quickly peeled off by razor blades and forceps, and the remaining parenchyma of testes as well as the brains were individually homogenized in lysis buffer containing protease inhibitor (Cat# 88665, Pierce; Rockford, IL, USA). Resultant homogenates were subjected to centrifugation at 12,000 rpm for 10 min to remove nuclei and debris. Supernatants were measured for the protein concentration using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Supernatants containing 20 μg proteins were mixed with an equal volume of 2X sodium dodecyl sulfate (SDS) sampling buffer (63 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromophenol blue) and denatured with boiling at 95 °C for 10 min. Proteins were separated using 10% SDS–polyacrylamide gel electrophoresis and were electrophoretically transferred to a PVDF membrane (GE Healthcare; Buckinghamshire, UK). After blocking with 5% skimmed milk for 1 h with shaking, blotted membranes were incubated with the rabbit anti-mouseEFA6D polyclonal primary antibody (0.1 μg/ml) overnight. The synthesis procedure of the antibody against the N-terminal region (amino acids 1–680) of mouse EFA6D and its specificity were already described in a previous study by one (HS) of the present authors (Fukaya et al., 2016). The membranes were then incubated with peroxidase-conjugated secondary Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP at dilution of 1:2500 for 1 h (Cat# 31460 Invitrogen/Thermo Fisher Scientific; Frederick, MD, USA). A Tris-buffered saline (10 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.3% Tween 20 was used as the dilution and washing buffer. Immuno-reactions were visualized with the ECL prime chemiluminescence detection system (Cat#RPN2232, Amersham/GE Healthcare; Buckinghamshire, UK) and captured using an Image Quant LAS 4000 mini (GE Healthcare; Tokyo, Japan).

2.3. Immuno-light microscopy

Five mice, under thiopental sodium anesthesia, were perfused first briefly with 10 ml of 0.1 M phosphate buffer and then with 10 ml of 4% paraformaldehyde in phosphate buffer. The testes were removed and further postfixed with the same fixative overnight at 4 °C. Specimens were dipped into 30% sucrose in phosphate buffer for cryoprotectioning. Cryosections of 20 μm thickness were incubated with 0.3% H_2O_2 in methanol for 10 min, then 10% normal goat serum in phosphate buffered saline (PBS) for 30 min. The sections were incubated with the same primary antibody against mouse EFA6D (1 μg/ml) in 0.1%TritonX-100/PBS, overnight at room temperature. The sections were subsequently incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (Cat# ab64256, Abcam; Cambridge, MA, USA). They were then treated for DAB (diaminobenzidine) reaction by Vectorstain elite ABC kit (Cat# PK-6100, Vector Laboratories; Burlingame, CA, USA). Some of the slides were counterstained with hematoxyline.

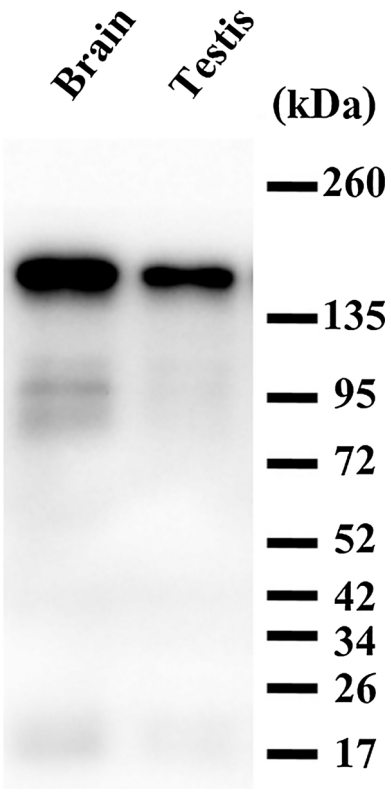

Fig. 1. Immunoblotting of testis together with total brain of adult mice. Note a distinct band in testis at the same size as that detected in brain.

2.4. Immuno-electron microscopy

Pre-embedding immuno-electron microscopic analysis was done in the same way as that already reported by the present authors (Chomphoo et al., 2016). Briefly stating, cryostat-sections were mounted on poly-L-lysine-coated plastic slides pretreated with 0.1% saponin, and incubated with the same primary antibody (5 μg/ml) overnight. They were subsequently reacted with goat anti-rabbit IgG covalently linked with ultra-small gold particles at 1:100 in dilution (Cat# 800.011, Aurion; Hatfield, PA, USA). After the silver enhancement using a kit (Aurion R-GENT SE-LM silver enhancement kit (Cat# 500.011, Aurion, Hatfield, PA, USA), the sections were osmicated, dehydrated, and directly embedded in Epon. Ultrathin sections, after stained with both uranyl acetate and lead citrate, were observed under a transmission electron microscope (Jeol; Tokyo, Japan).