

Ultrastructural Localization of Endogenous Exchange Factor for ARF6 in Adrenocortical Cells *In Situ* of Mice

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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 dynamics and actin re-organization of cells. The present study was attempted to localize EFA6 type D (EFA6D) in mouse adrenocortical cells *in situ* whose steroid hormone secretion is generally considered not to depend on the vesicle-involved regulatory mechanism. In immunoblotting, an immunoreactive band with the same size as brain EFA6D was detected in homogenates of adrenal cortical tissues almost free of adrenal capsules and medulla. In immuno-light microscopy, EFA6D-immunoreactivity was positive in adrenocortical cells and it was often distinct along the plasmalemma, especially along portions of the cell columns facing the interstitium. In immuno-electron microscopy, the gold-labeling was more dense in the peripheral intracellular domains than the central domain of the immunopositive cells. The labeling was deposited on the plasma membranes in a discontinuous pattern and in cytoplasmic domains rich in filaments. It was also associated with some, but not all, of pleiomorphic vesicles and coated pits/vesicles. No labeling was seen in association with lipid droplets or smooth endoplasmic reticulum. The present finding is in support of the importance of EFA6D for activation of Arf6 in adrenocortical cells.

Key words: EFA6D, ultrastructure, membrane dynamics, adrenal cortex, mice

I. Introduction

Arf6 (ADP-ribosylation factor 6) is a member of the Arf small GTPase family and implicated in the regulation of actin cytoskeleton rearrangement as well as endocytosis and recycling of the plasma membrane. Arf6 is activated by a 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 and all share a conserved SEC7 domain that is required for the GEP activity. Among them, a subfamily EFA6 has recently been shown to consist of at

least four isoforms, EFA6A, B, C and D [4, 5, 7, 8, 15].

Although the membrane dynamics and actin re-organization and the expression of Arf6 involved in these phenomena are ubiquitous in eukaryotic cells, it is considered that the selective employment of GEF (GDP/GTP exchange factor) molecules in a given cell is a key factor to regulate the activity of Arf6 in the cell [15]. It is also possible that these cellular phenomena are active in proteinaceous secretory cells which exert regulated or constitutive secretory action. Several studies have shown that Arf6 and some Arf6 GEFs are required for optimal regulated secretion in endocrine cells such as pancreatic islet cells and chromaffin cells [2, 11, 20]. Different from the proteinaceous secretion, it is generally known that steroid hormones are not stored in any membrane-limiting compartments of

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steroidogenic cells, and that the rate of the hormone secretion depends on the *de novo* synthesis of the cell-specific steroid. On the other hand, the regulation of steroidogenesis, essentially similar to that of proteinaceous hormone synthesis, occurs primarily through activation of G protein-coupled receptors (GPCRs) for external stimuli specific to individual hormone-secreting cells. The receptors not only exert stimulatory actions of the external stimuli in the synthesis, but also play roles in the desensitization of the same steroidogenic response, resulting eventually in decrease and loss of the receptors by their sequestration and internalization. There have been several reports showing the possibility that Arf6 is involved in the receptor internalization of GPCRs [9, 10].

Considering the regulation mechanisms of synthesis and secretion of steroid hormones described above, it would be significant to identify a molecular species of Arf GEFs selectively expressed and localized in steroidogenic cells. The present immuno-histochemical study was thus attempted to localize EFA6D, as an expected GEF, in adrenocortical cells of mice. These selections for the analysis were made by the two criteria: (1) that much wider tissue localization of EFA6D than other EFA6 isoforms has previously been reported by two (HS, HK) of the present authors [16] and a higher chance to encounter the most appropriate molecular species of GEFs was expected; and (2) that the adrenal cortex was expected to present the most appropriate tissue target clarifying the qualitative comparison in membrane dynamics between the steroidogenic cell and the proteinaceous exocrine one, the adrenal medullary chromaffin cell. As a result, ultrastructural localization of EFA6D was successfully clarified in the cortical cells *in situ*, but not the medullary cells, suggesting the importance of this molecule in the membrane dynamics and actin reorganization of the cortical cells.

II. Materials and Methods

Male ICR mice of postnatal week 8 were used in the present study. For immunohistochemical examination, mice, under deep anesthesia by pentobarbital (40 mg/kg body weight), were perfused with 10 ml PBS, followed by 10 ml 4% paraformaldehyde/0.1 M phosphate buffer. The adrenal glands were extirpated and postfixed with the same fixative for 2 hr. Specimens were dipped into 30% sucrose/PBS for cryoprotection. Cryosections of 20 μm thickness were mounted on glass slides and permeabilized with 0.1% TritonX-100/PBS for 30 min at room temperature, incubated with 0.3% H_2O_2 /methanol for 10 min, then 5% normal goat serum/PBS for 30 min. Sections were incubated with anti-mouse EFA6D rabbit IgG (1 $\mu\text{g}/\text{ml}$ each) overnight at room temperature. The specificity of the antibody was already described in our previous study [16]. The sections were subsequently incubated for 1 hr at room temperature with biotinylated anti-rabbit IgG secondary ready to use antibody (Abcam, Cambridge, MA, USA). They

were then treated for DAB reaction by VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). In addition, the specificity of the immunoreactivity was confirmed by disappearance of the immunoreactivity with use of EFA6D antigen-preabsorbed antibody.

For pre-embedding immuno-electron microscopy, the basic protocol was similar to that already reported by one (HS) of the present authors [18], and it was slightly modified in the present study. Cryostat-sections were mounted on poly-L-lysine-coated plastic slides, pretreated with 0.1% saponin, and incubated with the antibody (5 $\mu\text{g}/\text{ml}$) overnight, and subsequently reacted with goat anti-rabbit IgG covalently linked with ultra small gold particles (1:100 in dilution; Aurion, Hatfield, PA, USA). Following silver enhancement using a kit (Aurion R-GENT SE-LM silver enhancement kit, Aurion, Hatfield, PA, USA), the sections were osmicated, dehydrated, and directly embedded in Epon. Ultrathin sections were prepared and stained with both uranyl acetate and lead citrate for observation under an electron microscope (Jeol 1010, Tokyo, Japan). The specificity of the immunoreaction was confirmed by the disappearance of immunolabeling when the antibody was pre-incubated with the synthetic antigen.

For immunoblotting, mice were sacrificed by neck dislocation and the adrenal glands were extirpated. Subsequently, the capsule was scraped off by razor blades and the adrenal medulla was enucleated with spatulas. The remaining cortical parenchyma was homogenized in lysis buffer containing protease inhibitor (Pierce, Rockford, IL, USA). The resultant homogenate was subjected to centrifugation at 3,000 $\times g$ for 10 min to remove nuclei and debris. The supernatant was measured for the protein concentration using the BCA protein assay system (Pierce, Rockford, IL, USA). Homogenates containing 20 μg protein were mixed with an equal volume of 2 \times 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 $^\circ\text{C}$ for 10 min. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto an Immobilon-P Transfer Membrane (Immobilon, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 hr, blotted membranes were incubated with the primary antibody (0.1 $\mu\text{g}/\text{ml}$) overnight and then with peroxidase-conjugated secondary antibody for 1 hr (Invitrogen, Frederick, MD, USA; 1:2,500). 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. Immunoreactions were visualized with the ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) and captured using an Image Quant LAS 4000 mini (GE Healthcare, Tokyo, Japan).

All the procedures in the present study 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 3/2014.

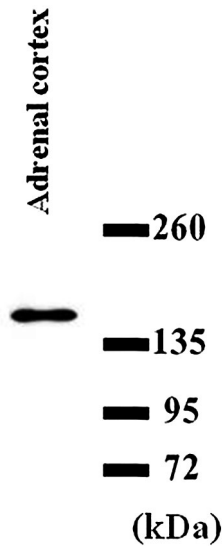


Fig. 1. Immunoblot for EFA6D of enucleated adrenal glands almost free of medullary and capsular tissues from adult mice. Note a band (145 kDa) which is similar to the established size of brain EFA6D.

III. Results

In Western blotting, a distinct immunoreaction band at the size corresponding to brain EFA6D [16] was clearly discernible in homogenates of the adrenal cortex almost free of the capsular and medullary tissues (Fig. 1).

In immuno-light microscopy, almost all the cortical cells were weakly to moderately immuno-positive for EFA6D with the zona reticularis cells more intense (Fig. 2). The immunoreactive materials were deposited rather widely throughout the cells except for the nuclei. The immunoreactivity was often distinct along portions of the plasmalemma facing the capillaries, especially in the zona fasciculate, resulting in frequent appearance of long straight and some transverse immunostained lines along the cortical cell columns. No significant immunoreactivity was discerned in the adrenal medulla.

In immuno-electron microscopy, gold-particles representing the immunoreaction tended to be more prominent in the cell periphery, that is, along the plasma membranes and in cell domains subjacent to the plasma membranes, than central domains of the cells. The particle-labeling was randomly focal/spot-like, but not continuous, along the plasma membranes (Figs. 3, 4). Some of the immuno-labeled cell domains were rich in filaments, while some others contained vesicles of various sizes and shapes. The gold-particles were deposited in association with the filaments, and they were found within or close to some, but not all, of the vesicles and coated pits/vesicles as well as endosomes and lysosome (Figs. 3, 4). The labeling were sometimes found within or close to some mitochondria (Figs. 3, 4), while no labeling was found in association with lipid droplets, smooth endoplasmic reticulum (Fig. 4), and adherens junctions between two adjacent cortical cells (data not

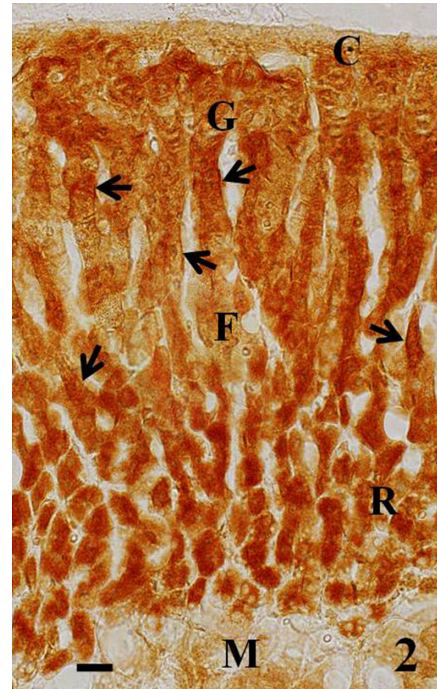


Fig. 2. Immuno-light micrograph of adrenal gland for EFA6D. The immunoreactivity is distinctly positive in the cortex composed of zonae granulosa (G), fasciculate (F) and reticularis (R), in contrast to no significant immunoreactivity in the medulla (M). Note a plasmalemmal pattern of the immunoreaction in several spots (arrows). C: adrenal capsule. Bar=10 μ m.

shown). No significant gold-labeling was discerned in the medullary chromaffin cells characterized by chromaffin granules (Fig. 5).

IV. Discussion

In the present immunohistochemical and immunoblot analyses, endogenous EFA6D was shown clearly for the first time to be present in steroidogenic adrenocortical cells *in situ* of adult mice. This finding indicates that the adrenocortical cells employ EFA6D to activate Arf6 which is known to be involved in the membrane dynamics including endo- and exocytosis and actin reorganization [4]. The results were further strengthened by the immuno-electron microscopic finding, which is the first ultrastructural localization of endogenous EFA6D in non-neuronal *in situ* cells, although there have been reports on ultrastructural localization of EFA6 and Arf6 exogenously expressed in *in vitro* cells by transfection of respective cDNAs [6, 7]. To briefly summarize the present ultrastructural finding, the gold-labeling was often found on the plasma membranes and in association with aggregations of filaments and some vesicles of various sizes and shapes in the peripheral intracellular domains facing the pericapillary interstitial spaces, but not in association with lipid droplets or smooth endoplasmic reticulum.

The present ultrastructural finding is compatible with

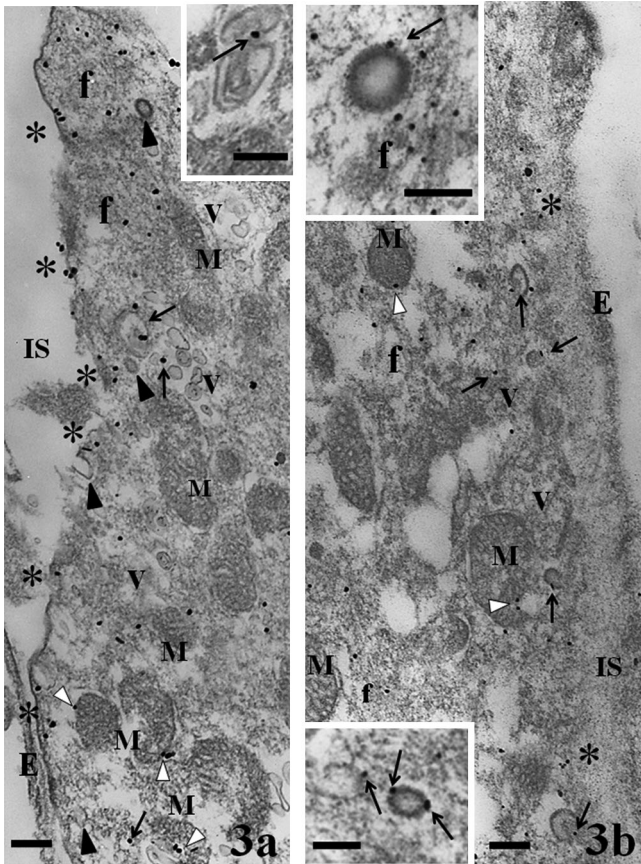


Fig. 3. a, b: Immunoelectron micrographs for EFA6D of adrenocortical cells *in situ*. Note gold-labeling in association with the plasma membranes (*), submembranous cytoplasm rich in filaments (f), and some vesicles and lysosomes (arrows) in vesicle-rich areas (v). Also note no labeling on some coated vesicles and pits (arrowheads) and some other vesicles. Further note labelings (white arrowheads) on some mitochondria (M). Inset of a shows higher magnification view of labeled pleiomorphic vesicles (endosomes), while insets of b show higher magnification views of labeled coated vesicles (arrows in upper & lower insets) and filaments (f in upper inset). E: endothelium, IS: interstitial space. Bars=200 nm (a, b) and 100 nm (insets).

the general understanding of the involvement of Arf6 activated by EFA6 in the membrane dynamics. In this regard, it is to be noted that the immuno-labeling was deposited on some, but not all, of coated pits/vesicles and vesicles of various sizes and shapes including those equivalent to endosomes. Several explanations are possible for this heterogeneous labeling. One of them is that there is a heterogeneity among those vesicular components in terms of their involvement in the membrane dynamics via the Arf6 GEF to the Arf6 signaling, and that vesicular components non-labeled for EFA6D may be driven in the signal using GEFs other than EFA6 in one and the same cell. The same may be the case for the focal/spot-like labeling on the plasma membranes.

The occurrence of the gold-labeling in association with aggregations of filaments in sub-membranous cytoplasmic domains is in accord with a previous immuno-

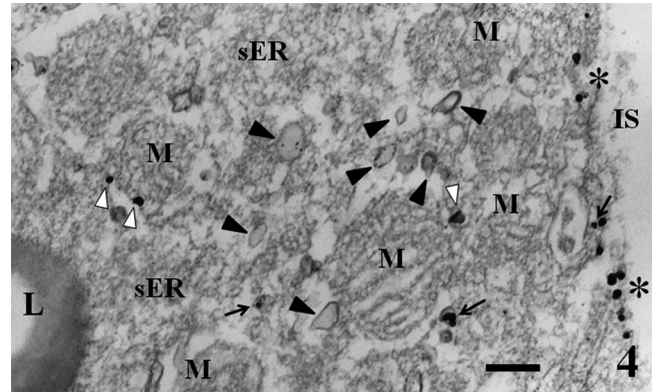


Fig. 4. Immunoelectron micrograph for EFA6D of adrenocortical cells *in situ*. Note no labelings on lipid droplets (L) or smooth endoplasmic reticulum domains (sER), both of which are features characteristic of adrenocortical cells with steroidogenesis. In the same way as Figure 3, asterisks, arrows and white arrowheads indicate gold-labeled plasma membranes, vesicles and mitochondria (M), respectively, whereas arrowheads indicate non-labeled vesicles. IS: interstitial space. Bar=200 nm.

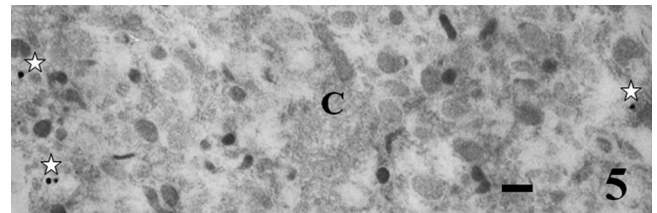


Fig. 5. Negative control in immunoelectron micrograph for EFA6D of adrenomedullary chromaffin cells (C) characterized by numerous chromaffin granules. No gold-labeling in the cells except for only a few (white stars) randomly appearing at background levels. Bar=200 nm.

precipitation finding that the PH domain of EFA6 interacted directly with F-actin [12]. The finding is also compatible with the general understanding that the EFA6 to Arf6 signaling is involved in the cortical actin remodeling via the coiled coil (CC) motif and PH domain in its C-terminal region as well as through increased phosphatidylinositol (PI)_{4,5}-bisphosphate synthesis by stimulation of PIP₃-kinase at the plasma membrane [3, 4, 12, 15, 17].

On the other hand, the gold-labeling in close association to some mitochondria is to be noted. Although no preceding molecular biological data are available in relation to the mitochondrial localization of any GEFs, the broader functional significance of this mitochondrial labeling may require taking into consideration some yet unknown Arf-independent functions of EFA6D. In this regard, there have recently been studies reporting possible roles of GEF members beyond Arf activation such as the effect of cytohesin GEFs on signaling through epidermal growth factor [1] and the regulation of microtubule dynamics by EFA6 of *C. elegans* [14].

To what degree the EFA6D to Arf6 coupling is implicated specifically in the regulation of steroidogenesis and/or secretion in the adrenocortical cells remains to be

elucidated. With regard to steroidogenesis regulation, as briefly stated in the Introduction, there have recently been studies showing that the Arf6-mediated membrane dynamics is involved in the internalization of G-protein-coupled receptors including that for luteinizing hormone, a representative steroid hormone in ovarian follicular cell membranes [9, 10, 13, 19]. Whether or not this is the case for melanocortin-2 receptor for corticosteroids remains to be elucidated. Regarding the secretion regulation of steroid hormones, on the other hand, there has recently been a study reporting the possibility of vesicle-mediated release of an insect steroid hormone termed ecdysone in contrast to the prevailing “free diffusion” model for mammalian steroid secretion [21]. Since their novel finding in *Drosophila* suggests a similar possibility in mammalian steroidogenic cells, the present localization of EFA6D in the adrenocortical cells may shed light on the regulation mechanisms in the adrenocortical steroidogenesis and secretion.

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VI. References

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