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# Localization and Changes of Tyrosine Phosphorylated Proteins and ß Actin in Epididymis of Rats Treated with Valproic Acid

## Localización y Cambios de las Proteínas Tirosina Fosforiladas y la ß Actina en Epidídimos de Ratas Tratadas con Ácido Valproico

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**SAWATPANICH, T.; ARUN, S.; TONGPAN, S.; CHAICHUN, A.; SAMPANNANG, A.; SUKHORUM, W.; MANEENIN, C.; BURAWAT, J. & IAMSAARD, S.** Localization and changes of tyrosine phosphorylated proteins and ß actin in epididymis of rats treated with valproic acid. *Int. J. Morphol., 36(3)*:835-840, 2018.

**SUMMARY:** Tyrosine phosphorylated proteins have been localized and identified in male reproductive tissues such as testis and capacitated/ acrosome reacted sperm except epididymis. The changes of such proteins are associated with decreased sperm quality of valproic acid treatment. This study aimed to investigate the presence and alterations of protein phosphorylation in epididymal epithelium and fluid of rats treated VPA. Sixteen adult male rats were divided into control and VPA-treated groups (n=8/ each). Treated rats were injected with VPA (500 mg/ kgBW, intraperitoneally) for 10 consecutive days. At the end of experiment, the monoclonal anti-phosphotyrosine (clone 4G10) was used for immunohistochemistry to probe tyrosine phosphorylated proteins and also to examine the expression of such proteins using immuno-Western blotting in epididymal tissue and fluid. The result showed that positive reactivity of phosphorylated proteins was clearly observed in cytoplasmic principle cells, nuclei of apical & basal cells and sperm mass surrounded with epididymal fluids. The profiles of phosphorylated proteins in epididymal fluid were 182, 127, 80, 70, 57, 45, 34, and 31 kDas, respectively. Interestingly, VPA affected the changes of phosphorylated proteins and β actin in head, body, and tail epididymal fluids. We conclude that tyrosine phosphorylated proteins were detected in epididymal epithelium and fluid. The expressions of those proteins and actin were altered under VPA treating.

**KEY WORDS: Localization; Beta actin; Tyrosine phosphorylated proteins; Epididymal fluid; Rats.**

## INTRODUCTION

In general, protein tyrosine phosphorylation is a posttranslational modification known to be essential in regulation of cellular proliferations, divisions, growths, and differentiations (Hunter & Cooper, 1985; Hunter, 1987; Hanks *et al*., 1988; Ullrich & Schlessinger, 1990). In male reproductive system, the tyrosine phosphorylated proteins are involved in epididymal sperm capacitation and acrosome reaction to succeed in fertilizing process (Yanagimachi, 1994; Visconti & Kopf, 1998; Stival *et al*., 2016). Tyrosine phosphorylation is assumed to play roles in sperm and testosterone productions since phosphorylated proteins have been localized in Interstitial cells (Leydig cells), Sustentacular cells Sertoli cells) permatogonia, spermatocytes, and spermatids (round and elongated), respectively (Arad-Dann *et al*., 1993; Chaichun *et al*., 2017). Previously, various studies have shown that the changes of testicular phosphorylated proteins are associated with the decrease of sperm concentration and testicular damages (Arun *et al*., 2016a,b; Sukhorum & Iamsaard, 2017; Iamsaard *et al*., 2013, 2014, 2017a,b; Sampannang *et al*., 2017). In functional maturation process of sperm, testicular sperm must be transited through the vas deferens, head and body of epididymis to be further stored in tail epididymis before ejaculation. During transit through parts of epididymis, sperm will be bound by various fertilizing factors secreted from epididymal epithelium onto sperm membrane. Such factors may include phosphorylated proteins; however, the localizations and identifications of phosphorylated proteins in epididymal epithelium and fluid have never been documented.

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Valproic acid (VPA) is widely used for treatments in many neurological disorders especially epilepsy and anticancer activities but it also has side effects on the gastrointestinal, neurological, hematological, endocrine, and reproductive systems (Berendsen *et al*., 2012; Glister *et al*., 2012; Taubøll *et al*., 2008; Bialer & Yagen, 2007; Göttlicher, 2004). Indeed, VPA causes male infertility in both epileptic men and experimental animal models (Bialer & Yagen; Isojärvi, 2008). In addition, VPA induces atrophy of the testis, epididymis, prostate gland, and seminal vesicles (Nishimura *et al*., 2000; Krogenaes *et al*., 2008; Sukhorum & Iamsaard). Moreover, it has been shown that VPA indeed affects the alterations of phosphorylated proteins in testis (Iamsaard *et al*., 2015, 2017a,b; Sukhorum *et al*., 2016; Sukhorum & Iamsaard). However, such effects of VPA on epididymal tissues and secreting fluids secreted from head, body, and tail epididymis have never been elucidated. Taken together, this study attempted to localize tyrosine phosphorylated proteins in epididymal epithelium and fluids with their charges affected by VPA treatment.

## MATERIAL AND METHOD

**Animals and treatment.** Sixteen adult male Sprague-Dawley rats (180-200 g) were purchased from the Nomura Siam International Co., Ltd., Pathumwan, Bangkok, Thailand and transferred to the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. All rats were housed under standard environmental conditions and received commercial pellet food and water *ad libitum*. Animals were acclimatized for 7 days before use. This study has used animals, duly approved by Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand (rec. no. IACUC-KKU-91/60). The rats were divided into 2 groups (control and valproic acid [VPA] - treated groups; n = 8 in each group). Control animals were intraperitoneally injected with normal saline while experimental rats were injected with a single dose of 500 mg/BW per day, VPA (sodium salt; Sigma-Aldrich) for 10 consecutive days (Hamza & Amin, 2007; Iamsaard *et al*., 2015; Sukhorum & Iamsaard). At the end of experiment, all rats were anesthetized by pentobarbital sodium before euthanasia by cervical dislocation. After euthanasia, the epididymis were dissected out. Fat pads surrounding the head, body, and tail epididymis were removed. All parts of right epididymis were immediately squeezed for their fluids and gently cut into small pieces in 1 ml PBS. Then, the mixtures were centrifuged to separate the epididymal fluid from tissue pellets. The epididymal fluid was measured for total protein concentration and kept in -80 °C before immuno-Western blot analysis. The left epididymis was rapidly fixed in 10 % phosphate buffered formalin (pH 7.4) to be used for immunohistochemistry.

**Immunohistochemistry.** The fixed- epididymal tissues were dehydrated in graded alcohols, cleared in xylene, infiltrated and embedded by paraffin using tissue auto-processor apparatus at Department of Pathology, Faculty of Medicine, Khon Kean University. The paraffinized-epididymal blocks were sectioned at 5-7 mm thickness (Semi-automatic Rotary Microtome, ERM 3100 HESTION, Australia). Sections of epididymis were placed on gelatin-coated glass slides, and then warmed in hot air oven (60 °C), deparafinized in xylene, and rehydrated in serial alcohols. For antigen retrieval, the sections were soaked in citrate buffer (10 mM citric acid, 0.05 % Tween-20, pH 6.0) and heated using microwave at 95°C. Then, such slides were cooled down and washed with PBS. The endogenous peroxidase activity on tissues was blocked with 30 % hydrogen peroxide and then washed with PBS. The non-specific proteins were blocked with 5 % bovine serum albumin (BSA; Millipore Co., USA) in PBS within moist chamber. Then, epididymal sections (4 slides) were probed with monoclonal anti-phosphotyrosine (clone 4G10, 1:200 (v/v); Millipore, CO., USA) diluted in PBS. In parallels, the negative control sections (4 slides) were omitted with primary antibody. All sections were washed with PBS for 5 min and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:300 (v/v); InvitrogenTM, USA) for 2 h within moist chamber at RT. The sections were washed and incubated with the Vector NovaRED peroxidase substrate kit (Vector Laboratories, USA). Then, all sections were counterstained with haematoxylin. Subsequently, the sections were washed with DW. After that, all sections were dehydrated with serial alcohols, cleared with xylene, and mounted with dibutylphthalate polystyrene xylene, respectively. The positive or negative immunoreactivity on testicular tissue sections was photographed using a Nikon light ECLIPSE E200 light microscope equipped with DXM1200 digital camera (Nikon, Japan).

**Immuno-Western blot.** The total protein concentrations of the epididymal fluids obtained from head, body and tail were measured by using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) at absorbance 280 nm. To determine the protein profiles, the epididymal fluid proteins (50 µg) were loaded and separated on 10 % sodium dodecyl sulfate (SDS) polyacrylamide gel followed by Comassie blue staining (SDS-PAEG). To examine the expression of tyrosine phosphorylated proteins or beta actin in all epididymal fluids, the separated proteins on SDS gel were transferred onto nitrocellulose membrane. Then, the membrane was incubated with 5 % skim milk (0.1 % Tween-

20, PBS, pH 7.4) for 1 h to block non-specific binding proteins and incubated with monoclonal anti-phosphotyrosine (clone 4G10, 1:2,000; Millipore Co., USA) or anti ß actin antibody. After washing such primary antibody, it was incubated with anti-mouse conjugated with HRP secondary antibody for 2 h and washed with 0.05 % PBST before detections of tyrosine phosphorylation by using enhanced chemiluminescence substrate under gel doct 4 (ImageQuant 400, GH Healthcare, USA). The epidermal growth factor stimulated A413 cell lysate (EGF; Millipore CO., USA) and bovine serum albumin (BSA; Millipore CO., USA) were used as positive and negative controls, respectively, to confirm the real reactivity of anti phosphotyrosine antibody.

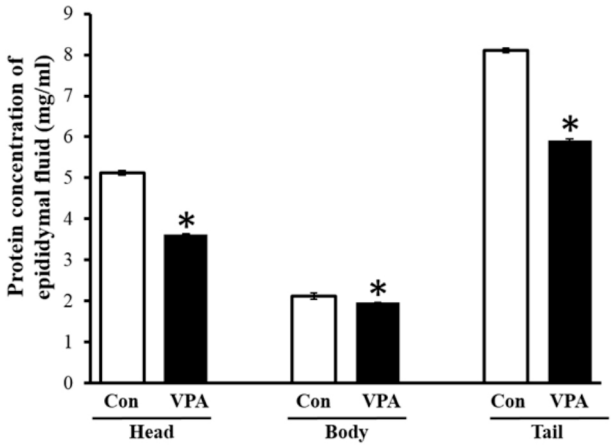


Fig. 1. Showing the total protein concentration of epididymal fluid obtained from head, body, and tail regions of rat epididymis compared between control and VPA-treated groups. *p < 0.05 compared to control.

## RESULTS

The total protein concentrations of epididymal fluid obtained from head, body, and tail regions of rat epididymis in both groups are shown in Figure 1. The result showed that total protein concentrations of the fluids in all parts of epididymis in VPA-treated group were significantly decreased as compared to those of control (Fig. 1).

Under light microscopic investigation, we found that tyrosine phosphorylated proteins are strongly localized in apical cytoplasm of the principal cells of epididymal epithelium as shown in Figure 2 B and C as compared to that of omitted-primary antibody control (Fig. 2A). Additionally, nuclei of apical cells and basal cells are

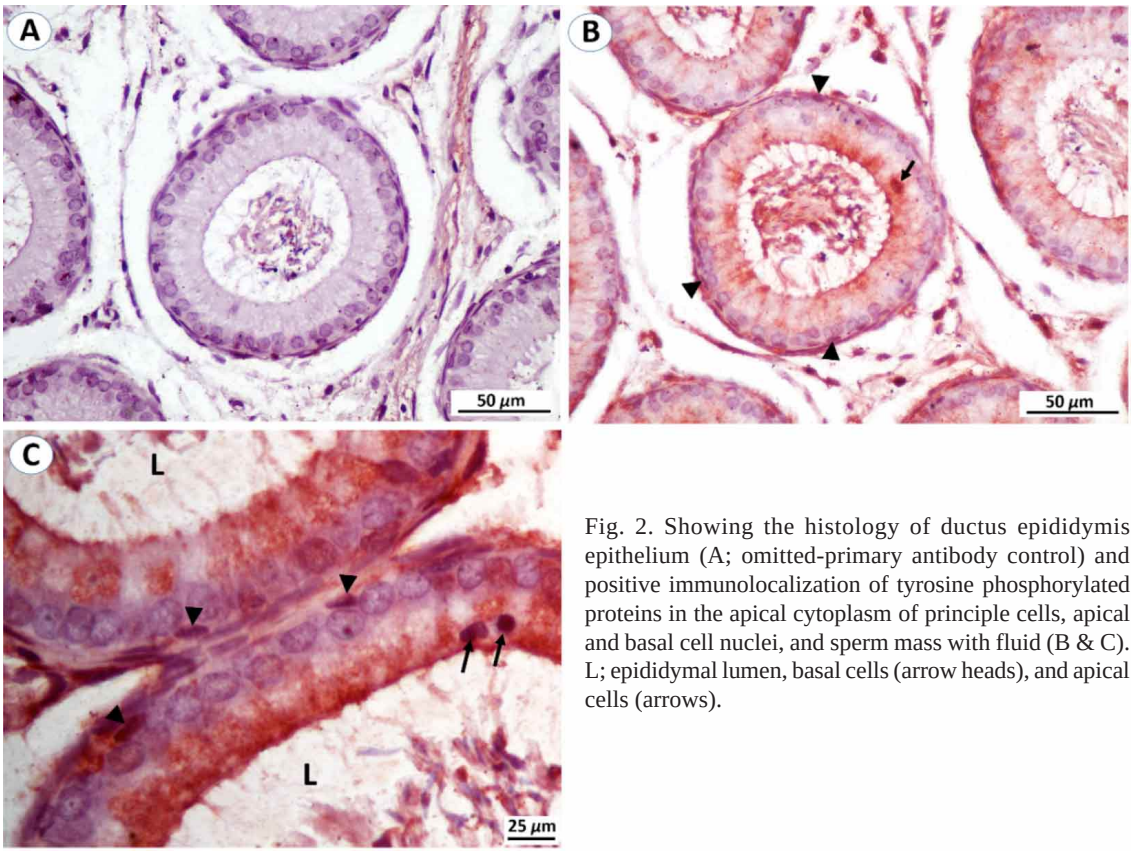


Fig. 2. Showing the histology of ductus epididymis epithelium (A; omitted-primary antibody control) and positive immunolocalization of tyrosine phosphorylated proteins in the apical cytoplasm of principle cells, apical and basal cell nuclei, and sperm mass with fluid (B & C). L; epididymal lumen, basal cells (arrow heads), and apical cells (arrows).

specifically positive to phosphotyrosine antibody (Fig. 2B and C) compared to negative control epididymal epithelium (Fig. 2A). Moreover, it was found that imuunopositive reactivity of such monoclonal antibody is strongly localized in the epididymal lumen containing mature sperm mass embedded with fluid (Fig. 2B and C) as compared to control (Fig. 2A).

In addition, we found that various proteins presented in rat epididymal fluid of control and VPA-groups (Fig. 3A). For immuno-Western blot results, eight tyrosine phosphorylated proteins were specifically detected by monoclonal-phosphotyrosine antibody in head, body, and tail of epididymal fluids (Fig. 3B). The profiles of these tyrosine phosphorylated proteins are 182, 127, 80, 70, 57, 45, 34 and 31 kDas, respectively (Fig. 3B).

Interestingly, the intensity of 182 and 70 kDa phosphorylated proteins in VPA group was increased in all regions of epididymis as compared to control (Fig. 3B). VPA also increased the expressions of a 127 kDa protein in only head and body epididymis. In the body epididymis, the expression of a 80 kDa protein was more intensive in VPA group (Fig. 3B). VPA could also increase the intensity of 57, 45, 34, and 31 kDas in head and tail epididymis, respectively as shown in figure 3B.

Moreover, it was found that VPA could increase the expression of the beta actin secreted into lumens of head, body, and tail of epididymis (Fig. 4). Indeed, the intensity of beta actin present in epididymal fluids of head, body, and tail in VPA rats was significantly increased as compared to that of control as shown in Figure 4.

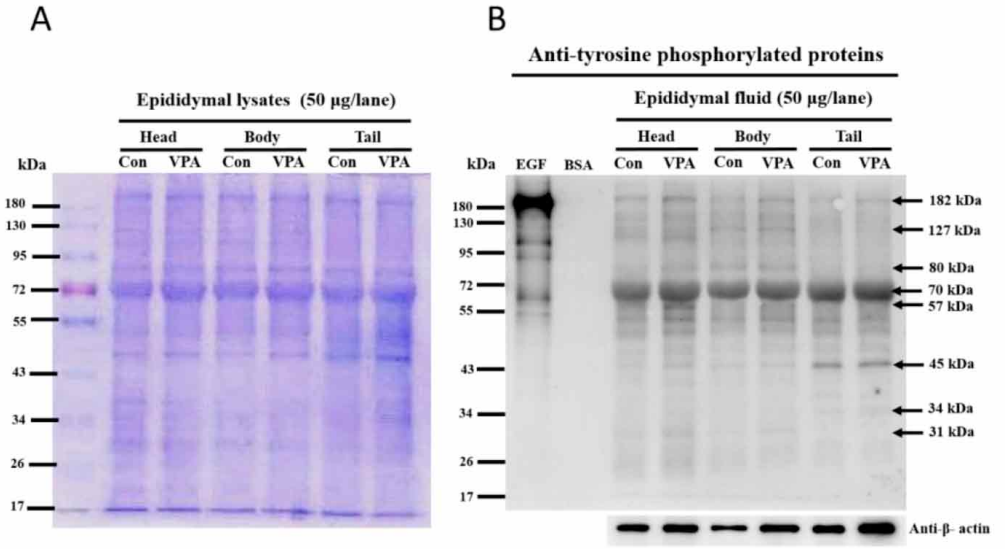


Fig. 3. Representative SDS-PAGE stained by Coomassie blue showing total epididymal fluid proteins (A) and the immuno-Western blot of their tyrosine phosphorylated proteins after transferring onto nitrocellulose membrane (B). Bovine serum albumin (BSA) and epidermal growth factors (EGF) were used as negative and positive controls, respectively (n = 4).

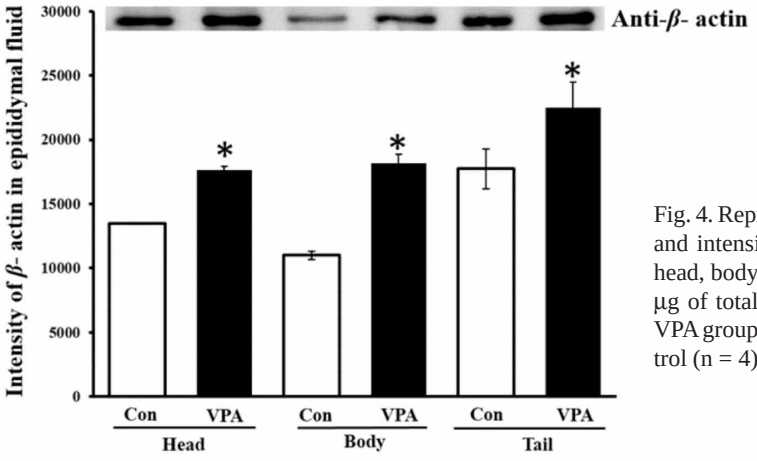


Fig. 4. Representative immuno-Western blot and intensity of the ß actin present within head, body, and tail of epididymal fluid (50 µg of total protein loading) of control and VPA groups. *p < 0.05 compared to the control (n = 4).

## DISCUSSION

The tyrosine phosphorylated proteins have been localized in only Sustentacular cells (Sertoli) and elongated spermatid cells (Arad-Dann *et al*.). Recently, the monoclonal antibody specific to the tyrosine phosphorylated proteins is available from companies including Millipore Co., USA. This antibody is widely used in Western blot analysis to examine the patterns of tyrosine phosphorylated proteins in many tissues including testis as previously described (Brewis *et al*., 1998; Kulanand & Shivaji, 2001; Ballester *et al*., 2004; Tomes *et al*., 2004; Iamsaard *et al*., 2013, 2014; Arun *et al*., 2016a,b; Sukhorum & Iamsaard; Chaichun *et al*.). Especially, Chaichun *et al*. have clearly demonstrated that the testicular tyrosine phosphorylated proteins are located in seminiferous epithelium including Sustentacular cells (Sertoli cells), spermatogonia, spermatocytes, and spermatids, respectively. Such proteins also are localized in interstitial tissues particularly within the Interstitial cells (Leydig cells) (Chaichun *et al*.). By immuno-Western blot, their results also showed that the patterns of tyrosine phosphorylated proteins including 200, 131, 93, 70, 60, and 48 kDas, respectively, are present in testicular lysate (Chaichun *et al*.). Indeed, those findings support the idea that protein phosphorylation in testis are important for spermatogenesis and androgen synthesis especially testosterone hormone. However, the immunohistochemical localization and protein patterns of tyrosine phosphorylated proteins using this antibody in other male reproductive tissues of rats have never been documented. Our recent study is the first study that demonstrated the presence of tyrosine phosphorylated proteins in epithelium and fluid of epididymis of Sprague-Dawley rats. Herein, we showed for the first time that tyrosine phosphorylated proteins were expressed in adult rat epididymal epithelium specifically in cytoplasm of principle cells, nuclei of apical cells and basal cells (Fig. 2). Those proteins are partially identified as 182, 127, 80, 70, 57, 45, 34, and 31 kDas, respectively in head, body, and tail epididymal fluids. We assumed that such proteins are secreted from epididymal epithelium and play roles in functionally sperm maturation process. Previously, VPA has been shown to alter the patterns of the testicular phosphorylated proteins corresponding to decrease of sperm quality (Iamsaard *et al*., 2015, 2017a,b; Sukhorum *et al*.; Sukhorum & Iamsaard). This study also demonstrated that VPA not only changed the phosphorylated proteins but also increased the beta actin expression in epididymal fluid (Fig. 4). We assumed that the changes of such proteins might be involved in epididymal sperm abnormality in VPA-treated rats as previously demonstrated (Sukhorum & Iamsaard).

## CONCLUSION

This study has already demonstrated that the tyrosine phosphorylated proteins were localized and identified in epididymal epithelium and fluids of adult Sprague-Dawley rats. In addition, VPA affects the changes of tyrosine phosphorylated protein intensity and beta actin expressions in head, body, and tail epididymal fluids.

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**RESUMEN:** Las proteínas tirosina fosforiladas han sido localizadas e identificadas en tejidos reproductores masculinos tales como testículos y espermatozoides, capacitados a nivel acrosómico, excepto en el epidídimo. Los cambios de estas proteínas están asociadas con una disminución de la calidad del esperma en el tratamiento con ácido valproico (AVP). Este estudio tuvo como objetivo investigar la presencia y las alteraciones de la fosforilación de proteínas en el epitelio epididimal y en el fluido espermático de ratas tratadas con AVP. Dieciséis ratas macho adultas se dividieron en dos grupos: control y tratadas con AVP (n = 8 / cada uno). A las ratas tratadas se les inyectó AVP por vía intraperitoneal (500 mg / kg de peso corporal) durante 10 días consecutivos. Al final del experimento, se realizó inmunohistoquímica con la anti-fosfotirosina monoclonal (clon 4G10) para sondear las proteínas tirosina fosforiladas y también para examinar la expresión de tales proteínas usando inmunotransferencia Western, en tejido y fluido epididimarios. El resultado mostró reactividad positiva de proteínas fosforiladas en células citoplásmicas principales, en los núcleos de las células apicales y basales y en la masa de esperma rodeada por fluidos epididimarios. Los perfiles de proteínas fosforiladas en el fluido epididimal fueron 182, 127, 80, 70, 57, 45, 34 y 31 kDas, respectivamente. El AVP provocó cambios en las proteínas fosforiladas y en la β actina de los fluidos epididimarios de cabeza, cuerpo y cola del epidídimo. Concluimos que las proteínas tirosina fosforiladas se detectaron en el epitelio y el fluido epididimarios. Las expresiones de esas proteínas y de la β actina se alteraron bajo tratamiento con AVP.

**PALABRAS CLAVE: Localización; Beta actina; Proteínas tirosina fosforiladas; Fluido epididimal; Ratas.**

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