Ezrin Distribution Is Abnormal in Principal Cells from a Murine Model of Autosomal Recessive Polycystic Kidney Disease

STEPHANIE A. ORELLANA, ANDREA M. QUIÑONES, AND MARYLOU L. MANDAPAT

Departments of Pediatrics [S.A.O., A.M.Q., M.L.M.] and Physiology and Biophysics [S.A.O.], Case Western Reserve University School of Medicine and The Rainbow Center for Childhood PKD, Rainbow Babies and Children's Hospital, University Hospitals of Cleveland, Research Institute, Cleveland, Ohio 44106-6003, U.S.A.

ABSTRACT

Abnormalities in cell proliferation and intracellular signaling are features of inherited human and murine polycystic kidney diseases (PKD), regardless of the primary genetic defects. Loss of protein kinase A regulation of cell proliferation has been reported in the murine C57BL/6J*Cys1^{cpk-/-}* (*cpk*) model of autosomal recessive PKD. Qualitative differences in protein kinase A subunit distribution were observed between filter-grown cultures of noncystic- (C57BL/6J mice) and cystic *cpk*-derived principal cells. It was hypothesized that protein kinase A subunit distribution differences were mediated by differences in A-kinase anchoring protein (AKAP) expression, so expression of four AKAPs was examined in filter-grown cultures of primary murine cystic- and noncystic-derived principal cells. AKAP-KL expression was ambiguous, but mAKAP, AKAP95, and ezrin were expressed at expected molecular sizes and cellular locations in noncystic-derived cells. Perinuclear mAKAP and nuclear AKAP95 were distributed normally in *cpk*-derived cells. Expression of AKAP95 in cystic epithelium was diminished relative to controls, and ezrin expression was modestly decreased and abnormally distributed within a region near the apical surface. Qualitative differences were observed in ezrin location in response to medium change or stimulation with epidermal growth

factor which suggested cell-specific differences may result from the *cpk* mutation or the abnormal epidermal growth factor receptor phenotype that characterizes PKD. Ezrin has been implicated in tubulogenesis, so altered ezrin expression or function could be disruptive. If PKD mutations that contribute to PKD pathogenesis are postulated to disrupt normal tubular development, perhaps the mechanism includes altered ezrin function and abnormal protein kinase A targeting. (*Pediatr Res* 54: 406–412, 2003)

Abbreviations

AKAP, A-kinase anchoring protein
ARPKD, autosomal recessive polycystic kidney disease
C57, C57BL/6J mouse
CLSM, confocal laser scanning microscopy
cpk, homozygous C57BL/6J*Cys1^{cpk-/-}* mouse
DAPI, 4',6-diamidino-2-phenylindole
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
ERM, ezrin-radixin-moesin
MDCK, Madin Darby canine kidney
PKA, cAMP-dependent protein kinase
PKD, polycystic kidney disease

Human PKDs, autosomal dominant PKD and ARPKD, is distinguished by epithelial proliferation and progressive fluid accumulation in renal tubules to form cysts, which can lead to renal failure [for review see Davis *et al.* (1)]. Six genes responsible for human, murine, and rat PKD family members have been identified (2–7), but whereas the known gene products (polycystin-1, polycystin-2, fibrocystin, cystin, and po-

laris) have been postulated to form membrane-associated signaling complexes involved in normal nephrogenesis, and polycystin-1 specifically mediates tubulogenesis in two renal cell lines (8, 9), a specific mechanistic pathway leading directly from abnormal gene expression to renal cyst formation has yet to be described.

Compartmentalization of cAMP action can occur via the association of individual AKAPs with specific PKA subtype activities. Inasmuch as AKAPs may permit assembly of signaling complexes (10–12), the association of individual AKAPs with specific PKA substrates could provide a mechanism for linking and targeting PKA subtype regulation [for review, see Steinberg and Brunton (13)] to control of polarized epithelial cell functions. Our previous reports demonstrated

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Correspondence: Stephanie A. Orellana, Ph.D., Department of Pediatrics, 11100 Euclid Avenue, Mail Stop 6003, Cleveland, OH 44106-6003, U.S.A.; e-mail: sao3@po.cwru.edu

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differences in PKA subunit distribution that predicted differences in PKA subtype distribution and function in collecting tubular epithelial cell cultures (14–17). Roles for cAMP in the family of inherited human and murine PKDs have been postulated by us and others [for reviews, see Marfella-Scivittaro *et al.* (15) and Calvet and Grantham (18)]; thus, the present study was undertaken to evaluate potential mechanisms for subcellular PKA distribution and differences in proliferation previously observed between noncystic and cystic renal epithelial cell models. Because AKAPs can mediate PKA subunit distribution and act as a foundation for signaling complexes, expression of AKAPs known to be associated with cytoskeletal and nuclear structures was examined in normal and cystic-derived murine principal cell cultures.

METHODS

Cell isolation and culture. An animal model of ARPKD, the *cpk* mouse (19), arose from spontaneous mutation in the C57 strain. The murine disease phenotype and its progression mimic the human disease ARPKD [for reviews, see Avner (20) and Sweeney and Avner (21)]. Enriched populations of untransformed principal cells from both C57 and *cpk* animals were isolated and cultured on Transwell-Clear filter inserts (Corning Costar Corp., Lake Placid, NY, U.S.A.) as described (15). All cultures were confluent when assayed. Use of animals for this study was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Immunologic reagents. Primary antibodies used were anti-ezrin (E8897, Sigma Chemical Co., St. Louis, MO, U.S.A.); anti-AKAP-KL (A81420, BD Transduction Laboratories, Lexington, KY, U.S.A.); anti-mAKAP (07–087, Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.); and anti-AKAP95 (sc-6447, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). The fluorescent-tagged secondary antibodies used were Alexa-Fluor 488 donkey anti-goat conjugate (A-11055); Alexa-Fluor 594 donkey anti-goat conjugate (A-11058); Oregon Green 488 goat anti-mouse conjugate (O-6380); Oregon Green 488 goat anti-rabbit conjugate (O-6381); Texas Red-X goat anti-mouse conjugate (T-6390); and Texas Red-X goat anti-rabbit conjugate (T-6391; all from Molecular Probes, Eugene, OR, U.S.A.). Peroxidase-linked secondary antibodies used were goat anti-rabbit (sc-2004); goat anti-mouse (sc-2005); and donkey anti-goat (sc-2020; all from Santa Cruz Biotechnology, Inc.).

Western analysis of protein expression. Protein samples (15 μ g each) were subjected to Western analysis as previously described (15). Equal sample loading was required because comparative expression of a protein like actin, constitutively expressed in many normal tissues, was unsuitable for cystic tissues in which actin expression was abnormal (15), and in which actin isoforms may be down-regulated (22). Specific primary and secondary antibody combinations are indicated in the legend (Fig. 1), and densitometric quantitation of immunoreactive bands was performed using Scion Image v.4.0.2 software (Scion Corp., Frederick, MD, U.S.A.) and a flatbed scanner as described (23). For additional analyses, blots were stripped with the Re-Blot Plus Strong antibody stripping solu-

Figure 1. Murine principal cell cultures expressed AKAPs. Confluent filter-grown cultures of cystic *cpk* and noncystic C57-derived principal cells were subjected to Western analysis as described in Methods. *A*, Western analysis: primary antibodies described in “Methods” were used at the following dilutions: anti-mAKAP (07–087), 1:750; anti-AKAP95 (sc-6447), 1:750; anti-ezrin (E8897), 1:750; and anti-AKAP-KL (A81420), 1:750. Appropriate secondary antibodies described in “Methods” were used at the following dilutions: sc-2004, 1:3000; sc-2020, 1:1500; and sc-2005, 1:1500. Apparent molecular weights are indicated as kilodaltons. Control analyses performed in the absence of primary antibodies resulted in no signal (data not shown). Protein loaded was 15 μ g/lane. Results are representative of three experiments. *B*, relative expression: specific immunoreactive bands shown in *A* were subjected to densitometric scanning and quantitation as described in “Methods.” Expression is shown in arbitrary units. For each separate experimental pair (C57 and *cpk*), the C57 value was set at 1, and the corresponding *cpk* value was expressed relative to the C57 value. Values shown are mean $\pm$ SEM, $n = 3$ experiments.

tion (2504, Chemicon International, Temecula, CA, U.S.A.) according to the manufacturer’s instructions, and stripping time was increased by 5 min for each subsequent incubation.

Localization studies. For cultures treated before fixation, growth medium was removed and cultures were incubated at 37°C, in fresh defined medium (21) lacking serum and containing additions indicated in figure legends. Paraformaldehyde fixation, immunostaining, and sample mounting were performed as described (15), using antibody combinations listed in figure legends (Figs. 2–4). Specificity was demonstrated using controls lacking primary antibodies, which in all cases resulted in no detectable fluorescent signal (data not shown). Actin labeling was performed using Oregon Green 514-phalloidin conjugate (O-7465, Molecular Probes), which labels F-actin (15). Nuclear staining was performed after immunofluorescent labeling was completed, using propidium iodide (Molecular Probes) (15) or VectaShield with DAPI (H-1200, Vector Laboratories, Inc.).

Two methods were used to localize specific proteins identified by immunofluorescence (14, 15). Immunoreactive signals for all AKAPs examined were detected by standard fluorescence microscopy, using an Optiphot-2 fluorescence microscope with fluorescein isothiocyanate, Texas Red, and DAPI filter sets (Nikon Corp., Tokyo, Japan). Samples were photographed with a SPOT digital color camera (Diagnostic Instruments, Sterling Heights, MI, U.S.A.). Semitransparent layered images were constructed with Adobe PhotoShop 5.5 software [Adobe Systems Incorporated, San Jose, CA, U.S.A.; Figs. 2 and 4 (15)].

CLSM was used to localize immunoreactive proteins as described (14, 15). A full z -series of x - y scans was performed from above the epithelial layer and into the filter support, a depth typically ranging from approximately 6 to 11 μ m. The full z -series was used to reconstruct the x - z cross-sections

Figure 2. Only *cpk* ezrin expression was clearly abnormal compared with C57 when the cellular distribution of each AKAP was examined in principal cell cultures that formed confluent epithelial layers. Confluent filter-grown cultures of cystic *cpk*- and noncystic C57-derived principal cells were subjected to fixation and immunostaining as described in “Methods.” Primary antibodies described in “Methods” (anti-mAKAP, 07–087; anti-AKAP95, sc-6447; anti-ezrin, E8897; and anti-AKAP-KL, A81420) were used at 1:100 dilutions. Appropriate (red or green) fluorescent-conjugated secondary antibodies described in “Methods” (T-6391 or O-6381; A-11058 or A-11055; and T-6390 or O-6380; respectively) were used at 1:250 dilutions. Controls performed in the absence of primary antibodies resulted in no detectable fluorescent signals (data not shown). After immunostaining by fluorescent red-conjugated antibodies, samples were stained with a fluorescent green-conjugated phalloidin (to detect F-actin and approximate the location of cell boundaries) and DAPI (to detect nuclei, blue), as described in “Methods.” Images shown are representative of three fields per sample, of one to two samples per experiment, of two to three experiments. Samples were viewed by conventional fluorescence microscopy, and images were captured, as described in “Methods.” Individual red, green, and blue images were layered using Adobe PhotoShop 5.5 software. Magenta corresponds to regions of overlap between red and blue images. Bar represents 25 μ m.

shown (Fig. 3). Enlargements shown were made using Adobe PhotoShop 5.5 software to select and increase the size of the regions between the white lines.

Tests of ezrin redistribution were performed by treatment of live cell cultures, fixation, and staining, as indicated above and in the legend to Figure 4. For each sample, 10 separate regions were viewed by conventional fluorescence microscopy as de-

Figure 3. Optical sectioning of confluent principal cell cultures showed that only *cpk* ezrin expression was abnormal compared with C57 when the sub-cellular distribution of each AKAP was examined. Samples were prepared and indirect immunofluorescence staining was performed as described in the legend to Figure 2, but only immunoreactive AKAP signals are shown. Samples were viewed by CLSM, and images were captured, as described in “Methods.” Large panels are single *x-y* scans and small panels are *x-z* cross-sections reconstructed from the full *z*-series of *x-y* scans. Arrowheads (>, <) indicate the location of the *x-y* plane shown in the *x-y* scans. No ezrin immunostaining was detected at the basal surfaces of either C57 or *cpk* cell cultures, and all cultures were confluent when fixed. Bar represents 25 μ m in all CLSM images except for the gray-bordered images. To show apparent apical immunostaining, regions of the ezrin cross-sections between the white lines were enlarged as described in “Methods.”

scribed above, and separate red (ezrin immunostaining) and green (phalloidin staining to locate cell boundaries, shown only for C57 + EGF, as a combined gray-scale image) images were collected for each region. Images shown are for the predominant phenotype present in each treatment condition.

Figure 4. Only *cpk* ezrin was redistributed within the cell in response to a medium change, but both C57 and *cpk* ezrin were redistributed in response to EGF treatment, and a cAMP analog had no effect. Confluent filter-grown cultures of cystic *cpk*- and noncystic C57-derived principal cells had either fresh medium (Control), or were treated with fresh medium containing 10 μ M 8-bromo-cAMP (8BrcAMP) or 15 ng/mL EGF (added to both sides) for 30 min, before fixation as described in "Methods." Immunofluorescent staining for ezrin was performed, samples were viewed by conventional fluorescence microscopy, and 10 images per sample were viewed and prepared, as described in "Methods." Bar corresponds to 25 μ m. Data shown are the predominant phenotype observed in three *cpk*, and one C57, experiments each performed in duplicate. Control conditions at 0 min were identical to Figure 2 ezrin immunostaining. All cultures were confluent when fixed, as demonstrated by the panel labeled +actin, which shows a gray-scale conversion of the combined red (ezrin immunostaining) and green (phalloidin conjugate staining) layered Adobe Photoshop image of the data from the C57 + EGF condition directly above it.

Other information. Statistical determinations and protein quantitation were performed, and reagents not specifically listed above were obtained, as described (15).

RESULTS

To determine whether noncystic- and cystic-derived principal cells expressed AKAPs that might mediate differences in PKA subunit location and regulation of cAMP-mediated cell proliferation previously observed (15), expression of four known AKAPs was examined. Two AKAPs associated with nuclear structures (mAKAP, AKAP95), and two AKAPs associated with the cytoskeleton (ezrin, AKAP-KL) were targeted. The mAKAP has been reported to occur at the nuclear membrane of differentiated myocytes (24) and was reported to provide a base for multiprotein regulation of the cardiomyocyte ryanodine receptor (24–26). AKAP95 is a primarily nuclear matrix protein whose subcellular distribution and ability to associate with the PKA regulatory subunit type II α isoform is cell cycle-dependent (27). AKAP95 has been reported to bind to an RNA helicase, supporting its role in modulating multiprotein regulation of nuclear matrix proteins and assembly of hormonally responsive transcription complexes (27–29). Ezrin is a member of the ERM family, is both an AKAP and an EGFR substrate, is present at the apical cytoplasmic side of many polarized cells, links plasma membrane proteins to the cytoskeleton, and binds other proteins [for reviews, see Bretscher (30) and Weinmann *et al.* (31)]. AKAP-KL proteins are highly expressed in kidney and lung, have a polarized apical distribution in epithelia, and interact with and modulate actin cytoskeletal structure (32).

As shown (Fig. 1), immunoreactive mAKAP, AKAP95, ezrin, and AKAP-KL proteins were expressed in confluent filter-grown cultures of both noncystic C57- and cystic *cpk*-derived principal cells. Single forms of AKAP95 and ezrin were detected at appropriate apparent molecular weights (95 and 80 kD, respectively). An mAKAP protein of 300 kD is the primary form found in brain and heart, but a 255-kD form was also reported (24–26). In principal cell cultures, both mAKAP 300-kD and 255-kD forms were expressed. AKAP-KL has been reported to migrate by SDS-PAGE as several differently sized proteins (105–133 kD) resulting from alternative splicing in whole tissue lysates, although calculated molecular weights for six isoforms ranged from 74 to 98 kD (32). Three immunoreactive AKAP-KL bands of approximately 145, 112, and 68 kD were variably observed in the untransformed C57- or *cpk*-derived principal cell filter-grown cultures (Fig. 1). Densitometric quantitation of protein expression in each 15- μ g sample revealed that cystic-derived samples had lower relative expression levels compared with noncystic values (set arbitrarily at 1) of AKAP95, and modestly decreased expression of ezrin, but mAKAP expression was similar (Fig. 1). Owing to the variability observed in AKAP-KL immunoreactivity, quantitation of AKAP-KL expression was not performed.

Cystic- and noncystic-derived cell culture growth and fixation conditions were identical, and all cultures were confluent when fixed for microscopic evaluation, as shown by the green phalloidin staining of lateral F-actin (Fig. 2). Some immuno-

reactive AKAP-KL was detected by conventional fluorescence microscopy, and although noncystic and cystic expression appeared qualitatively different with some apparent nuclear immunostaining in C57, AKAP-KL localization studies were not pursued further (Fig. 2). The AKAPs mAKAP, AKAP95, and ezrin were detected at appropriate cellular locations within noncystic-derived principal cell cultures (Fig. 2, conventional microscopy; Fig. 3, CLSM). Location in cystic-derived cultures was similar for the perinuclear mAKAP and primarily nuclear AKAP95. In both cell types, ezrin immunostaining was detected by a full *z*-series of CLSM *x-y* scans only between 6 and 11 μm above the filter support, indicating expression at the apical side of the epithelium. Normal ezrin immunostaining differed from *cpk* ezrin, which was distributed in a nonuniform manner within an apparently narrower apical plane (Fig. 3).

Because ezrin is an EGFR tyrosine kinase substrate (33), cystic principal cells have an abnormal apical EGFR phenotype (34), *cpk* ezrin is located differently from C57 ezrin within the apical region (Figs. 2 and 3), and inhibition of ezrin tyrosine dephosphorylation was reported to alter its distribution in MDCK cells (35), ezrin distribution was evaluated by examining conventional fluorescence microscopic images of confluent cystic- and noncystic-derived principal cell cultures after stimulation by EGF. As shown (Fig. 4), a 30-min control incubation did not alter ezrin distribution in C57 cells. However, the same control removal and replacement of medium resulted in more diffuse *cpk* ezrin immunostaining (compare with Fig. 2). Addition of 8-bromo-cAMP, a cell-permeable cAMP analog, had no effect on ezrin distribution in either cell type, compared with the 30-min controls. Incubation of live cultures with EGF resulted in ezrin redistribution within each confluent cell type, and effects were qualitatively different. EGF-treated C57 cells appeared to have an ezrin phenotype comparable to untreated *cpk* cells (compare with Figs. 2 and 3). As shown (Fig. 4, *bottom left*), EGF-treated C57 cells maintained normal cell boundaries as indicated by phalloidin staining of lateral actin, as did all other samples (data not shown). EGF-treated *cpk* cells had a different ezrin phenotype altogether, in which ezrin immunostaining appeared concentrated at lateral edges and within the cell in a unique pattern.

DISCUSSION

Four AKAPs (mAKAP, AKAP95, AKAP-KL, and ezrin) previously reported to mediate PKA anchoring were expressed in primary principal cell cultures from murine noncystic and ARPKD kidneys. These data demonstrate renal epithelial expression of mAKAP, an AKAP originally identified in skeletal muscle whose role in renal epithelia is unknown. Qualitatively more AKAP95 was detected in cystic nuclei, in spite of lower overall expression, perhaps reflecting a difference in cell cycle status between the two cell types, which have been shown to have strikingly different basal proliferation levels (15). As it is likely that altered gene expression regulates the altered cell proliferation observed in PKD, perhaps the apparent AKAP95 down-regulation observed for *cpk* resulted in loss of a normal PKA-regulated proliferation control mechanism (15). The variable AKAP-KL expression observed suggests that a more

detailed analysis is required to characterize the many AKAP-KL forms reported to arise from alternative splicing (32). The qualitatively different subcellular distribution observed for *cpk* ezrin suggested abnormal distribution within the apical microvillar or cytoskeletal region of the principal cell epithelium. Apical actin appeared normal (15), so it is likely that characteristic blunt, sparse microvilli were present in both principal cell cultures. We speculate that in the absence of a functional PKD gene product, *cpk* cell ezrin may associate abnormally with apical PKA regulatory subunits. More or less PKA at the *cpk* cell apical surface that is associated with ezrin, or with another apical AKAP, could change PKA-dependent regulation of epithelial transport (and perhaps proliferative) functions to mediate cystogenesis.

Full-length ERM proteins are present in soluble cell fractions in a dormant, folded form in which an intramolecular interaction prevents binding to other proteins. Conformational activation, by Rho-kinase-dependent phosphorylation of ezrin on T567, to the actin-binding oligomeric form unmasks binding sites for other proteins, such as the Rho-GDP-dissociation inhibitor RhoGDI, phosphatidylinositol 3-kinase regulatory subunit p85, and a family of ezrin binding proteins, findings that have led to the suggestion that ERMs have potential roles in signaling pathways [for reviews, see Weinmann *et al.* (31) and Bretscher *et al.* (36)]. Support for a signaling role of ezrin was demonstrated by EGF- and hepatocyte growth factor-induced phosphorylation of the same two residues, Y146 and Y354 [for review, see Bretscher *et al.* (36)]. Tyrosine phosphorylation of ezrin by hepatocyte growth factor through its receptor, c-met, was implicated in regulation of branching tubulogenesis in the epithelial cell line, LLC-PK1 (37).

In plastic-grown MDCK cells, pervanadate inhibition of Y146 and Y354 dephosphorylation led to ezrin redistribution (35), and some of the resulting microscopic data were qualitatively similar to those reported here. The previous report demonstrated altered ezrin distribution within the apical region and the cytoplasm, and increased intensity of immunostaining at cell borders, in response to pervanadate incubation. The authors concluded that the change indicated cytosol to plasma membrane redistribution of tyrosine-phosphorylated ezrin (35). For experiments in both the previous report (35) and the present study, growth medium was removed from epithelial layers and replaced with treatment medium for 30 min before fixation. Therefore, it is possible that the apparent ezrin redistribution observed in both studies was partly the result of disruptions of the apical epithelial surface as a result of acute medium changes. However, if cystin, the *Cys1* gene product disrupted in *cpk*, is associated with apical cilia, which have been postulated to act as flow sensors (2), then perhaps ciliary malfunction could account for the greater apparent ezrin disruption by medium change observed in *cpk*- compared with C57-derived cultures. However, a quantitative evaluation of CLSM ezrin immunostaining and phosphorylation status in cultures treated without a complete medium change will be required to demonstrate whether ezrin redistributes in epithelial cells in response to tyrosine phosphorylation, as suggested previously (35).

Evidence for cellular signaling through the AKAP feature of ezrin (38) has been demonstrated by its ability to provide PKA regulation of: the Na-H exchanger through an interaction with an EBP50 family member, Na-H exchanger regulatory factor 1 [for review, see Weinmann *et al.* (31)]; the cystic fibrosis transmembrane conductance regulator through an interaction with E3KARP (39); and the β -adrenergic receptor through an interaction with Na-H exchanger regulatory factor 2 (40). Nothing is known about the cellular function of this feature in tubulogenesis or cystogenesis. However, PKA phosphorylates RhoA on S188 to inhibit and then block activation of Rho kinase, leading to loss of stress fibers [for reviews, see Bretscher *et al.* (36), Diviani *et al.* (41), and Sah *et al.* (42)]. We previously reported disrupted actin stress fibers in the same *cpk* principal cell culture system used in the present study (15), and also have observed disrupted stress fibers in a conditionally immortalized principal cell culture system for another murine ARPKD model (43, 44). Perhaps altered PKA targeting by ezrin has the potential to interfere with the activation of ezrin by Rho kinases and subsequent membrane-cytoskeletal linkage and function.

CONCLUSIONS

There have been several recent reports that other PKD models have altered stress fibers (45) and altered expression of cytoskeletal organization genes (22, 46); that Rho family proteins are involved in renal tubulogenesis (47) and cytoskeletal regulation of cAMP-mediated aquaporin-2 translocation (48); and that polycystin-1 induced morphologic changes in MDCK cells that required phosphatidylinositol 3-kinase (49). These seemingly disparate phenomena led us to speculate that in combination with the abnormal apical EGFR phenotype common to all PKDs [for review, see Sweeney *et al.* (50)], ezrin could provide an integration point for abnormal signaling in PKD.

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