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RNA interference silencing of DRAL affects processing of amyloid precursor protein

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Abstract

In a previous study, we reported that Alzheimer's disease-associated presenilin-2 interacts with a LIM-domain protein, namely, DRAL/FHL2/SLIM3. In this study, we investigated whether DRAL modifies the metabolism of the amyloid precursor protein (APP). We used small interfering RNA (siRNA) to knockdown DRAL in COS7 and HEK293 cells that stably overexpress APP695. We found that the knockdown was accompanied by a decrease in the amount of secreted α-secretase-cleaved APP and the membrane-bound C-terminal fragment C83 and an increase in the amount of secreted β-amyloid peptide (Aβ) from the cells. We also found that in addition to a disintegrin and metalloprotease (ADAM)-17, DRAL binds to ADAM-10. Thus, DRAL may be involved in the processing of APP through the α-secretase pathway.

Key words: alpha-secretase; Alzheimer's disease; amyloid precursor protein (APP); ADAM-10/Kuzbanian; ADAM-17/TACE; DRAL/FHL2/SLIM3

Introduction

Alzheimer's disease (AD) is the most common type of senile dementia. It is characterized by the progressive formation of insoluble amyloid plaques consisting of β-amyloid peptides (Aβ), which comprise 40 to 42 amino acids in the brain. Aβ is sequentially cleaved from the amyloid precursor protein (APP) by 2 proteolytic enzymes, namely, the β- and γ-secretases [5]. In an alternate pathway, APP is cleaved within the Aβ-domain (the major site of cleavage is between Lys-16 and Leu-17 in the Aβ domain) by α-secretase, releasing a large (105-125 kDa) N-terminal ectodomain fragment, namely, sAPPα from the cell surface. A shorter C-terminal transmembrane stub, i.e., C83, is further cleaved by γ-secretase to generate a soluble p3 fragment. Thus, the processing of APP by the α-secretase pathway precludes the formation of Aβ. In many cell types, the activation of protein kinase C (PKC) by the PKC agonist phorbol 12-myristate 3-acetate (PMA) has been shown to increase sAPPα release and reduce the secretion of Aβ [1]. Two members of a disintegrin and metalloprotease (ADAM) family of proteases, namely, ADAM-10/Kuzbanian and ADAM-17/TACE, have been reported to exhibit α-secretase activity [1,3,8]. ADAM-10 is considered to be involved in constitutive α-secretase activity, while ADAM-17 is thought to be involved in PMA-stimulated activity. γ-Secretase consists of at least 4 proteins – presenilin (PS)-1 or PS-2 at the catalytic core, nicastrin, APH1 and PEN-2 [5]. We previously found that PS-2 interacts with DRAL (alias FHL2 or SLIM3), and the overexpression of PS-2 in cells resulted in an increase in the amount of PS-2-associated DRAL in the membrane fraction [12]. DRAL is a member of a family of 5 proteins containing four and a half LIM domains and expressed in various tissues including brain [12]. It participates in

various cellular processes, including regulation of cell survival, transcription, and signal transduction [6,7,9,11]. Recently, Canault *et al.* have reported that ADAM-17 interacts with DRAL. Under PMA stimulation, DRAL-deficient macrophages had a lower ability to release ADAM-17 substrates, tumor necrosis factor receptor-1 and receptor-2 (TNFR-1 and TNFR-2), when compared with wild-type macrophages [4]. Since ADAM-17 is an α-secretase candidate of APP, we investigated whether DRAL modifies APP metabolism. We used small interfering RNA (siRNA) in DRAL-knockdown experiments and found that the knockdown was accompanied by a decrease in sAPPα secretion and an increase in Aβ secretion. We also found that in addition to ADAM-17, DRAL binds to ADAM-10. Thus, DRAL may be involved in the processing of APP through the α-secretase pathway.

Materials and Methods

Plasmid construction

The entire coding sequences of DRAL, ADAM-10, and ADAM-17 cDNA were amplified and cloned into the pFLAG-CMV™-2 (Sigma), pEF6/V5-His (Invitrogen), and pcDNA™3.1/Zeo(-) (Invitrogen) vectors, respectively. The cDNA containing the last 100 residues of the C termini of APP (C100), and an initiating methionine and the last 83 and 89 residues of the APP C terminus (MetC83 and MetC89, respectively) were amplified and inserted into the pcDNA™3.1/Zeo(-) vector.

siRNA targeting of the DRAL gene

The siRNA duplexes composed of 21-nucleotide (nt) sense and 21-nt antisense strands with a 2-nt 3' overhang, were designed by using the siRNA user guide of the Tuschl

laboratory (http://www.rockefeller.edu/labheads/tuschl/sirna.html). The target sequences used in this study are as follows: siRNA B sense: 5'-gccuguuuccacugcucgctt-3' and antisense: 5'-gcgagcaguggaaacaggctt-3'; siRNA C sense: 5'-gugccaggaaugcaagaagtt-3' and antisense: 5'-cuucuugcauuccuggcactt-3'; siRNA D sense: 5'-cugcuucugugacuuguautt-3' and antisense: 5'-auacaagucacagaagcagtt-3'. These sequences are conserved between human and COS7 cells. We used control (nonsilencing) siRNA (Qiagen-Xeragon) as a negative control.

Transfection for siRNA

The COS7 or HEK293 cells that stably overexpress APP695 (HEK293-APP695 cells) [13] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells in a 12-well plate were transfected with siRNA using the LipofectAmine2000® transfection reagent (Invitrogen) with 2 μl/160 pmol (reagent/siRNA), according to the manufacturer's instructions. Transfection with siRNA involved 2 consecutive rounds in which cells underwent an initial transfection (to achieve more complete silencing), and then 2 days later, the cells were replated and next day transfected again. Five days after the first transfection, the cells were washed with serum-free DMEM and cultured in serum-free DMEM in the absence or presence of 30 nM PMA or dimethyl sulfoxide (DMSO) (vehicle) for 6 or 12h; the cells and the conditioned medium were then harvested.

Western blot analysis

The cells were lysed in sodium dodecyl sulfate (SDS)-sample buffer. The collected medium was treated with 125 μg/ml sodium deoxycholate and 6% (w/v) trichloroacetic acid [2]. Proteins were precipitated by centrifugation at 20,800 × g for 30 min, and the

pellets were washed with 80% ice-cold acetone. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis using the anti-Aβ1−17 mouse monoclonal antibody 6E10 (Signet). the anti-APP C-terminus rabbit polyclonal antibody A8717 (Sigma), the anti-ADAM-17 rabbit polyclonal antibody CT (AnaSpec), the anti-ADAM-10 rabbit polyclonal antibody A2851 (Sigma), a horseradish peroxidase (HRP)-conjugated anti-V5 mouse monoclonal antibody (Invitrogen), an anti-FHL2/DRAL mouse monoclonal antibody 11-134 (MBL), and an anti-actin rabbit polyclonal antibody (Sigma). Western blots were visualized and quantitatively analyzed by using the luminescent imaging analyzer LAS-1000 (Fujifilm). The antibody 6E10 does not recognize the β-cleaved secreted ectodomain APP and p3.

Sandwich enzyme-linked immunosorbent assay for Aβ

The concentration of Aβ40 in the conditioned medium was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) performed using the Signal Select™ Human Aβ40 kit (Biosource), according to the manufacturer's protocols. The capture antibody in this kit is against Aβ1-12 and does not recognize the p3 fragment. The resulting values were normalized to the amount of total protein in each cell extract.

Immunoprecipitation

To investigate the interaction of DRAL with ADAM-10 or ADAM-17, COS7 (6-cm dish) cells were cotransfected with the indicated expression plasmids using FuGENE 6 transfection reagent (Roche). Twenty-four hours after the transfection, the cells were lysed for 30 min on ice in 800 μl of a lysis buffer (50 mM Hepes, pH7.5; 5 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM NaCl, 1% NP-40, protease inhibitors CelLytic™M [Sigma], and

phosphatase inhibitors [10 mM sodium β-glycerophosphate and 1 mM sodium orthovanadate]). Insoluble materials were removed by centrifugation (20,800 × g for 30 min). Lysates (400 μl) were precleared with protein G-agarose (Roche) and immunoprecipitated with mouse anti-FLAG monoclonal antibody M2 (Sigma) and protein G-agarose overnight at 4^{o}C. The beads were washed 3 times with lysis buffer, and the bound material was eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE and western blot analysis.

Results and Discussion

Knockdown of DRAL leads to decreased sAPPα release and increased Aβ secretion

Since the ectopic expression of DRAL has been reported to induce apoptosis in some cell lines [11] and DRAL functions as a transcriptional co-activator [6,7,9], we used siRNA to knockdown DRAL and examined the effect of this knockdown on the metabolism of APP. The transfection of each of the 3 siRNAs against human DRAL (siRNA B, siRNA C, and siRNA D) into the COS7 and HEK293-APP695 cells had no significant effect on the viability of the cells, as determined by MTS assay (Promega), when compared with the viability of mock-transfected and control siRNA transfected cells (data not shown). In COS7 cells, different siRNAs against DRAL efficiently reduced the DRAL protein level by 92 to 93% (normalized to β-actin) as compared with control siRNA treatment (Figs. 1 and 2) without significantly altering the expression of full-length APP (mature and immature) and actin. The amounts of α-secretase-cleaved sAPPα in the conditioned medium and the C-terminal fragment C83 in the cell lysates were detected by western blot analysis (Figs. 1 and 2). β-Secretase-cleaved C89 and C99 were rarely detected by this analysis (Fig.2). Under the constitutive (unstimulated)

condition, the siRNAs against DRAL slightly reduced the amounts of sAPPα by 12 to 22% in the conditioned medium and a single 10-kDa C83 by 10 to 13 % in the cell lysates. However, the PMA-stimulated condition, a reduction in endogenous DRAL by the siRNA was accompanied by a significant decrease in the amount of sAPPα by 28 to 38% and C83 by 22 to 27% (Figs. 1 and 2). To determine whether DRAL down-regulation influences the amount of Aβ secretion, each of the 3 DRAL siRNAs was introduced in the HEK293-APP695 cells, and the levels of Aβ in the conditioned medium were determined by sandwich ELISA (Fig. 3). In the HEK293-APP695 cells, DRAL siRNA treatment significantly reduced DRAL levels by 87 to 89% (normalized to β-actin) as compared with control siRNA treatment (Fig. 3A). While the secretion of Aβ was not affected by the transfection of control siRNA, the gene knockdown of DRAL by each of the 3 DRAL siRNAs resulted in increased Aβ secretion under the PMA-stimulated (by 30 to 34%) and unstimulated (by 21 to 22%) conditions; this indicated that DRAL is involved in regulating the production of Aβ under both conditions, and under the stimulated condition, the amount of Aβ secretion was more significantly influenced by DRAL down-regulation. ADAM-17 has been reported to interact with DRAL, and under PMA stimulation DRAL-deficient macrophages exhibit decreased ability to release ADAM-17 substrates, TNFR-1, and TNFR-2; however, this is not observed under the unstimulated condition [4]. Thus, under PMA-stimulated condition, DRAL may have considerable effect on α-secretase cleavage.

DRAL binds with ADAM-10 in addition to ADAM-17

ADAM-10 and ADAM-17 have been considered to be involved in constitutive α-secretase activity and PMA-stimulated activity, respectively. Since we observed a

slight decrease in sAPPα and C83 following the down-regulation of DRAL under the unstimulated condition, we examined the interaction between DRAL and ADAM-10 and ADAM-17. As shown in Fig. 4, V5-tagged ADAM-10 and ADAM-17 were co-immunoprecipitated with Flag-tagged DRAL in Flag-tagged DRAL overexpressing COS7 cells but not in empty-vector transfected cells. These results suggest that DRAL binds with ADAM-10 and ADAM-17 and stimulates α-secretase activity for APP.

Influence of DRAL on ADAM-10 and ADAM-17 expression

We investigated whether the down-regulation of DRAL alter the expression of ADAM-10 and ADAM-17. The knockdown of DRAL in COS 7 cells did not affect the amounts of ADAM-17 and ADAM-10 levels, and there was no difference in the unprocessed (proform) or mature (active) forms of either ADAM-17 or ADAM-10 between lysates from the cells treated with DRAL siRNA and the control siRNA (Fig. 5). Canault *et al.* have reported low amounts of ADAM-17 at the surface of wild-type mouse macrophages compared to those on the surface of DRAL-deficient macrophages and suggested that DRAL is involved in the regulation of the cellular localization of ADAM-17 [4]. However, we were unable to determine whether the knockdown of DRAL in the COS7 cells influences the cell surface localization of ADAM-10 and ADAM-17 (data not shown). A discrepancy in the results may be caused by differences between null expression and knockdown or due to the difference between the cell types used. We examined the effect of exogenous DRAL expression on the α-secretase activity of ADAM-10 and ADAM-17. However, the overexpression of DRAL had a negligible effect on the release of sAPPα and the production of C83 and did not enhance the α-secretase activity of exogenous ADAM-10 and ADAM-17 expression (data not shown). DRAL is an adaptor/docking protein, which interacts with various

proteins [6,10]. DRAL may require other factors for the regulation of α-secretase activity. The results of our study indicate that siRNA-mediated inhibition of endogenous DRAL expression is accompanied by a reduction in the α-cleavage of APP and results in an increase in the amount of Aβ secreted. Thus, our data suggest that DRAL regulates APP metabolism by modulating α-secretase activity. Since DRAL is expressed in the skeletal muscle [12], and overexpression of APP and intracellular accumulation of Aβ in vacuolated muscle fibers is considered a central mechanism in the pathogenesis of sporadic inclusion body myositis (sIBM) [14], DRAL may be involved in APP metabolism in the muscle and the pathogenesis of sIBM.

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Figure legends

Fig. 1. Effect of DRAL siRNA on sAPPα secretion. COS7 cells were transfected with siRNA indicated in the figure. Five days after the first transfection, the cells were treated with 30 nM PMA or DMSO (vehicle) for 6 h. The proteins, concentrated by trichloroacetic acid precipitation of the culture medium, and the total cell lysates were analyzed using western blot analysis. (A) Western blot analysis of sAPPα, full-length APP (mature and immature), actin, and DRAL. (B) (C) The amounts of DRAL and full-length APP (mature and immature) in the cell lysates (normalized to β-actin) were determined by western blot analysis. (D) The amounts of sAPPα in the conditioned medium. The mean of the control siRNA-transfected control in each independent experiment was set at 100%. The results are mean ± SD of 4 independent experiments. $*P < 0.05$, $***P < 0.005$, and $****P < 0.001$, compared with the control siRNA-transfected cells (paired *t* test).

Fig. 2. Effect of DRAL siRNA on C83 production. COS7 cells were transfected with siRNA indicated in the figure. Five days after the first transfection, the cells were treated with 30 nM PMA or DMSO (vehicle) for 6 h. The proteins, concentrated by trichloroacetic acid precipitation of the culture medium, and the total cell lysates were analyzed by using western blot analysis. (A) Expression levels of C83, actin, and DRAL. (B) The amounts of C83 were determined by western blot analysis, and the values were normalized to the level of actin in the same sample. The mean of the control siRNA-transfected control in each independent experiment was set at 100%.

The results are mean ± SD of 4 independent experiments. *$P < 0.05$, **$P < 0.01$ compared with the control siRNA-transfected cells (paired *t* test).

Fig. 3. Aβ sandwich ELISA. HEK293 cells that stably overexpress APP695 were transfected with siRNA indicated in the figure. Five days after the first transfection, the cells were treated with 30 nM PMA or DMSO (vehicle) for 12 h. Aβ sandwich ELISA was performed as described in the Materials and Methods. (A) Effects of DRAL siRNA on DRAL levels in HEK293-APP695 cells. The DRAL levels assessed by quantifying DRAL in the western blot analysis (normalized to β-actin). Transfection with DRAL siRNA did not significantly alter the expression of full-length APP, compared with control siRNA treatment (data not shown). (B) The quantity of Aβ40 was normalized to the protein content of the cells. The mean of the control siRNA-transfected control in each independent experiment was set at 100%. The results are mean ± SD of 4 independent experiments. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.001$ compared with the control siRNA-transfected cells (paired *t* test).

Fig. 4. Co-immunoprecipitation of DRAL with ADAM-10 and ADAM-17. Lysates (400 μl) from the COS7 cells transfected with plasmids indicated in the figure (1 μg each) were immunoprecipitated using an anti-Flag antibody. The immunoprecipitates (IP) and 10 μl of each lysate (input) were then analyzed by western blot performed using HRP-conjugated anti-V5 (for ADAM-10), anti-ADAM-17, anti-actin, or anti-DRAL antibodies.

Fig. 5. Effect of DRAL siRNA on ADAM-10 and ADAM-17 expression. COS7 cells were transfected with siRNA indicated in the figure. Five days after the first transfection, the cells were treated with 30 nM PMA or DMSO (vehicle) for 6 h. The

total cell lysates were analyzed using western blot analysis performed using anti-ADAM-10, anti-ADAM-17, anti-actin, and anti-DRAL antibodies. Circles and asterisks denote unprocessed (proform) and mature (active) forms of either ADAM-17 or ADAM-10, respectively.

Figure

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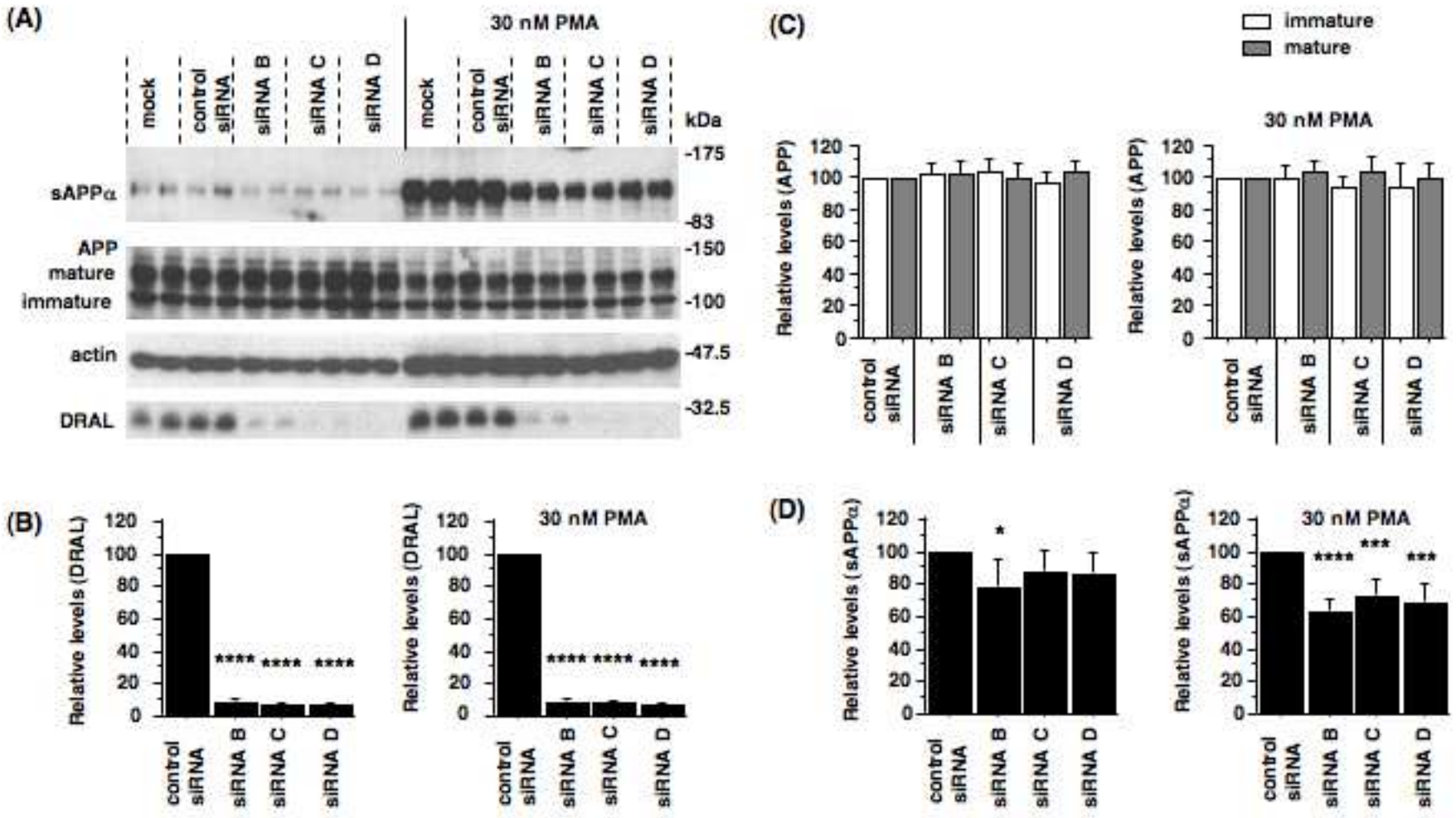

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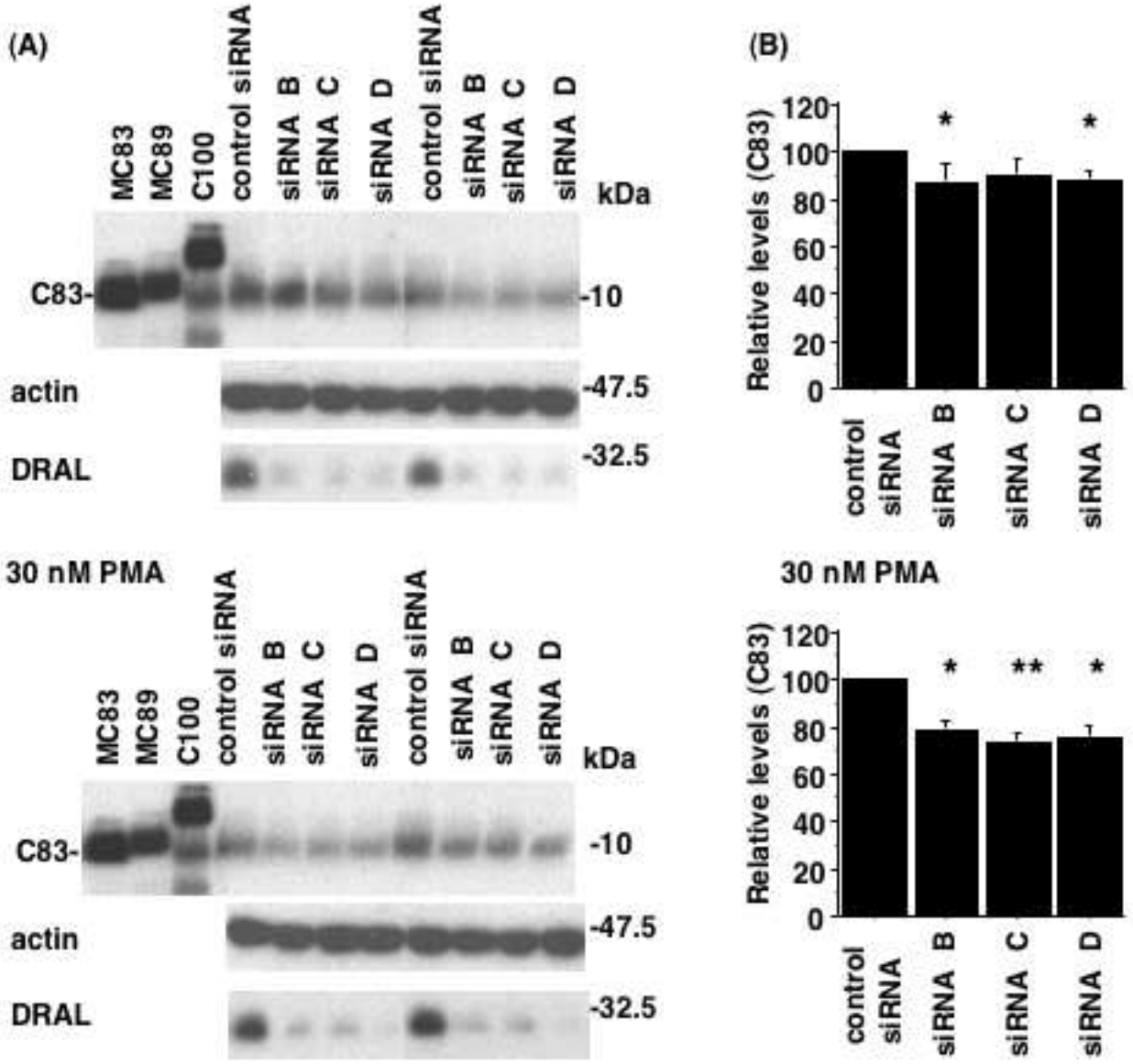

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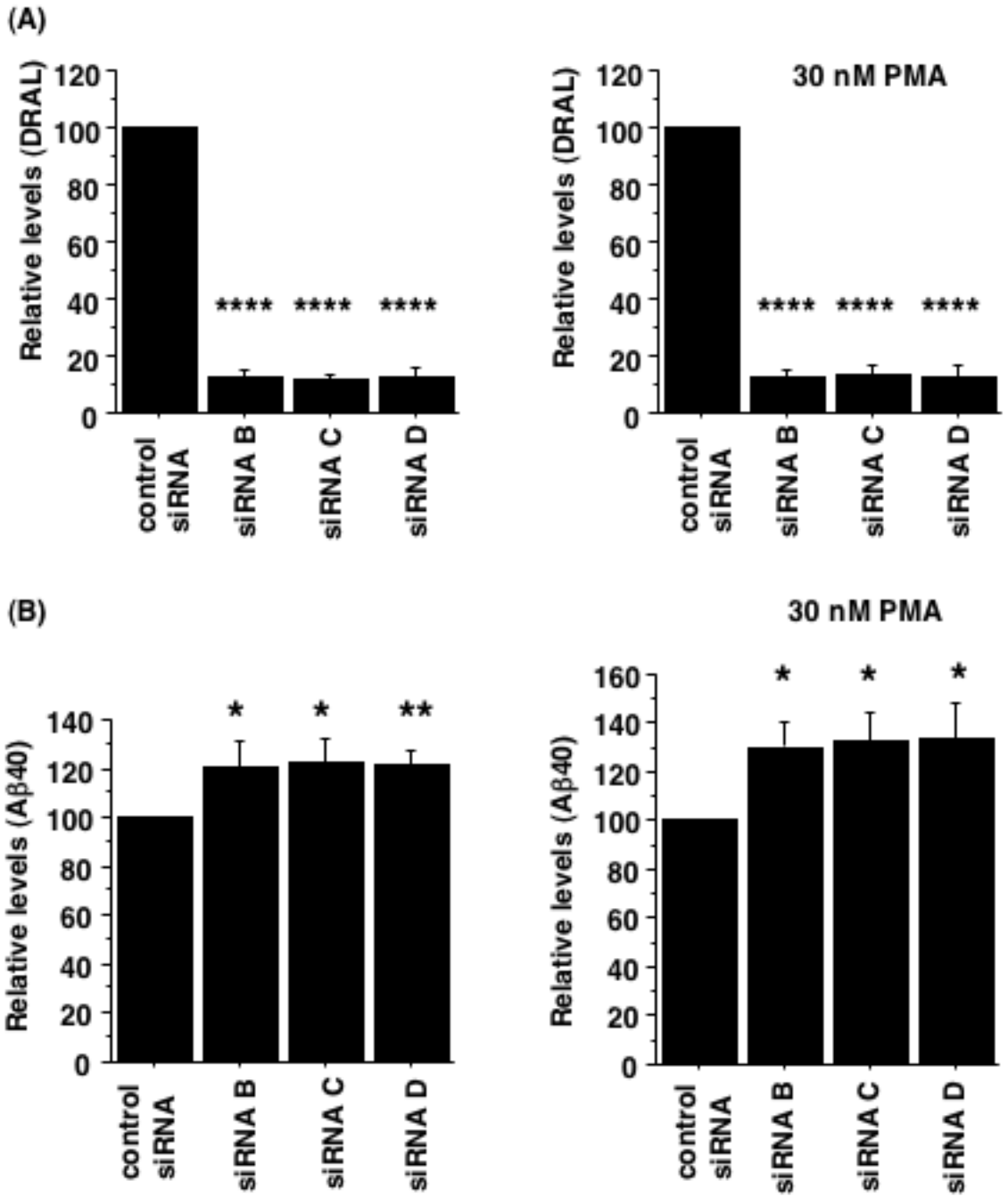

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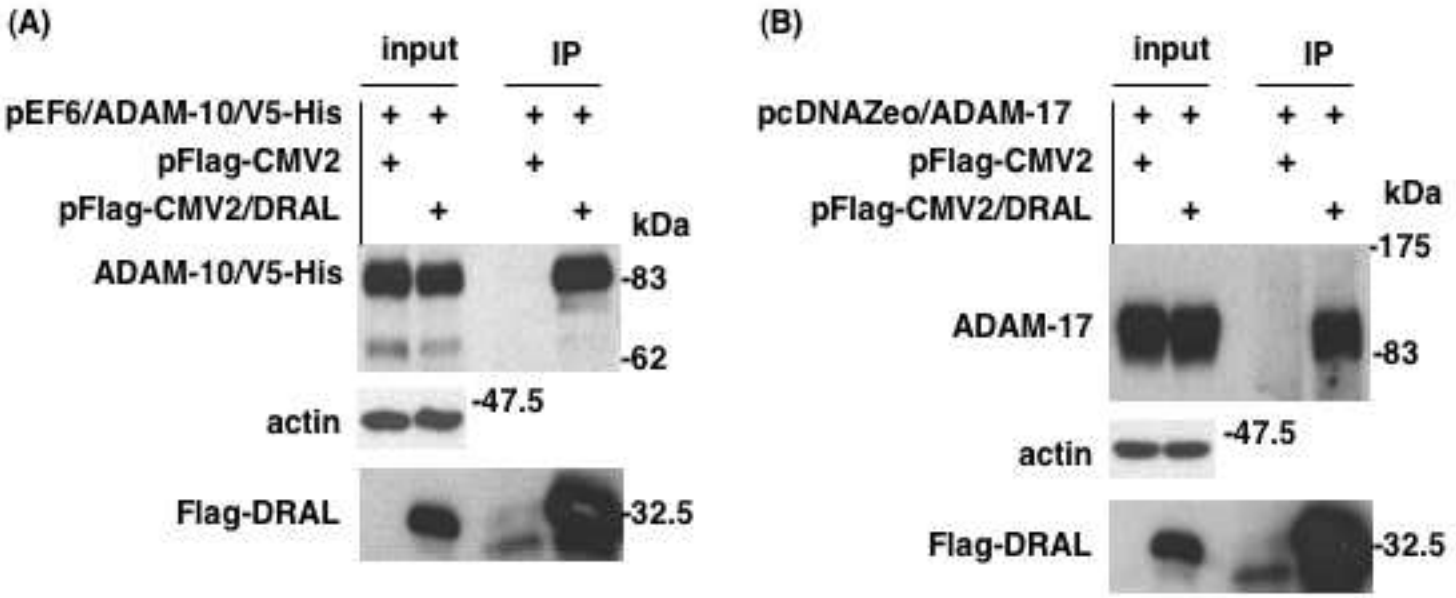

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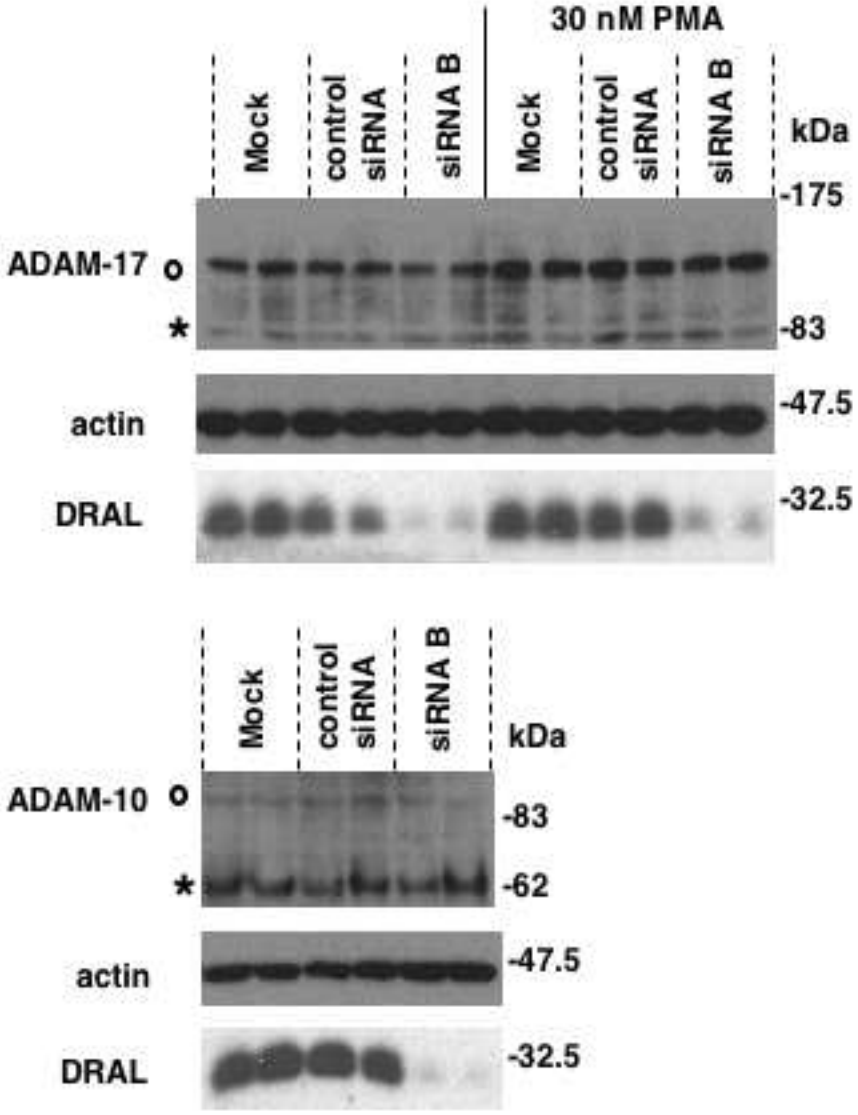
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