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Redox Signaling Regulates Transcriptional Activity of the Ca^{2+}-Dependent Repressor DREAM

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Abstract

DREAM/KChIP3 (Downstream Regulatory Element Antagonist Modulator) is a multifunctional Ca^{2+}-binding protein that acts in the nucleus as a Ca^{2+}-dependent transcriptional repressor. Binding to DNA and repressor activity of DREAM is regulated by Ca^{2+}, specific post-translational modifications as well as by protein–protein interactions with several nucleoproteins. Here, using the yeast two-hybrid assay, we characterized the interaction of DREAM with peroxiredoxin 3 (Prdx3), an antioxidant enzyme that uses the thioredoxin system as electron donor. Importantly, the DREAM/Prdx3 interaction is Ca^{2+} dependent and is blocked by DTT. Coexpression of Prdx3 enhances DREAM binding to DRE sites and its repressor activity *in vivo*. Two cysteine residues in the N-terminal domain of DREAM are responsible for the redox modulation of its activity. Double Cys to Ser substitution results in a mutant DREAM with stronger repressor activity. Finally, we show that transient DREAM knockdown sensitizes PC12 cells to H_2O_2-induced oxidative stress, suggesting a protective role for DREAM against oxidative damage. *Antioxid. Redox Signal.* 14, 1237–1243.

Introduction

Incomplete reduction of oxidizing agents such as free radicals and other reactive oxygen species (ROS) derived from cellular metabolism leads to oxidative stress (22). Pernicious effects of accumulation of ROS in the cells involve protein and DNA damage and subsequent cellular apoptosis (22). In neurons, oxidative stress has been associated with neurodegeneration in Alzheimer's, Huntington's and Parkinson's diseases, as well as neuronal decay during aging (36). To avoid the negative effects of ROS accumulation, a finely tuned redox balance is critical. Several oxidant and antioxidant systems coexist within the cell to maintain proper functionality and redox homeostasis.

Peroxiredoxins (Prdx) are thiol peroxidases that belong to the thioredoxin/thioredoxin-reductase (Trx/TR) antioxidant system. The Prdxs act as neuroprotective antioxidant enzymes protecting neurons against oxidative stress (26). In mammals, there are six *Prdx* genes (*Prdx* 1 to 6) encoding six enzymes that share a common catalytic mechanism that involves oxidation of a conserved peroxidatic cysteine to a sulfenic acid group by a peroxide molecule (27, 39). Prdxs are abundant enzymes with a wide tissue distribution and subcellular localization in cytosol (Prdx1, 2, 5, and 6), mitochondria (Prdx3 and 5), endoplasmic reticulum (Prdx4), and the nucleus (Prdx 1, 2, and 5) and extracellular (Prdx4) (18).

Transcription factors regulate cellular fate through changes in the gene expression profile. A number of transcription factors are regulated by redox signaling through modulation of their DNA binding capacity, oligomerization, or subcellular location. Thereby cells can adjust their transcriptome in response to physiological and pathophysiological changes in ROS levels (22).

The DREAM (Downstream Regulatory Element Antagonist Modulator) protein, a member of the DREAM/KChIP family of neuronal calcium sensors, is preferentially expressed in the CNS, immune system, gonads, and thyroid gland (6) and has specific functions in different subcellular compartments. In the nucleus, DREAM is a calcium- and cAMP-sensitive transcriptional repressor that regulates transcription by binding to specific sites, DREs (Downstream Regulatory Elements) in the promoter of target genes (6, 7, 16, 32), or through the interaction with other nucleoproteins (15, 28). In neurons, DREAM represses basal expression of the prodynorphin gene, and DREAM knock-out mice show reduced pain sensitivity, suggesting a critical role for DREAM in pain modulation (8). Outside the nucleus, DREAM, also named KChIP-3 (K^+ channel interacting protein-3) and calsenilin, participates in the trafficking of several membrane proteins, including Kv4 potassium channels (3) and the TSH receptor (29), and interacts with presenilins (5). Whereas the interactions with Kv4 channels and the TSH receptor have functional

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consequences, the meaning of the interaction between DREAM and the presenilins has been obscured by conflicting reports showing i) either a reversal of presenilins-induced calcium release (17) or the enhancement of presenilins-induced calcium release and apoptosis (13, 20) and, ii) an increased APP processing and Aβ42 accumulation has been reported either after calsenilin overexpression (13) or in the brain of calsenilin deficient mice (19).

In this study, we investigated the redox modulation of the DREAM protein after a yeast two-hybrid screening revealed a protein-protein interaction between DREAM and Prdx3. We found that the transcriptional activity of DREAM is modulated through the redox state of two cysteines, located at positions 45 and 46, and that DREAM is involved in neuronal survival in response to oxidative stress.

Materials and Methods

Yeast two-hybrid screen

The yeast two-hybrid assay was performed as recommended (Matchmaker protocol handbook; Clontech, Mountain View, CA) using the yeast strain *Saccharomyces cerevisiae* AH109. The bait plasmid contained a Ca^{+2}-insensitive DREAM mutant (EFmDREAM) in the pAS2-1 vector. A pretransformed bone marrow cDNA library cloned in the pACT2 vector was used for the screening. Positive interactions were selected by sequential plating on low selective media S.D.DDO and high selective media S.D.QDO. Selected clones were subsequently subcloned in the eukaryotic expression vector pCS2+MT.

Cells

CHO and Cos7 cells were maintained in Ham's F12 medium supplemented with Glutamax, fetal bovine serum (5%), and penicillin/streptomycin. PC12 cells were maintained in DEMEM medium supplemented with Glutamax, fetal bovine serum (5%), horse serum (10%), and penicillin/streptomycin.

Plasmids and transfection

The pGLHD3-*luc* reporter plasmid contains a 300 bp (−150 to +150) of the regulatory region of the human prodynorphin gene including one DRE (centered at position +40) and one CRE (Dyn CRE4) centered at +61 (7). Plasmids for wild-type DREAM, EFmDREAM, and antisense (AS)-DREAM have been reported elsewhere (6, 15). Prdx5 plasmids were a generous gift from Dr. Bernard Knoops (University of Louvain). The mutation C45,46S-DREAM was done using the QuickChange method (Stratagene, La Jolla, CA) in the pcDNA3 expression vector. Transfections were carried out with the JetPEI transfection reagent following the manufacturer instructions. The data are mean ± SEM of at least three independent experiments in triplicates.

DNA-binding assays

EMSAs using 100 ng of recombinant DREAM or 10 μg of nuclear extracts from PC12 cells were performed as previously described (15). The oligonucleotide containing the DRE from the prodynorphin (Dyn DRE) promoter has been previously described (6, 7). Protein or extracts were incubated for 20 min with 80,000 cpm of probe in a final volume of 20 μl.

Western blot analysis

Thirty μg of protein were resolved in SDS-PAGE and transferred to PVDF membranes (Millipore, Temecula, CA). Antibodies against β-actin (0.2 μg/ml) and PARP (0.2 μg/ml) were from Santa Cruz (Santa Cruz, CA). Polyclonal antibody for Prdx5 (37) was a generous gift from Dr. Bernard Knoops (University of Lovain). For detection of DREAM, we used affinity-purified Ab1014 (0.5 μg/ml) (21).

Immunoprecipiation

For coimmunoprecipitation experiments, cells were lysed in NP40 lysis buffer containing 50 m*M* Tris pH 8.0, 150 m*M* NaCl, 1% NP40, and a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ). Coimmunoprecipitation was performed overnight at 4°C using DREAM monoclonal antibody 1B1 (15) or Myc antibody (Abcam, Cambridge, UK). Immunocomplexes were captured with protein G (1B1) or A (Myc) Sepharose beads for 1 h and beads were washed three times in NP40 lysis buffer. Protein complexes were eluted in SDS sample buffer and subjected to Western blot analysis.

Statistical analysis

Student's unpaired two-tailed *t*-test was used for statistical analyses. p values of 0.05 or less were considered significant. Asterisks represent statistical significance versus the appropriate control in each case $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

DREAM interacts with Prdx3

To disclose new protein–protein interactions involving DREAM that could reveal new insights in the molecular mechanisms governing DREAM activity, we screened a pretransformed bone marrow library using a yeast two-hybrid assay. Previously, this approach had resulted exclusively in detection of the Ca^{2+}-independent interactions between DREAM and presenilins (5) and Kv4 potassium channels (3). Thus, we reasoned that intracellular concentrations of luminal Ca^{2+} in the yeast possibly conditioned these earlier screenings, and we decided to use a DREAM mutant insensitive to Ca^{2+} (EFmDREAM) (6) as bait. Several positive interacting clones were selected and fully sequenced (31). Among them, one interaction corresponding to a clone encoding Prdx3 was further analyzed. The interaction between DREAM and Prdx3 was confirmed by coimmunoprecipitation assays in Cos7 cells cotransfected with Myc-tagged Prdx3 and wild-type DREAM (Fig. 1A). The interaction was specific and no interaction was observed between DREAM and Prdx5, another member of the Prdx family (Fig. 1A). Binding of calcium to EF-hands of DREAM modifies its conformation, blocking its capacity to bind DRE sites (6) and the ability to interact with other proteins such as CREB (15). Similarly, the presence of calcium greatly reduced the DREAM-Prdx3 interaction (Fig. 1B). Furthermore, the interaction between DREAM and Prdx3 was completely blocked in the presence of 5 m*M* DTT, suggesting

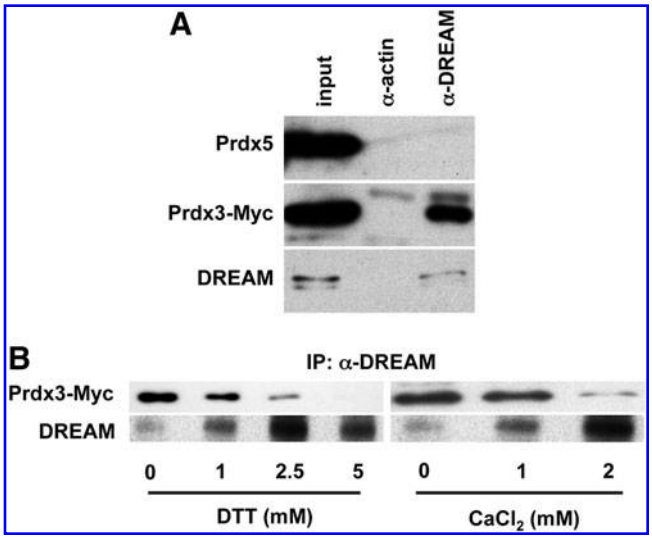

FIG. 1. DREAM specifically interacts with Prdx3. (A) Coimmunoprecipitation of endogenous DREAM in CHO cells transfected with Prdx3-Myc using monoclonal antibody 1B1 against DREAM. No coimmunoprecipitation was observed using a nonrelated antibody (anti-actin). No coimmunoprecipitation was observed with DREAM and endogenous Prdx5. Input corresponds to 5% of the total. **(B)** Coimmunoprecipitation of DREAM and Prdx3-Myc after incubation of total extracts with different concentrations of DTT or CaCl2 as indicated. Blots were developed using specific antibodies for DREAM, Myc and Prdx5.

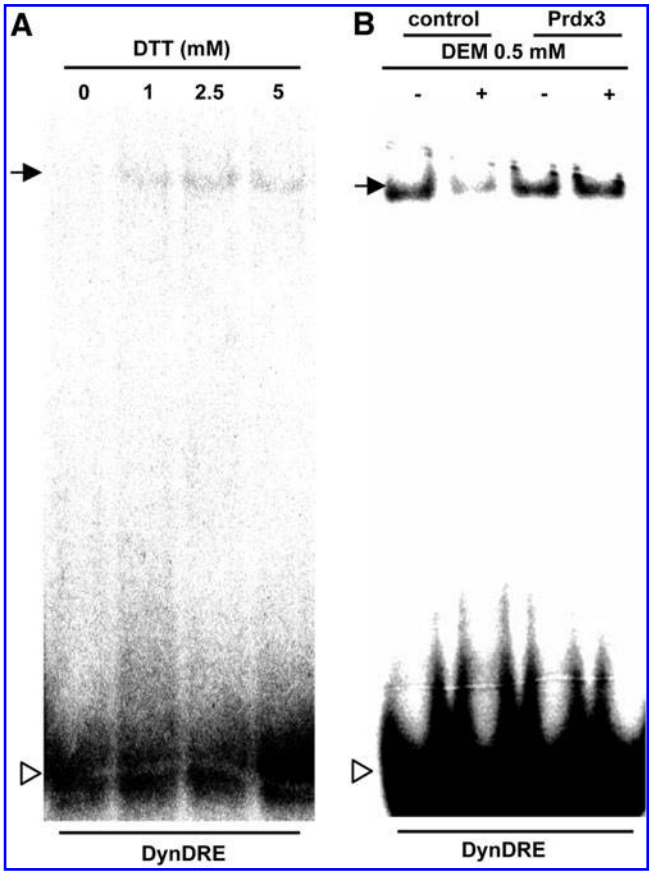

FIG. 2. Redox signaling modulates the DNA-binding ability of DREAM. (A) EMSA using purified DREAM protein, an oligonucleotide probe containing the DynDRE sequence and increasing concentrations of DTT, as indicated. **(B)** EMSA using nuclear extracts from PC12 cells transfected with Prdx3 or mock transfected cells and incubated in the presence or absence of DEM (diethylmaleate) as indicated. The *arrow* indicates the specific retarded band. *White arrowhead* indicates the migration of unbound probe.

that Prdx3 preferentially interacts with DREAM in oxidizing conditions (Fig. 1B).

Binding of DREAM to DRE sites is modulated by the redox state

Earlier *in vitro* studies demonstrated that redox controls the DNA-binding activity of a number of transcription factors such as c-jun (1) and CREB (10) via cysteine residues located at their DNA-binding domains. In both cases, DNA-binding activity was enhanced in reducing conditions. Similarly, incubation of recombinant DREAM in the presence of DTT enhanced its binding activity to the DRE site (Fig. 2A). The same increase in DNA-binding activity was observed in the presence of DTT using nuclear extracts from PC12 cells (data not shown). By contrast, binding of DREAM to the DRE site was reduced by the thiol-oxidizing agent diethyl maleate (DEM) (Fig. 2B), an effect that was not observed in nuclear extracts overexpressing Prdx3 (Fig. 2B). These results indicate that the DREAM-Prdx3 interaction influences the redox state of DREAM and that changes in the redox state of DREAM affect binding to DNA and suggest that redox signaling may control DREAM-dependent transcription.

Prdx3 modulates DREAM repressor activity

DREAM behaves as a transcriptional repressor through binding, as a homo- or hetero-oligomer with other KChIP proteins, to DRE sites in the promoter of its target genes. Upon stimulation with calcium or cAMP, DREAM is released from the DRE and transcriptional activation occurs via a derepression mechanism (6, 7). Thus, we investigated whether Prdx3 modulates transcriptional repression of the minimal promoter of prodynorphin, a "bona fide" DREAM target gene (8). Transient transfections in Cos7 cells showed that overexpression of Prdx3 reduced basal activity of the prodynorphin reporter up to 60% (Fig. 3A). In PC12 cells, induction of the prodynorphin reporter using forskolin alone, a PKA activator, or together with KCl, that stimulates calcium entry into the cells, was also partially blocked by Prdx3 (Fig. 3B). By contrast, Prdx3 did not change the induction of the prodynorphin reporter following activation of tyrosine-kinase receptors by NGF or EGF (Fig. 3B). Cotransfection experiments with Prdx5 did not modify basal or induced expression of the prodynorphin reporter (data not shown), further strengthen the specificity of the redox regulation of DRE-mediated transcription.

Two N-terminal Cys residues regulate DREAM redox state

Reduction of Cys residues prevents the formation of either intra- or intermolecular disulfide bonds and enhances DNA

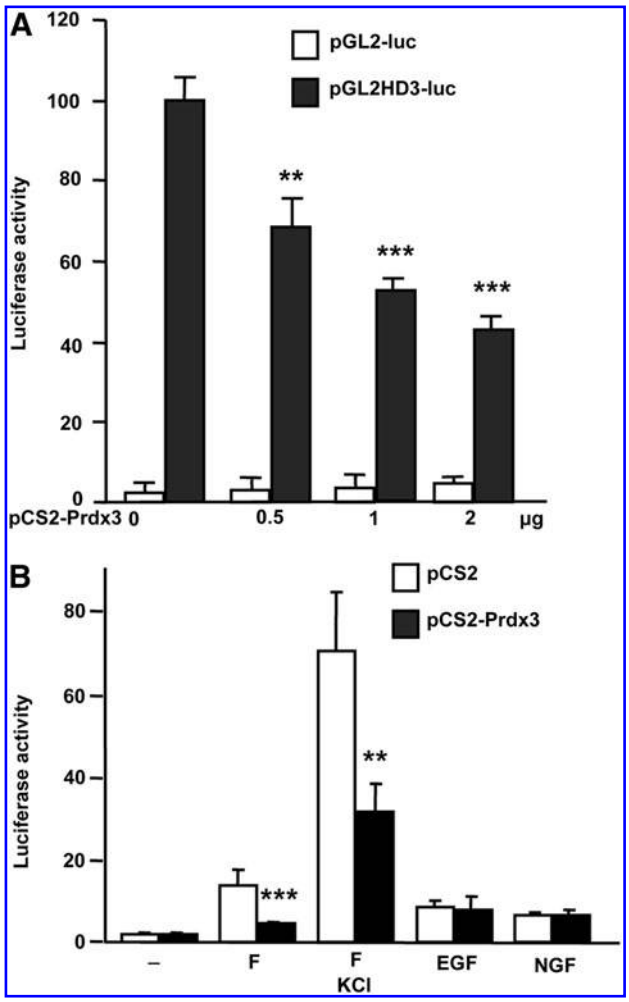

FIG. 3. Prdx3 enhances DRE-dependent transcriptional repression and blocks forskolin-induced derepression. (A) The repression of the basal activity of the DRE-containing pGL2HD3-luc reporter by increasing amounts of the Prdx3 expression vector (pCS2-Prdx3) in COS cells is shown. An empty reporter was included as control. **(B)** Derepression of the pGL2HD3-luc reporter in PC12 cells after forskolin (F, 1 μM) or forskolin plus KCl (65 mM) for 24 h, was blocked by pCS2-Prdx3. Prdx3 did not block transactivation by EGF or NGF (25 ng/ml, for 24 h). The empty pCS2 vector was included as control. Luciferase activity is shown as arbitrary units. In each case, the results are the mean ± SD of three experiments performed in triplicates. *Asterisks* represent statistical significance versus luciferase activity in the absence of Prdx3 $^{**}p < 0.01$ and $^{***}p < 0.001$.

binding activity of several transcription factors including c-Jun, CREB, or the glucocorticoid receptor (22). DREAM binds with higher affinity to DNA as a dimer or tetramer (23). Interestingly, DREAM contains two Cys residues at positions 45 and 46 within the first leucine charged residue-rich domain (LCD), a domain that is involved in protein–protein interactions (16) and is likely to contribute to DREAM oligomerization (unpublished observations). Therefore, we hypothesized that reduction of these Cys residues might prevent disulfide bond formation and enhance oligomer formation of DREAM. To test this hypothesis, we mutated cysteine residues to serine in mutant C45,46S-DREAM, a mutation that mimics the reduction of Cys residues (10). Western blot analysis using total extracts from CHO cells overexpressing DREAM, C45,46S-DREAM or S95A-DREAM as a control, showed that wild-type DREAM and the S95A-DREAM mutant migrated as monomers at approx. 30 kDa (6). C45,46S-DREAM, however, was migrating as two major bands at between 50 and 60 kDa (Fig. 4A). This shift in migratory pattern of C45,46S-DREAM indicates changes in the conformation of DREAM and reflects the formation of dimers resistant to dissociation by the reducing condition during PAGE.

Enhanced oligomer formation of the constitutively reduced C45,46S-DREAM mutant should result in increased DNA binding and transcriptional repression. We tested this in PC12 cells, a cell line with high level of endogenous DREAM (15), where further overexpression of wild-type DREAM does not modify forskolin-induced derepression of the prodynorphin

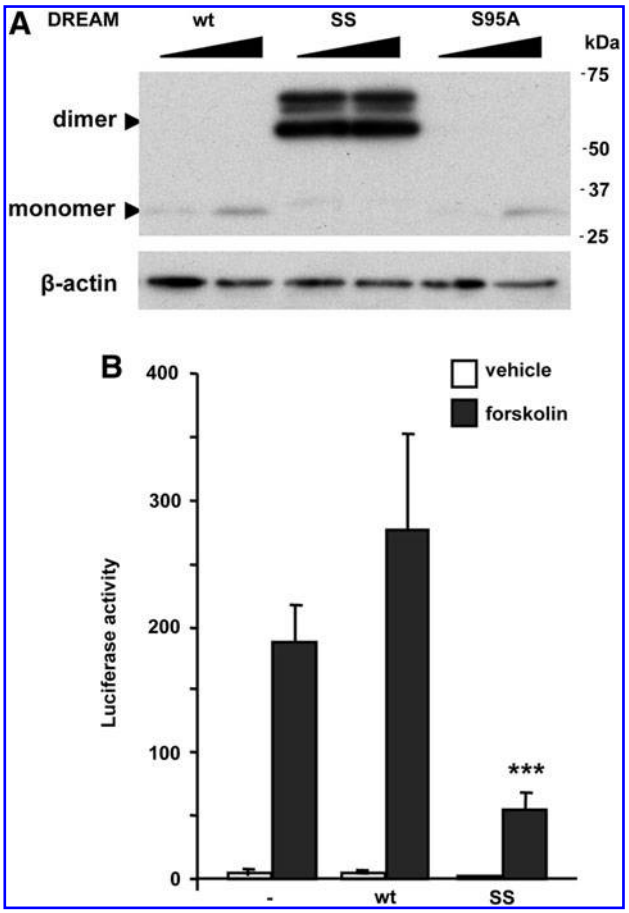

FIG. 4. The C45,46S-DREAM mutant forms dimers and blocks forskolin-mediated derepression. (A) Western blot analysis of CHO cells transfected with wild-type DREAM, C45,46S-DREAM (SS), or S95A-DREAM (S95A) mutants. Samples were resolved under reducing conditions by SDS-PAGE. **(B)** PC12 cells were transfected with wild-type DREAM, C45,46S-DREAM, or a control vector (pcDNA3), together with the pGL2HD3-luc reporter. Luciferase activity (arbitrary units) was measured after incubation of the cells with forskolin (1 μM) or vehicle. The results are the mean ± SD of three experiments performed in triplicates. *Asterisks* represent statistical significance versus cultures transfected with empty vector or wtDREAM $^{***}p < 0.001$.

promoter (Fig. 4B). However, overexpression of the C45,46S-DREAM mutant significantly blocked forskolin-induced transactivation (Fig. 4B). These results indicate that the redox state influences the transrepressor activity of DREAM.

Constitutively reduced C45,46S DREAM mutant prevents oxidative stress-mediated neuronal apoptosis

DREAM was reported to induce apoptosis through the interaction with presenilins (13, 20). More recently, it was shown that DREAM influence calcium homeostasis and neuronal viability through the regulation of NCX3 gene expression in cerebellar granules (9). Therefore, we next aimed to investigate the physiological consequence of the redox-modulation of DREAM on neuronal survival in response to oxidative stress.

For this, we exposed PC12 cells to H_2O_2, a model of oxidative stress widely used to study neurotoxicity and neuroprotection (12, 25, 38) and we monitored PARP cleavage as a marker of apoptosis. Exposure of PC12 cells to conditions of mild oxidative stress (10 and 30 μM H_2O_2, for 5 h) was not enough to induce detectable levels of apoptosis (Fig. 5). However, transient knockdown of endogenous DREAM with an antisense DREAM vector (15, 33) rendered PC12 cells more sensitive to the mild apoptotic stimulus showing increased levels of p85 PARP fragment (Fig. 5). This result indicates that endogenous DREAM plays a neuroprotective role in response to oxidative stress.

Discussion

In the present work, we have characterized the modulation of DREAM activity by redox signaling and its physiological consequences for neuronal survival. The interaction between DREAM and Prdx3, revealed in yeast two-hybrid screening, led us to disclose that redox signaling regulates DREAM repressor activity and that two cysteine residues in the N-terminal region of DREAM mediate this effect.

Prdx3 has been described as an exclusively mitochondrial protein, which is not located in the cytosol or in the nucleus in basal conditions. Since mitochondrial localization for DREAM has so far not been reported, it seems unlikely that in basal conditions endogenous Prdx3 mediates the redox regulation of DREAM. The short form of Prdx5, which is constitutively present in the cytosol, peroxisomes, and nucleus (4, 14, 34), does not interact with DREAM and seems neither to be responsible for redox regulation of DREAM *in vivo*. Thus, the subcellular compartment where the redox regulation of DREAM *in vivo* takes place and the Prdx subtype responsible are not yet characterized.

Binding to DRE sites and transcriptional activity of DREAM/KChIP proteins depends on homo- or hetero-oligomerization. Here, we show that dimer formation is greatly enhanced in mutant C45,46S-DREAM, a constitutively reduced form of DREAM, which correlates with enhanced repressor activity. Importantly, sequence analysis of the other KChIP proteins indicates the existence of a double cysteine in the equivalent position in KChIP2, isoforms a and b, while KChIP1 and KChIP4 have only one cysteine residue at the same position and none is present in the short KChIP2 isoform c. This indicates that redox signaling might as well regulate the repressor activity of all KChIP proteins and suggest that redox signaling might favor the formation of specific

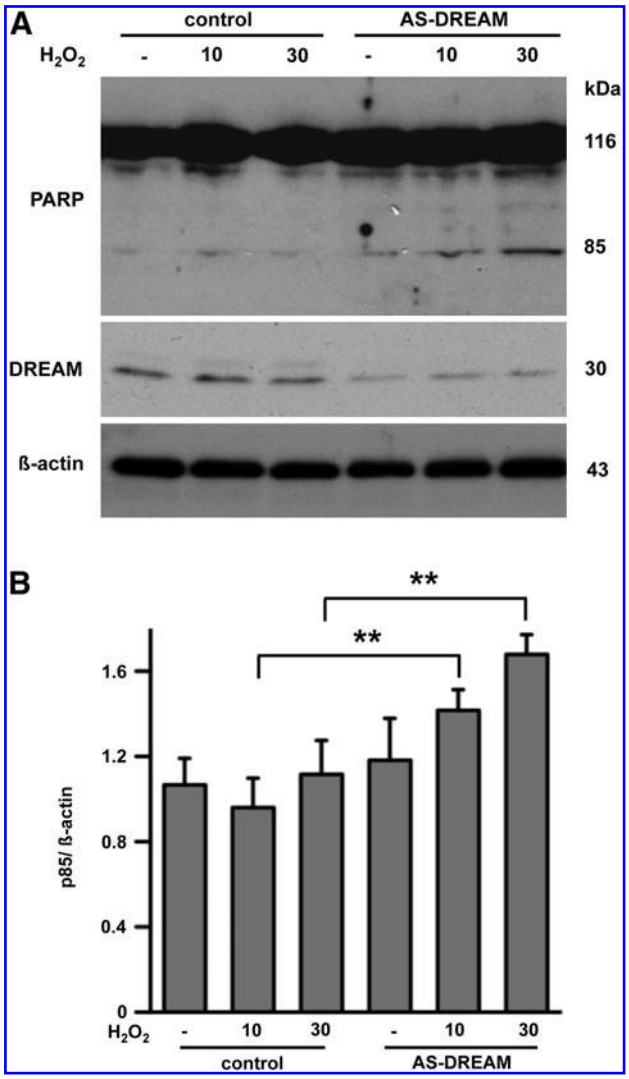

FIG. 5. DREAM knockdown sensitizes PC12 cells to H_2O_2-induced oxidative stress. (A) Western blot analysis of PARP fragmentation in PC12 cells transiently transfected with a DREAM antisense construct (AS-DREAM) or with a control vector (pcDNA3). Cells were exposed for 5 h to H_2O_2 (10 or 30 μM) or H_2O as control. Specific antibodies for DREAM, PARP (recognizing the intact 116 kDa protein and the cleaved p85 fragment), and β-actin were used. **(B)** The experiment was carried out three times and the specific band corresponding to the p85 cleaved PARP fragment was quantified, normalized by β-actin values; the results were plotted as arbitrary units. *Asterisks* represent statistical significance versus corresponding control $^*p < 0.05$ and $^{**}p < 0.01$.

DREAM/KChIP homo- and hetero-oligomers. Of note, exclusive expression of DREAM and KChIP2, without KChIP1 and 4, has been reported in the thyroid (29) and the immune system (33), while all four KChIP genes are expressed in brain and other organs (21).

Membrane expression of DREAM and KChIP2 depends on palmitoylation of the same cysteine residues (35) that regulate their redox state and transcriptional activity. Thus, two different and mutually incompatible post-translational modifications of DREAM and KChIP-2 determine their subcellular compartment and biological activity.

Mitochondria are the major source of cellular ROS, mostly due to aerobic respiration (24) and accumulation of H_2O_2 and the hydroxyl radical OH is elevated in mitochondria in neurodegenerative disorders (2, 11, 30). Also, it has been shown that during oxidative stress mitochondria release to the cytosol a number of factors that contribute to the cell death process. This includes initiator procaspase 9, Bax, and cytochrome c, as well as calcium that, by itself, activates several downstream mechanisms. In this scenario, it is attractive to speculate that Prdx3 is released from stressed mitochondria to the cytosol, where interaction with DREAM takes place, triggering a neuroprotective cascade to oppose the oxidative damage.

Sensitization to H_20_2-induced oxidative stress following transient knockdown of DREAM indicates that endogenous DREAM may elicit a neuroprotective function under oxidative stress conditions. Whether this effect is at the transcriptional levels and the nature of the putative DREAM target genes that could mediate this neuroprotective effect are presently unknown. DREAM is also involved in the endogenous response to pain (6, 8) and in learning and memory (2) through the regulation of target genes [*e.g.,* prodynorphin (6–8)]. Whether the regulation of DREAM activity by redox signaling is restricted to mechanisms that are related to the endogenous response to oxidative stress and neurodegeneration is currently unknown.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

APP = amyloid precursor protein
CREB = cAMP response element-binding protein
DEM = diethylmaleate
DRE = downstream regulatory element
DREAM = downstream regulatory element antagonist modulator
DTT = dithiothreitol
EGF = epidermal growth factor
EMSA = electrophoretic mobility shift assay
KChIP = potassium channel interacting protein
LCD = leucine-charged residue-rich domain
NGF = nerve growth factor
PARP = poly (ADP-ribose) polymerase
PKA = protein kinase A
Prdx3 = peroxiredoxin 3
PVDF = polyvinylidene fluoride
ROS = reactive oxygen species
SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis
Trx/TR = thioredoxin/thioredoxin-reductase
TSH = thyroid-stimulating hormone

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