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Original Contribution

Redox response of the endogenous calcineurin inhibitor Adapt78

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Abstract

Adapt78 (DSCR1/calcipressin/MCIP1) is a potent natural inhibitor of calcineurin, an important intracellular phosphatase that mediates many cellular responses to calcium. We previously reported two major cytosolic isoforms (1 and 4) of Adapt78, and that isoform 4 is an oxidative and calcium stress-response protein. Using a higher cell culture density and new antibody, we again observed that both major isoforms localized to the cytosol, but a significant level of isoform 4 (but not isoform 1) was also detected in the nucleus where it was present in the nonsoluble region and not associated with RNA. Exposure of cells to hydrogen peroxide led to the significant loss of isoform 4 from the nucleus with a moderate increase in cytosolic localization. The change in isoform 4 phosphorylation state in response to oxidative stress, characterized by a loss of the lesser (hypo) phosphorylated Adapt78, was not due to accelerated degradation, although general Adapt78 degradation was proteosome mediated. Finally, stimulation of Jurkat and primary T-lymphocyte signaling led to isoform 4 induction. This induction was BAPTA, diphenylene iodonium, and *N*-acetylcysteine inhibitable, and accompanied by induction of the classic immune response mediator and calcineurin-pathway-stimulated interleukin-2. These studies reveal new redox-related activities for Adapt78 isoform 4, which may contribute to its known calcineurin-regulating and cytoprotective activities, and further suggest that Adapt78 plays a role in basic T-cell response.

Keywords: Calcineurin; Redox; Oxidative stress; Hydrogen peroxide; Stress-response; Subcellular localization; T-cell response; Free radicals

Introduction

adapt78, also referred to as DSCR1/calcinpressin/MCIP1, was originally identified as an oxidative stress-inducible mRNA using hydrogen peroxide [1]. Subsequent studies determined that this mRNA is induced by multiple stresses, and is cytoprotective when overexpressed in hamster HA-1 cells [2–4]. It has subsequently been determined that Adapt78 protein inhibits calcineurin, an intracellular phosphatase that mediates many cellular responses to calcium [5–8]. Identification of this inhibitory activity has led to a surge of interest in Adapt78, since calcineurin is involved in many cellular and tissue functions, and its abnormal expression is associated with multiple pathologies [9–12]. Understanding the mechanism of Adapt78 action will therefore contribute to a basic understanding of calcineurin regulation and cellular stress response, and identify clinical targets for calcineurin- and stress-related pathologies.

Since *adapt78* mRNA was originally identified as a hydrogen peroxide-inducible mRNA, we considered the possibility that the translational protein product of *adapt78*, Adapt78, also responds to oxidative stress. Treatment of

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; 3/28, anti-CD3 plus anti-CD28; Cy, cytoplasm; NS, nuclear supernatant; NP, nuclear pellet; DPI, diphenylene iodonium; NAC, *N*-acetylcysteine; HP, hydrogen peroxide; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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HeLa and U251 human cells with several oxidants, including hydrogen peroxide, peroxynitrite, and menadione, all led to a shift in the phosphorylation status of two major isoforms, 1 and 4, from dual bands for each isoform, designated hypophosphorylated and hyperphosphorylated, to hyperphosphorylated following oxidant treatment [13,14]. These studies thus revealed that Adapt78 protein was also oxidative stress responsive. In addition, it was observed that isoform 4 (29-kDa form) exhibits a strong stabilization following oxidant exposure compared with isoform 1 (41-kDa Adapt78 form) [14]. p42/44 MAP kinase, known to be induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS), phosphorylates Adapt78 [15]. Combined, these observations suggest that ROS and RNS are important in Adapt78 regulation. Interestingly, calcineurin is also sensitive to oxidation [16–19] and nitrosation [19], and both superoxide and hydrogen peroxide inhibit its activity [16–18]. This suggests that Adapt78 and calcineurin represent a newly uncovered redox-sensitive couple whose activities depend, at least in part, on cellular redox state.

Here, we extend these previous studies to investigate the role of redox in Adapt78 regulation in more detail. These studies include the effect of oxidative state on subcellular localization and the mechanism behind our previously reported shift in the phosphorylation status of Adapt78 isoforms following oxidative stress. We also extend our previous observation that Adapt78 is induced by serum growth factors [13] by using the well-defined and physiologically relevant T-cell receptor-mediated signaling to assess Adapt78 response in T-lymphocytes. This is an ideal system to study Adapt78 and regulatory mechanisms, as calcineurin is very important in T-cell signaling [9,11,20,21], and ROS and calcium have been identified as important mediators of this response [22–25].

Materials and methods

Cell culture and treatment

HeLa epithelial-like, U251 astroglioma, and HT-1080 fibrosarcoma human cell lines were cultured in DMEM media containing 10% fetal bovine serum (FBS) and pen/strep (100 units/ml penicillin and 100 μg/ml streptomycin) in a humidified incubator atmosphere of 95% air and 5% CO_2 at 37°C. Jurkat human T-lymphocytes and primary lymphocytes were cultured in RPMI containing 10% FBS and pen/strep. For primary human T-lymphocyte isolation, peripheral blood was collected and T-lymphocytes were isolated using a combination of lymphocyte separation medium (Mediatech Inc., Herndon, VA) and negative selection with MHC Class II Dynabeads (Dynal Biotech, Brown Deer, WI) according to the manufacturers. Further enrichment was achieved using a glass bead column and lysing remaining red blood cells in the eluate. Cytotoxicity assays, using the monoclonal anti-T cell antibody OKT3 and rabbit complement, indicated that these preparations were greater than 99% pure T lymphocytes.

For cell treatments, monolayer cells were trypsinized, passaged, and exposed to the test reagents 2–3 days later. Suspension Jurkat cells were diluted 1:6 and then treated 2 days later. Primary human lymphocytes were collected, prepared, and treated the same day. For oxidant studies, the cells were divided into two groups, one receiving hydrogen peroxide (Sigma Chemical Company, St. Louis, MO) and the other solvent (phosphate-buffered saline) only (control). For studies involving the pretreatment of cells with MG132, cultures were removed from the incubator and MG132 (Sigma) added to the required concentration. Inhibitor solvent only (DMSO) was added to control cultures. All cultures were then returned to the incubator for 60 min prior to addition of peroxide.

Cell fractionation

For nuclear/cytoplasm separation, HeLa cells were washed twice with PBS and then pelleted by centrifugation. The cells were then resuspended in cold hypotonic buffer A (20 mM Tris, pH 7.4; 20 mM NaCl; 5 mM $MgCl_2$) for 15 min and homogenized, and the lysate was spun for 5 min at 600*g*. The supernatant was respun and that supernatant collected as the cytoplasmic fraction. The first-spin nuclear pellet was washed once with Buffer A and respun 5 min more, the supernatant discarded, and the pellet resuspended in buffer A containing broad specificity mammalian protease inhibitor cocktail (Sigma) plus 0.5% Nonidet P40 (NP40) to generate the nuclear lysate. This nuclear lysate was then centrifuged for 5 min at 1000*g* to generate the supernatant "NS" (nuclear soluble) fraction. The pellet was washed with Buffer A plus 0.5% NP40 and respun, the supernatant discarded, and the pellet ("NP" or nuclear pellet fraction) resuspended in Buffer A plus 0.5% NP40. The success of this nuclear/cytoplasm separation protocol and purity of fractions has been previously reported [14] using marker antibodies to the nucleus (PARP) and cytoplasm (α-tubulin).

Nuclear NP fraction subfractionation

The above NP fraction was divided into separate aliquots. A larger volume aliquot was extracted for 15 min on ice with 100 μl Buffer B (20 mM Tris, 7.4; 0.5 mM $MgCl_2$; 0.3 M sucrose) plus 100 mM NaCl and centrifuged 5 min at 1000*g*, and the supernatant collected for Western blot analysis. Extraction of the remaining pellet was then repeated, successively, with Buffer B plus 250 mM NaCl, then Buffer B plus 450 mM NaCl, and finally, RIPA buffer (PBS containing 1% NP40, 0.5% deoxycholate, and 0.1% SDS). After centrifugation to generate the supernatant RIPA extract, the final pellet was resuspended in 9:1 (v/v) Buffer B:RIPA.

For RNase treatment, equal volume nuclear lysate aliquots were treated with either Buffer B plus RNase A or Buffer B only (control) and then incubated 30 min at 37°