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ARTICLES

Mouse Translation Elongation Factor eEF1A-2 Interacts With Prdx-I to Protect Cells Against Apoptotic Death Induced by Oxidative Stress

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Abstract eEF1A-1 and eEF1A-2 are two isoforms of translation elongation factor eEF1A. In adult mammalian tissues, isoform eEF1A-1 is present in all tissues except neurons, cardiomyocytes, and myotubes, where its isoform, eEF1A-2, is the only form expressed. Both forms of eEF1A have been characterized to function in the protein elongation step of translation, and eEF1A-1 is shown to possess additional non-canonical roles in actin binding/bundling, microtubule bundling/severing, and cellular transformation processes. To study whether eEF1A-2 has similar non-canonical functions, we carried out a yeast two-hybrid screening using a full sequence of mouse eEF1A-2 as bait. A total of 78 hits, representing 23 proteins, were identified and validated to be true positives. We have focused on the protein with the highest frequency of hits, peroxiredoxin I (Prdx-I), for in-depth study of its functional implication for eEF1A-2. Here we show that Prdx-I coimmunoprecipitates with eEF1A-2 from extracts of both cultured cells and mouse tissues expressing this protein, but it does not do so with its isoform, eEF1A-1, even though the latter is abundantly present. We also report that an eEF1A-2 and Prdx-I double transfectant increases resistance to peroxide-induced cell death as high as 1 mM peroxide treatment, significantly higher than do single transfectants with either gene alone; this protection is correlated with reduced activation of caspases 3 and 8, and with increased expression of pro-survival factor Akt. Thus, our results suggest that eEF1A-2 interacts with Prdx-I to functionally provide cells with extraordinary resistance to oxidative stress-induced cell death. J. Cell. Biochem. 100: 267–278, 2007.

Key words: eEF1A-2; Prdx-I; yeast-two-hybrid; protein interaction; oxidative stress

Translation elongation factor eEF1A has two isoforms, eEF1A-1 and eEF1A-2, of which eEF1A-1 has been well characterized as playing a prominent role in protein synthesis: it binds and transports aminoacyl-tRNA to the A site of the ribosome in a GTP-dependent fashion, and as a result elongates the synthesizing polypeptide chain by one amino acid residue from each aa-tRNA translocation. Besides this role in peptide elongation, eEF1A-1 has been shown to bind and bundle actin filaments and to bind/sever microtubules [Shiina et al., 1994], and thus is important in maintaining the dynamic structure of cytoskeleton. eEF1A-1 has also been reported to function in embryogenesis [Krieg et al., 1989], cellular senescence [Shepherd et al., 1989], oncogenic transformation [Tatsuka et al., 1992], cell proliferation [Sanders et al., 1996], nucleocytoplasmic export/import [Gangwani et al., 1998], and oxidative defense [Chen et al., 2000].

eEF1A-2, first isolated from rat, is a isoform gene of eEF1A-1. It shows high homology in its cDNA and amino acid sequences to eEF1A-1, but is distinct from eEF1A-1 in its intronic and 3′ untranslated regions [Ann et al., 1992]. eEF1A-2 is expressed only in brain, heart and muscle, specifically the neurons, cardiomyocytes, and myotubes of these tissues, respectively; the eEF1A-2:eEF1A-1 ratio increases during development [Lee et al., 1992]. Using specific antibodies against either eEF1A-1 or eEF1A-2, we showed that whereas eEF1A-1 decreases in brain, heart and skeletal muscle

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during development, eEF1A-2 increases concomitantly [Khalyfa et al., 2001], indicating that the two isoforms of eEF1A are alternate forms that function at different developmental stages; eEF1A-1 is the embryonic, whereas its isoform eEF1A-2 is the adult, form. Spontaneous deletion of eEF1A-2-containing genomic segments in wasted mice, resulting in a failure to initiate gene expression at postnatal day 21, when it is predicted to replace the embryonic form eEF1A-1, may be responsible for the lethal phenotype at this developmental stage [Chambers et al., 1998]. Beyond the reported restriction of expression to brain, heart and muscle, eEF1A-2 has also been shown to be highly expressed in primary ovarian tumors, and to render NIH 3T3 fibroblasts tumorigenic [Anand et al., 2002].

Besides a report on its interaction with the M4 muscarinic acetylcholine receptor (mAChR) [McClatchy et al., 2002], a guanine exchange factor important in the recruitment of aminoacylated tRNA to the ribosome, few biochemical partners have been documented for eEF1A-2. In this context, we used the yeast two-hybrid approach to identify in vivo interacting partners of eEF1A-2. The screening resulted in panoply of interacting proteins, functioning in cytoskeleton organization, vesicular transport, transcription/translation regulation, metabolism, protein maturation, etc.

Of all the eEF1A-2 partners, peroxiredoxin I (Prdx-I) had the most hits, 10, in the yeast two-hybrid experiments. This interaction was further validated by co-immunoprecipitation assays; our results showed that Prdx-I indeed interacts with eEF1A-2, but not with eEF1A-1. Thus, we pursued further to investigate the functional impact of this interaction. Prdx-I is a typical 2-Cys peroxiredoxin, ubiquitously expressed in all mammalian cells [Wood et al., 2003]; it functionally protects cells from oxidative stress by reducing a range of reactive oxygen species (ROS) through its redox-active cysteine residue to yield a sulfenic acid derivative, which is further reduced by thioredoxin to regenerate active peroxiredoxins [Hofmann et al., 2002; Wood et al., 2003]. Our previous results have shown that over-expression of eEF1A-2 also protects cells from oxidative stress [Chen et al., 2000]. Although individually each of these two proteins shows functional protection from oxidative stress, no functional studies yet have shown whether the partnership between Prdx-I and eEF1A-2 can further magnify the oxidative resistance. Here, we show that the two proteins act together to increase several fold the protection from oxidative stress-induced death, and that this protection is correlated with suppression of cleavage of caspases 3 and 8, and elevated expression of Akt, a key pro-survival factor in protecting cells against apoptotic death. In all, our results lead us to suggest that the partnership of eEF1A-2 with Prdx-I may provide cells a fail-proof resistance against apoptotic death, a physiological state that must be prevented in long-lived cell types such as neurons, myotubes and cardiomyocytes.

MATERIALS AND METHODS

Yeast Two-Hybrid Assay

An eEF1A-2 cDNA sequence was released from a pCMV-SPORT6 IMAGE clone (Invitrogen) by digestion with EcoRI/NotI, and subcloned into an EcoRI/PstI digested pGBKT7 vector (BD Clontech). A fused ORF encoding the GAL4 DNA binding domain and the full-length eEF1A-2 ORF was verified by sequencing, ORF confirmation and conserved domain searches using relevant NCBI programs at http://www.ncbi.nlm.nih.gov/. The GAL4-eEF1A-2 bait construct was used to screen a Matchmaker pretransformed 9–12 week-old mouse brain cDNA library (BD Clontech) using the Matchmaker Yeast Two Hybrid 3 kit (BD Clontech), essentially according to the manufacturer's instructions. A medium-stringency screening was carried out using SD/-His/-Leu/-Trp selection medium, with 5 mM of 3-AT (3-amino-1,2,4-triazole) to prevent false positives from leaky expression of His. Candidate colonies were subsequently streaked onto low-stringency, and then re-streaked onto high-stringency, medium plates, both with X-α-Gal. Plasmid was isolated from the high stringency plates using a lyticase approach, according to the two-hybrid manual. The resulting plasmid was transformed into bacterial JM109 (Promega) competent cells. A single plasmid clone from each original positive colony was partially sequenced at the University of Tennessee Health Sciences Center.

PCR, In Vitro Transcription/Translation, and Co-Immunoprecipitation (Co-IP)

The pre-transformed library was constructed in pACT2 lacking a T7 promoter; therefore,

to generate translation products from these interacting library clones, we first introduced a T7 promoter sequence to the 5′ region of these clones. Primers were designed by modifying those recommended in the Co-IP kit manual to accommodate sequence differences in pACT2 (ADT7HA, 5′aaaattgtaatacgactcactatagggcgagccgccaccatgtacccatacgatgttccagattacgct; ADCoIPR, 5′acttgcggggtttttcagtatctacgat). PCRs were carried out with the ADT7HA primer and a reverse primer ADCoIPR, with Dynazyme DNA polymerase (M J Research, Bio-Rad), with cycling conditions essentially according to those suggested in the Co-IP manual. Interacting clones were transcribed/translated in vitro from PCR products, and eEF1A-2 from a pGBKT7 bait construct already containing a T7 promoter and c-myc epitope fused to eEF1A-2, in the presence of ^{35}S-methionine, using the TnT T7 Coupled Reticulocyte Lysate system (Promega). Briefly, translated eEF1A-2 protein was incubated alone or with each of the interacting target proteins for 2 h on ice; c-myc antibody against the epitope fused to eEF1A-2 was added and incubated for another hour at room temperature; Protein A beads (Santa Cruz Biotech) were then added to the reactions and incubated with shaking for 1 h at room temperature. The beads carrying the protein complexes were washed a total of 4 times with 500 μl of phosphate-buffered saline (PBS) each wash, followed by SDS–PAGE to visualize the interactions. Antibodies were provided with the Co-IP kit, against either the c-myc epitope on the pGBKT7 bait vector, or the HA epitope on the PCR products.

SDS–PAGE, Protein, and Western Analyses

In vitro transcription/translation and co-immunoprecipitation products were separated by SDS–PAGE on 12% polyacrylamide by standard techniques [Sambrook et al., 1989]. Upon finishing, gels were fixed, enhanced with Amplify Fluorographic Reagent (Amersham), soaked in 7% methanol, 7% Tris and 3% glycerol, and dried under vacuum to completion followed by autoradiography. Protein primary structure analysis was performed using the ProtParam software at http://us.expasy.org. For Western analysis, 50 μg of protein extracts were loaded in each lane. Antibodies against eEF1A-1 and eEF1A-2 are previously described [Khalyfa et al., 2001]. Other antibodies were purchased from Santa Cruz Biotechnology (anti-Pag for Prdx-I, Akt, Casp 3, and Casp 8). For protein extraction, cells were washed twice in 3 ml cold PBS, scraped in another 1.5 ml cold PBS, centrifuged, and then lysed with triple-detergent lysis buffer as described [Sambrook et al., 1989]. RIPA buffer [Sambrook et al., 1989] was used for mouse tissue protein extractions.

Cell Culture, H_2O_2 Treatment, and Cell Viability and Growth Assays

Mouse cell line NIH 3T3 fibroblasts were cultured in 10% serum MEM (Invitrogen) at 37°C in a 5% CO_2 incubator. For H_2O_2 treatment, cells were grown to 80–90% confluence, and then starved in 0.05% serum medium for 48 h to synchronize cell cycle progression; appropriate amounts of pre-diluted H_2O_2 in starvation medium were directly added to the plates without changing medium, followed by culturing for another 24 h. Both supernatant and adherent cells were collected and resuspended in 1 ml PBS. Equal volumes of the resuspended cells and 0.4% Trypan Blue (Sigma) were mixed for dye exclusion assays. Stained (dead) and non-stained (viable) cells were counted with a hemacytometer by photomicroscopy.

For MTT assay [Mosmann, 1983], cells were seeded in 24-well plates in triplicates, and treated as described above. After H_2O_2 treatment, 15 μl of MTT (methylthiazoletetrazolium, Sigma, 5 mg/ml) was directly added to each well containing 450 μl starvation medium. After 4 h incubation at 37°C, medium was removed and 200 μl DMSO was added. After shaking for 5 min at room temperature, the whole plates were scanned on a regular scanner, and the contents were transferred to a 96-well plate to obtain readings at 544 nm wavelength on a FluoStar plate reader (BMG Labtech GmbH). Viable cells take up the MTT dye, resulting in purple color, whereas dead cells do not.

Transfection and Stable Cell Line Selection

Constructs harboring either eEF1A-2 or Prdx-I were constructed in expression vector pcDNA3.1$^+$ neo (Invitrogen). DNA constructs were delivered with the Polyfect reagent (Qiagen), according to the manufacturer's guidelines. After 24 h of transfection, cells were passed to regular medium containing 400 μg/ml of G418. Cells were continuously passed until resistant colonies were formed (about 2 weeks). The colonies were then lumped and kept in the same selection medium.

RESULTS

To functionally characterize eEF1A-2, we screened a mouse brain cDNA library using a GAL4-eEF1A-2 fusion bait construct in a yeast two-hybrid assay. We screened approximately 3×10^6 diploid colonies using an intermediate-stringency selection medium, in the presence of 3-AT; a total of 181 positive colonies were obtained. One hundred twenty-eight well-grown blue colonies were obtained from re-streaked colonies. After transformation into bacteria and plasmid sequencing, 101 of these yielded high homology (close to 100%) to either cDNAs or known genes in the database. A total of 37 interacting proteins and 8 cDNAs were identified from the 101 sequenced clones. All single-hit clones were retested by co-transformation into yeast strain AH109 and subsequent selection on high-stringency plates. Clones that did not show robust blue colonies in the retest were eliminated, along with the 8 cDNA clones. Altogether we identified 23 interacting proteins relating to cytoskeleton, vesicular transport/signaling, transcription/translation regulation, etc. (Table I).

TABLE I. Interacting Partners of eEF1A-2 Identified in Yeast Two-Hybrid Assay by Functional Groupings

Protein	Number of clones
Cytoskeleton	
ABP280	1
Fascin	2
Keap1	1
mRif	6
RanBPM	3
Vesicular transport/signaling	
ZNF259	3
Contactin 2	5
Copine V	2
Death receptor 6	3
$GABA_A$ receptor $\alpha 4$	6
Klc1	3
LC3	4
RanBPM	3
ZPR1	4
Prdx-I	10
Transcription/translation	
E25	3
EfaPK	7
Keap1	1
RNA helicase	3
ZNF140	1
Metabolism	
COX6B	4
Enolase 2	2
PGAM-B	1
Protein maturation	
Ggm2	3
SDF2	1
Total positive clones	78

The number of clones shows independent isolations representing the same protein. Gene names and relevant references: ABP280 [Actin binding protein 280, Cunningham et al., 1992]; Fascin [Otto et al., 1980]; Keap1 [Itoh et al., 1999]; mRIF [mouse Rho in *Filopodia*, Ellis and Mellor, 2000]; RanBPM [Nakamura et al., 1998]; ZNF259 [El-Hussein and Vincent, 1999]; Contactin 2 [CNTN2, Lee et al., 2000]; Copine V [Creutz et al., 1998]; Death Receptor 6 [Pan et al., 1998]; $GABA_A$ receptor $\alpha 4$ [Mu et al., 2002]; Klc1 [Kenesin light chain 1, Rahman et al., 1998]; LC3 [MAP1-LC3, Kabeya et al., 2000]; ZPR1[Gangwani et al., 1998]; Prdx-I [Ishii et al., 1993]; E25 [Deleersnijder et al., 1996]; EfaPK (Named here for eEF1A protein kinase, GenBank accession no. BC027296); RNA helicase (putative, GenBank accession no. Q9UNV9); ZNF140 [Margolin et al., 1994]; COX6B (GenBank accession no. BC024343); Enolase 2 (GenBank accession no. BC031739); PGAM-B [Sakoda et al., 1988]; Ggm2 [Sango et al., 1995]; SDF2 [Hamada et al., 1996].

Co-Immunoprecipitation

We performed co-immunoprecipitation assays to verify the results obtained by the two-hybrid screening. Interacting clones, as well as the eEF1A-2 bait clone, were in vitro transcribed/translated in the presence of ^{35}S-methionine. The translated proteins were then incubated individually with the translated eEF1A-2 protein, and an antibody against the c-myc epitope on the eEF1A-2 construct was then added to the reactions to pull down the interacting proteins. Validation for two interacting proteins (SDF2 and Prdx-I) is shown in Figure 1. The SDF2 protein was co-immunoprecipitated with eEF1A-2 polypeptide by c-myc antibody (lane 3), indicating that these proteins interact directly, whereas SDF2 was not identified in the anti-myc immunoprecipitates when eEF1A-2 was absent (lane 4). Similarly, Prdx-I co-sediments with eEF1A-2 in the presence of c-myc antibody (lane 6), whereas Prdx-I alone did not react to the same antibody (lane 7).

eEF1A-2 Interacts With Prdx-I in Tissues Where eEF1A-2 is Present

Among all the candidates identified by the yeast 2-hybrid screening, Prdx-I had the highest number (10) of hits. Therefore, we chose to focus our study on this particular interaction. We performed immunoprecipitation assays using eEF1A-2-positive (brain, muscle) and -negative (liver) mouse tissues. Protein extracts of the above tissues were processed for pull-down experiments with a polyclonal eEF1A-2 specific antibody. The protein complex pulled down by eEF1A-2 antibody was analyzed by Western blotting and probed with antibody to peroxiredoxin I (Pag). As shown in Figure 2, panel A1, Prdx-I was identified in the immunoprecipitated complex from brain (lane B) and muscle (lane M), but not from liver (lane L), tissue extracts. Moreover, the amount of Prdx-I

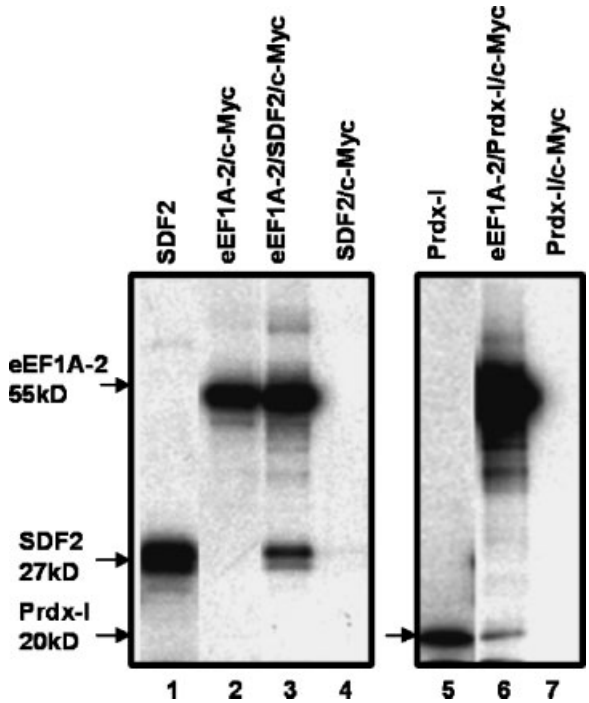

Fig. 1. Co-immunoprecipitation assays. In vitro translated products are shown for SDF2 (**lane 1**), Prdx-I (**lane 5**) and eEF1A-2 (**lane 2**). Arrows indicate proteins interacting with eEF1A-2. Whereas lanes 1 and 5 show in vitro translated proteins, bands at corresponding positions of **lanes 3** and **6** show the same proteins in complex with eEF1A-2.

co-precipitated with eEF1A-2 was proportional to the levels of endogenous Prdx-I in brain and muscle extracts, as shown in lanes B and M (panel A1, Fig. 2). Interestingly, even though an ample amount of Prdx-I was present in the liver extract, we could not identify any trace of Prdx-I in the immunoprecipitated protein complex from liver, where eEF1A-2 is not expressed. This result further confirmed that the presence of Prdx-I in immunoprecipitates was due to a specific interaction between this protein and eEF1A-2.

The high sequence homology between eEF1A-1 and eEF1A-2 suggest the possibility that both isoforms might react with Prdx-I. We thus performed pull-down experiments similar to those described above, using a peptide-derived polyclonal antibody specific to eEF1A-1. Interestingly, as shown in Figure 2 panel B, little or an undetectable amount of Prdx-I is identified by Western blotting in the eEF1A-1 pull-down precipitates of liver extract, as indicated by the arrow in lane L. This absence is also true with buffer or eEF1A-1-negative brain (lane B) or muscle (lane M) tissue extracts. eEF1A-1 is a major protein in liver [Fig. 2 panel B; Khalyfa et al., 2001], and as shown in Panel A, Prdx-I is present in ample quantity in this tissue

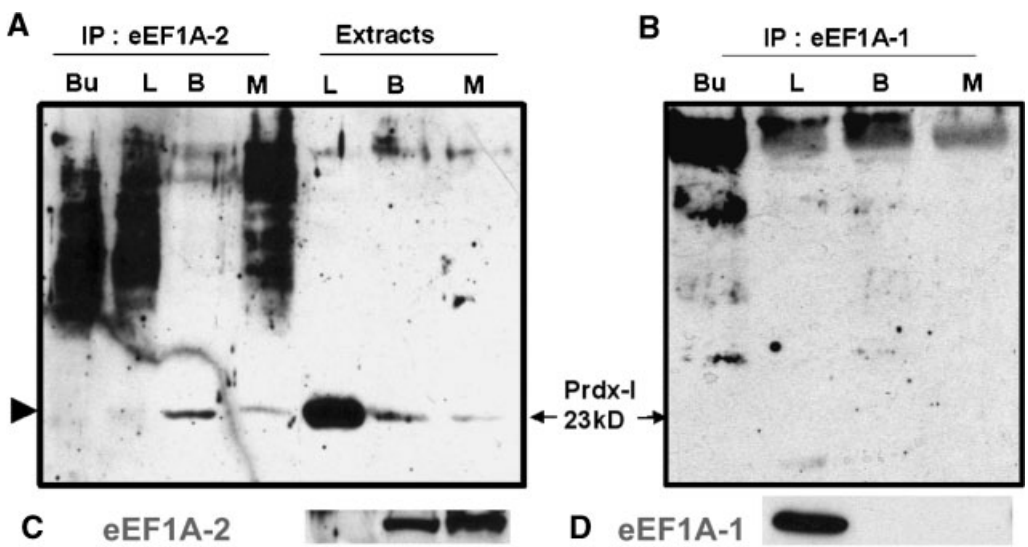

Fig. 2. Co-immunoprecipitation of Prdx I with eEF1A-2 from tissues. **Panel A** shows that Prdx-I (arrow) is co-precipitated with eEF1A-2 from muscle (M) and brain (B), but not from liver (L) or buffer alone (Bu) in lanes depicted by IP. The amounts of Prdx-I pulled down correspond to the levels of abundance of endogenous Prdx-I expression in the panel depicted as extracts for brain (B) and muscle (M) tissues. Note that Prdx-I is not pulled down from liver, where eEF1A-2 is not expressed, even though liver has abundant presence of Prdx-I (extract L). **Panel B** shows the absence of Prdx-I in immunoprecipitated product when antibody to eEF1A-1 is incubated with the same tissues, liver (L), brain (B) and muscle (M). **Panels C** and **D** show expression levels of eEF1A-2 and eEF1A-1, respectively, in these three tissues.

(Panel A, Fig. 2); should there be any physiological interaction between this protein and eEF1A-1 in liver, we would have been able to coprecipitate the two proteins as a complex, as we did for eEF1A-2 in brain and muscle extracts. Thus, this result suggests that Prdx-I may react with eEF1A-2 only, but not with eEF1A-1, or the interaction might be too weak to be detected by our current pull-down method. This suggestion was further supported by our finding that in the yeast two-hybrid screening with eEF1A-2, Prdx-I had the most hits (10/78), whereas a similar screening with eEF1A-1 yielded only 1 out of 17 hits for Prdx-I (Y. Liu and EW, unpublished data).

Functional Impact of eEF1A-2 and Prdx-I Interaction on Cells

To elucidate the biological significance of the interaction between eEF1A-2 and Prdx-I, we expressed eEF1A-2 in mouse NIH 3T3 fibroblast cells that normally lack this protein. Full-length cDNAs of Prdx-I and eEF1A-2 were subcloned into a mammalian expression vector pcDNA3.1^{+}neo (Invitrogen). NIH 3T3 cells were transfected with these constructs, alone or together, as well as with pcDNA3.1 vector alone as control; stable lines were established by neomycin selection. We chose stable transfection since pilot experiments with transient transfection did not show significant differences among these transfectants, likely because the transgene expression time was too short to exhibit a physiological impact. In contrast, with stable transfectants (4 stable lines: Prdx-I, eEF1A-2, Prdx-I/eEF1A-2, and vector alone), as shown in Figure 3 Panel A, cultures doubly transfected with Prdx-I and eEF1A-2 exhibit readily detectable differences in monolayer cell culture topography; specifically, individual cells show flattened and enlarged cell morphology (Fig. 3 upper panel). Stable transfectants with eEF1A-2 or control vector alone show very little difference from the original NIH 3T3 cells (data not shown), while those with Prdx-I alone exhibit morphology similar to that observed in the double transfectants.

Since Prdx-I is known to function in oxidative defense, and our previous work with eEF1A-2 showed that this elongation factor might exhibit non-canonical functions in protecting cells against apoptotic death, we hypothesized that interaction between eEF1A-2 and Prdx-I might function to elevate this protection against oxidative insults. To test this hypothesis, we subjected the four stably transfected cell cultures to H_2O_2 treatment. As shown in Figure 3 Panel AII, Prdx-I and eEF1A-2 double transfectants were able to resist treatment with 200 μM H_2O_2, allowing the majority of cells to maintain their viable, non-refractile cell morphology. Cultures transfected with either Prdx-I or eEF1A-2 alone had partial protection to peroxide insults, whereas virtually all cells transfected with control vector alone showed typical dying refractile morphology, as did non-transfected 3T3 cells (not shown).

Resistance to oxidative insults of these stable lines was quantified by Trypan blue exclusion assays, in which live cells exclude the dye color whereas dead cells stain positive. As shown in panel B of Figure 3, single transgenes, Prdx-I, and eEF1A-2, provide 50 and 40% survivability, respectively, while transfectant with double transgenes showed survivability as high as 80% with 200 μM H_2O_2. Transgene expressions in all three stable transfectant cultures were verified by immunoblotting with antibodies either to Prdx-I (panel C, Fig. 3), or to eEF1A-2 (Panel D, Fig. 3), using actin as loading control.

The above results showed that an eEF1A-2/Prdx-I double transfectant indeed confers resistance to NIH 3T3 cells against oxidative stress, and the conferred resistance is correlated to over-expression of these proteins. We confirmed the above findings further with MTT assays, in which all growing cells should stain blue, whereas dead cells show no dye uptake. We seeded cells in 24-well plates and cultured them close to confluence, then changed to low-serum (0.05%) medium and maintained them in this condition for 72 h to synchronize the cell division progression, and subjected the cells to a full range of peroxide treatment, from 0 to 1,000 μM.

Panel A of Figure 4 shows one of three 24-well culture plates. As expected, the vector transfectant showed virtually no viability at 0 μM after 72 h of 0.05%-serum culturing condition. Single transfectants of Prdx-I and eEF1A-2 showed significant degrees of survivability under this low serum condition, with the Prdx-I-transgene stable transfectant showing slightly higher resistance than the eEF1A-2-transgene stable transfectant alone. The stable transfectant expressing both transgenes showed the greatest protection against low-serum culture conditions; this protection persists after adding

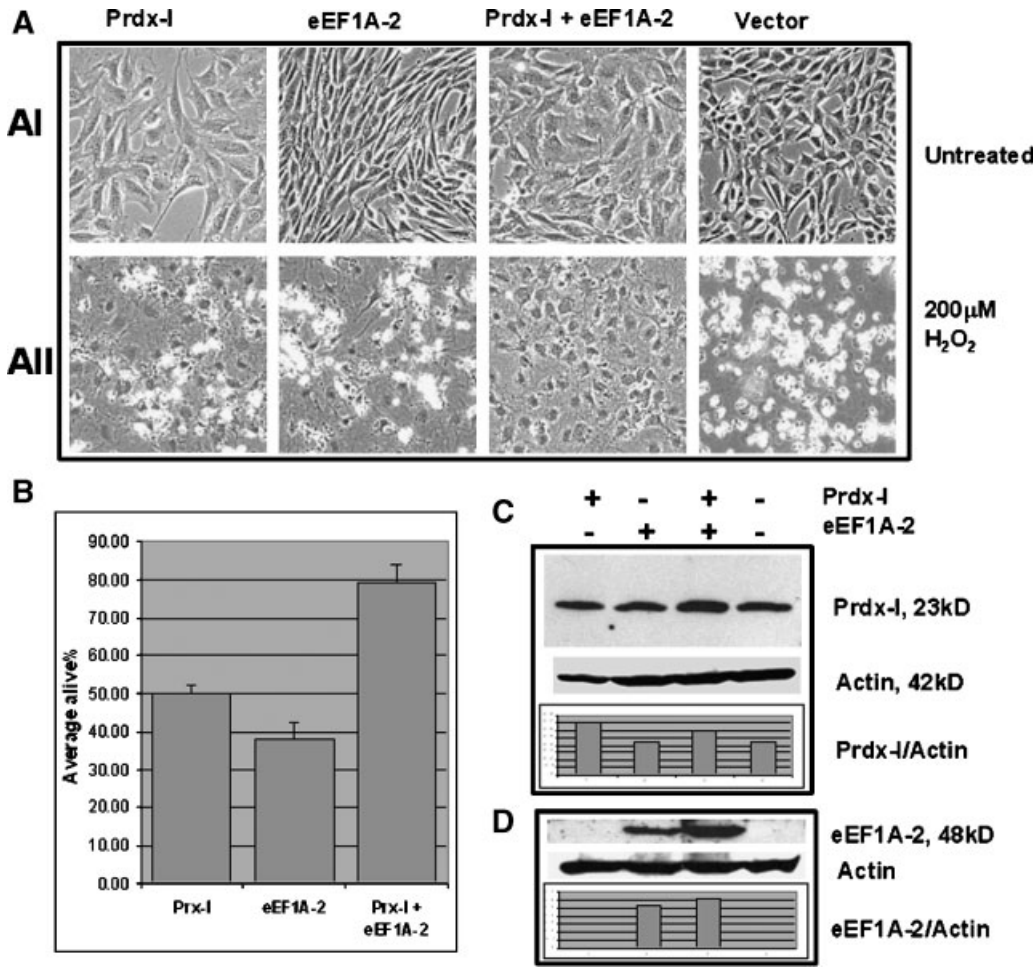

Fig. 3. Morphology and viability of four stable transfection lines after 24 h H_2O_2 treatment. **Panel A 1** shows the morphology of stable cell lines without any treatment, whereas **A II** shows morphology after 200 μM H_2O_2 treatment. **Panel B** shows cell viability counts by Trypan-blue exclusion assays, with percent survival derived from the average of three replicates. **Panel C** shows expression of Prdx-I transgene by Western blot, normalized to actin; the presence of endogenous Prdx-I is shown in the last lane, and the vector-alone transfectant is used as control. **Panel D** shows expression of eEF1A-2. Fibroblast cells do not express this protein in the absence of the eEF1A-2 transgene.

200 μM peroxide to the culture. The differential resistance among the three stable transfectants becomes evident as the concentration of H_2O_2 increases, with the Prdx-I transfectants continuing to survive to some extent at 400 μM. Remarkably, stable transfectants expressing both Prdx-I and eEF1A-2 show survivability to treatment as high as 1,000 μM.

Figure 4 panel B shows the quantification of the MTT assays for the four stable transfectant cultures. Indeed, the stable transfectant expressing both genes shows the highest ratio of MTT uptake, with decreasing values as the concentration of peroxide increases. As expected, the Prdx-I stable transfectant showed slightly better MTT uptake than the eEF1A-2 transfectant; 0% survivability for the former occurred at 400 μM, and for the latter at 200 μM, whereas 0% survivability was observed at 0 μM for vector alone. Taken together, these experiments strongly suggest roles for both eEF1A-2 and Prdx-I in protecting cells against oxidative stress, as well as their additive effect of heightened protection against extremely high concentrations of peroxide insults.

Changes in Apoptosis-Related Factors

We further investigated whether the resistance to peroxide-induced cell death is due to a signaling mechanism controlling key modulators vital to this cellular process. We characterized three proteins, caspases 3 and 8 and Akt, changes in which are well known to reflect apoptotic signaling. For these experiments,

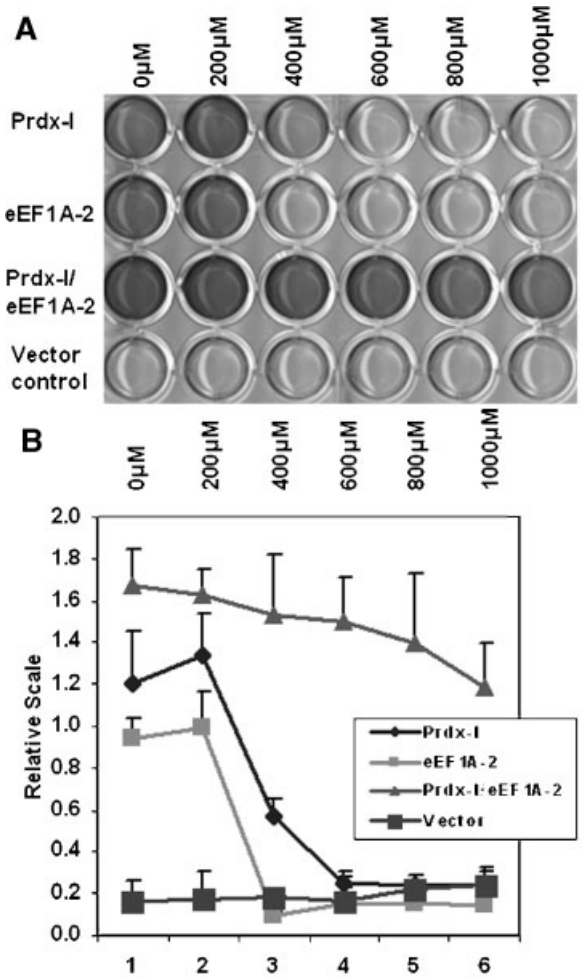

Fig. 4. Co-expression of Prdx-I and eEF1A-2 confers resistance to peroxide-induced apoptotic death additively. **Panel A** shows one plate of MTT staining for viability determination in three separate 24-well culture plates, with stable cell lines expressing Prdx-I, eEF1A-2, both transgenes together, and vector alone. **Panel B** shows quantitative analysis of the MTT assays for three independent plates.

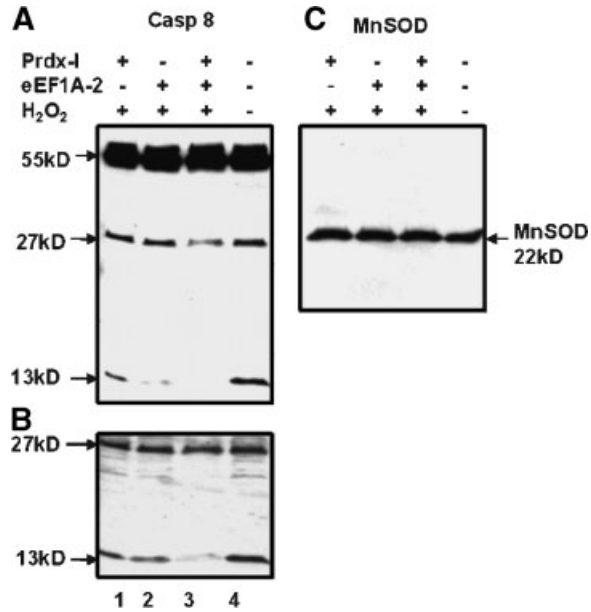

Fig. 5. Inhibition of Caspase-8 activation in stable transfectant expressing both eEF1A-2 and Prdx-I. **Panel A** shows that transfectant expressing both transgenes exhibits a diminished level of cleaved caspase 8 product, the polypeptide of 12–13 kDa (arrow), low levels in either single transfectant, and a high level in transfectant expressing vector alone. **Panel B** shows the same blot with longer exposure. **Panel C** shows the expression of MnSOD as an independent event of caspase-8 activation, and as loading control.

cells were first maintained for 48 h in low-serum (0.05%) culture conditions, and then treated with 200 μM H_2O_2 for another 24 h. Proteins were then extracted from these treated cells. With this manipulation, all three cultures of stable transfectants expressing either Prdx-I or eEF1A-2, alone or together, showed significant survivability, while no viable cells were detected in the control stable transfectant carrying vector alone. Therefore, protein for vector-alone transfectant was extracted at 48 h after low-serum culturing.

As shown in Figure 5, caspase 8 exhibits dramatic changes in activation levels, inversely correlated with resistance levels to H_2O_2 treatment. Panel A shows that stable transfectant carrying vector alone shows the highest amount of cleaved products (lane 4), while single transfectants with either Prdx-I or eEF1A-2 show lower amounts of cleaved products (lanes 1, 2). In contrast, eEF1A-2 and Prdx-I double transfectants (lane 3) exhibit little or no caspase 8 activation, as evidenced by the absence of the cleaved products at 27 and 12 kDa; the cleaved products were barely detectable even with extended exposure, as shown in Panel B. The selective absence of cleaved products was further verified by probing the same blot with an antibody to MnSOD, as a control for protein loading as well as for possible non-specific immuno-interactions. As shown in Panel C, MnSOD levels did not change significantly among the four stable lines, indicating that the absence or reduced amounts of caspase 8 cleaved products in the three stable transfectants indeed reflects a lack of endogenous apoptosis-associated activity. A similar trend of reduced cleaved 17 kDa product is observed for caspase 3 activation in stable transfectants expressing either Prdx-I or eEF1A-2 alone, and

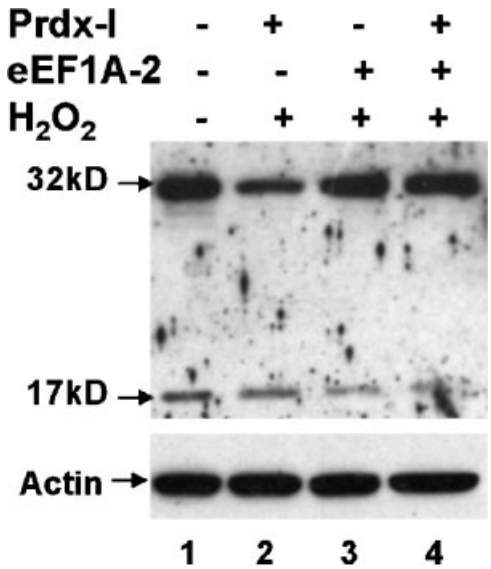

Fig. 6. Inhibition of Caspase-3 activation in stable transfectant expressing both eEF1A-2 and Prdx-I. The upper panel shows a Western blot probed with caspase 3 antibody, showing that procaspase 3 (31 kDa) is present in all four stable transfectant protein extracts, and that the cleaved active form (17 kDa) is present most abundantly in the transfectant expressing vector alone, decreased levels when expressing either Prdx-I or eEF1A-2 alone, and only a very faint band when expressing both transgenes. The lower panel shows the same blot probed with antibody against actin.

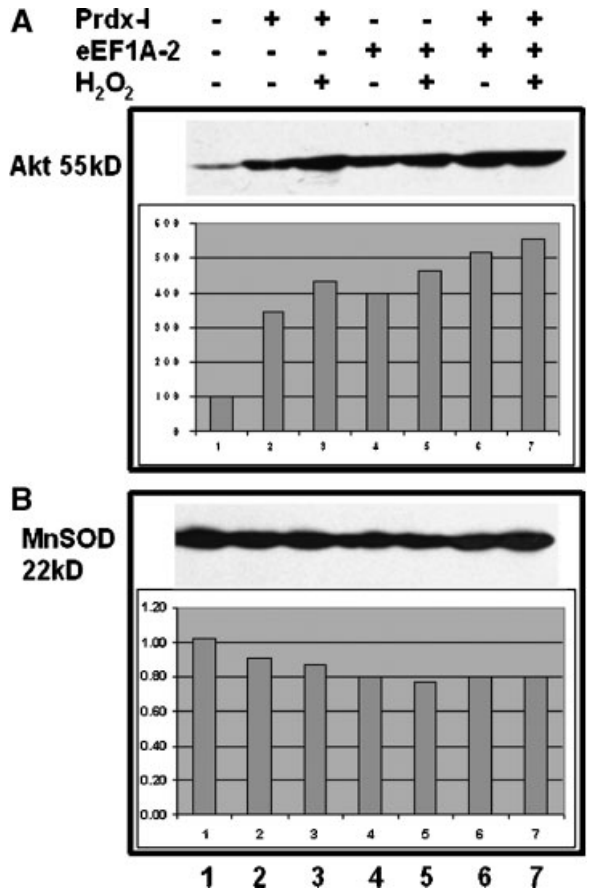

Fig. 7. Increased pro-survival protein factor Akt expression in stable transfectant expressing both eEF1A-2 and Prdx-I. **Panel A** top panel shows elevated expression of Akt in transfectants expressing either Prdx-I or eEF1A-2, or both, and this increase is further heightened by treatment with 200 μM H_2O_2. The lower panel of Panel A shows the quantification of this increase to approximately 5-fold in stable transfectants expressing both transgenes, compared with a baseline level of Akt in cells transfected with vector alone. **Panel B** shows that expression of MnSOD is independent of transgene expression, and is also used as loading control.

further reduced cleavage is observed in stable transfectant expressing both of these transgenes (Fig. 6).

Transgene-inhibited pro-apoptotic activity in stable transfectants was further investigated in terms of the presence of pro-survival activity, the level of Akt. Akt is a crucial mediator of cell survival signals, and may target several factors related to apoptosis in the cell [reviewed in Datta et al., 1999]. As shown in Panel A of Figure 7, all three stable transfectants expressing either Prdx-I, eEF1A-2 or both increase Akt abundance significantly above the baseline in vector-alone transfectant, and this increase is further accentuated by peroxide treatment. The increase reaches almost 5-fold in stable transfectant expressing both transgenes, and increases another half-fold upon treatment with peroxide. The specificity of the increase in the level of Akt is further evidenced by the fact that no such increase is observed in any of the conditions examined when the same blot was probed with antibody to MnSOD, although transfectant with vector alone shows a slightly higher level. Taken together, these results suggest that protection against apoptotic death in the stable transfectants is indeed associated with increased expression of pro-survival factor Akt, proportionally corresponding to the absence of pro-apoptotic activity, that is, the cleavage of caspases 3 and 8 to their activated protein products.

DISCUSSION

The function of eEF1A-2, an isoform of eEF1A-1, has not been characterized functionally beyond its traditional role in translation elongation. Here, we report that eEF1A-2 may have multiple molecular partners, as does its

isoform, for possible functional involvement in several key cellular processes. Among all the interacting partners identified by the yeast 2-hybrid study, in-depth analysis shows that eEF1A-2 interacts strongly with Prdx-I, a protein involved in oxidative defense, both in vitro and in vivo. This interaction, shown by pull-down experiments with brain and muscle extracts, suggests that Prdx-I and eEF1A-2 may be complexed together in these tissues. That Prdx-I is not pulled down from eEF1A-2-negative liver tissue further validates the specificity of the interaction between the two proteins. Interaction of Prdx-I with eEF1A-1, however, is not detected under the same experimental conditions with eEF1A-1-positive tissues, indicating that the interaction is specific to eEF1A-2. Further functional analysis of the interaction between Prdx-I and eEF1A-2 shows that while individually each exerts protection against oxidative stress-induced apoptotic death, together they foster extremely high resistance to oxidative insults, as high as 1 mM peroxide treatment. Thus, we suggest that the unique presence of eEF1A-2 in adult neurons, cardiomyocytes and myotubes provides not only necessary support for peptide elongation, but also supports extreme protection from oxidative stress by working together with peroxiredoxin in these long-lived cells, where accidentally triggering of apoptotic death would be catastrophic.

Few examples of eEF1A-2 interacting partners have been reported so far. In a brain pull-down assay using mAChR as bait, McClatchy et al. [2002] identified eEF1A-2 as an interacting partner. In another study [Mansilla et al., 2002], eEF1A-2 was shown to interact with the translation elongation eEF1H complex. In the present study, we used eEF1A-2 as bait to identify interacting partners in a two-hybrid assay. To our knowledge, this is the first such comprehensive study performed for eEF1A-2. Our result, identifying 23 partners, presents strong implications that eEF1A-2, like its isoform gene eEF1A-1, may (1) possess multiple non-canonical functions beyond its known role in translation elongation; and (2) have multiple functions in adult neurons, cardiomyocytes, and myotubes, to facilitate these cells' functions in their host tissues: brain, heart, and skeletal muscle, respectively.

The three major functional groups of interacting partners identified in this study are related to cytoskeleton, vesicular transport/signaling and transcription/translation control. Our yeast two-hybrid screening indicates that eEF1A-2, like its isoform eEF1A-1, is a versatile protein possessing multiple functions, not only participating in translation elongation but also in other important biological pathways, including vesicle trafficking, cytoskeletal organization, cell signaling, etc. Elucidating exactly what these biochemical interactions mean in functional terms will have to await future experiments to characterize the partnerships individually. These multiple interactions suggest that eEF1A-2 may participate in complex cellular processes by regulating the availability of many regulatory proteins, by either sequestering or facilitating the transport of the partners to strategic cellular target locations.

We have shown that interaction between eEF1A-2 and Prdx-I may cause downstream effect on elevating the expression level of Akt, especially under oxidative conditions, indicating that these proteins act additively, if not synergistically, to protect NIH 3T3 cells against apoptotic death by mediating the level of Akt expression. Our characterization of eEF1A-2 and Prdx-I as a complex in brain and muscle tissue extracts suggests that these two proteins may work together to provide extraordinary protection against activating the apoptosis pathway, by raising the abundance of pro-survival factor Akt. The low levels of cleavage of caspases 3 and 8 further validate that apoptosis signaling is indeed blocked when both eEF1A-2 and Prdx-I are present.

It is not surprising that over-expression of Prdx-I is pro-survival, since it has been shown to protect thyroid cells against H_2O_2-induced apoptosis [Kim et al., 2000], nor is it surprising that eEF1A-2 protects cells from apoptotic death, as our previous result demonstrated that it prevents myotubes from dying [Ruest et al., 2002] However, it is surprising in the present result that simultaneous expression of the two genes functionally endows cells with extraordinary resistance to peroxide treatment, as high as 1 mM for 24 h. Our results identifying these two proteins as interacting partners in brain and muscle, as well as the additive interaction against oxidative stress, have laid the first step in explaining the functional significance of the unique presence of eEF1A-2 in long-lived adult neurons and myotubes, and have paved the way to future studies elucidating the functional

mechanism behind the additive action of eEF1A-2 and Prdx-I.

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