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SPATIAL AND FUNCTIONAL RELATIONSHIP BETWEEN POLY(ADP-RIBOSE) POLYMERASE-1 AND POLY(ADP-RIBOSE) GLYCOHYDROLASE IN THE BRAIN

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Abstract—Poly(ADP-ribose) polymerases (PARPs) are members of a family of enzymes that utilize nicotinamide adenine dinucleotide (NAD^+) as substrate to form large ADP-ribose polymers (PAR) in the nucleus. PAR has a very short half-life due to its rapid degradation by poly(ADP-ribose) glycohydrolase (PARG). PARP-1 mediates acute neuronal cell death induced by a variety of insults including cerebral ischemia, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism, and CNS trauma. While PARP-1 is localized to the nucleus, PARG resides in both the nucleus and cytoplasm. Surprisingly, there appears to be only one gene encoding PARG activity, which has been characterized *in vitro* to generate different splice variants, in contrast to the growing family of PARPs. Little is known regarding the spatial and functional relationships of PARG and PARP-1. Here we evaluate PARG expression in the brain and its cellular and subcellular distribution in relation to PARP-1. Anti-PARG (α-PARG) antibodies raised in rabbits using a purified 30 kDa C-terminal fragment of murine PARG recognize a single band at 111 kDa in the brain. Western blot analysis also shows that PARG and PARP-1 are evenly distributed throughout the brain. Immunohistochemical studies using α-PARG antibodies reveal punctate cytosolic staining, whereas anti-PARP-1 (α-PARP-1) antibodies demonstrate nuclear staining. PARG is enriched in the mitochondrial fraction together with manganese superoxide dismutase (MnSOD) and cytochrome C (Cyt C) following whole brain subcellular fractionation and Western blot analysis. Confocal microscopy confirms the co-localization of PARG and Cyt C. Finally, PARG translocation to the nucleus is triggered by NMDA-induced PARP-1 activation. Therefore, the subcellular segregation of PARG in the mitochondria and PARP-1 in the nucleus suggests that PARG translocation is necessary for their functional interaction. This translocation is PARP-1 dependent, further demonstrating a functional interaction of PARP-1 and PARG in the brain.

Key words: NMDA, mitochondria, nucleus, immunostaining, subcellular fractionation.

Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) is a member of a growing family of enzymes that utilize nicotinamide adenine dinucleotide (NAD^+) as substrate to transfer ADP-ribose moieties onto glutamic acid residues of proteins to form ADP-ribose polymers (PAR) of various lengths and complexity (Ame et al., 2004). Accounting for 80–90% of cellular PARP activity, PARP-1 is activated by DNA strand breaks due to a variety of genotoxic stressors, including oxygen radicals, ionizing radiation, or alkylating agents (Lautier et al., 1993; de Murcia et al., 1994; Shall and de Murcia, 2000). Many nuclear proteins are poly-(ADP-ribosyl)ated by PARP-1, including histones, topoisomerase, p53, and PARP-1 itself in an automodification reaction (D'Amours et al., 1999). The actions of PARP-1 are significant to a number of cellular events, including transcriptional activation (Hassa and Hottiger, 1999; Ju et al., 2004), chromatin relaxation (de Murcia et al., 1986; Kim et al., 2004), mitosis (Kanai et al., 2003; Chang et al., 2004), and DNA maintenance (D'Amours et al., 1999; Dantzer et al., 2000). Because PARP-1 activation in response to DNA damage facilitates DNA repair and cellular recovery (Lautier et al., 1993; de Murcia et al., 1994; Shall and de Murcia, 2000; Rouleau et al., 2004), poly(ADP-ribosyl)ation has the important role of maintaining genomic integrity.

In the nervous system, PARP-1 activation plays a critical role in acute neuronal cell death elicited by a variety of insults, including cerebral ischemia (Zhang et al., 1994; Eliasson et al., 1997; Endres et al., 1997), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism (Cosi and Marien, 1999; Mandir et al., 1999), and

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Abbreviations: AIF, apoptosis inducing factor; CSS, control salt solution; Cyt C, cytochrome C; GFP, green fluorescent protein; HEK, human embryonic kidney; KO, knockout; MEM, modified Eagle's medium; MnSOD, mitochondrial manganese superoxide dismutase; NAD^+, nicotinamide adenine dinucleotide; NGS, normal goat serum; NLS, nuclear localization signal; NMDA, *N*-methyl-D-aspartate; PAR, poly(ADP-ribose) or ADP-ribose polymer; PARG, poly(ADP-ribose) glycohydrolase; PARPs, poly(ADP-ribose) polymerases; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TS, trophoblast stem; WT, wild type.

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traumatic brain injury (Whalen et al., 1999, 2000; LaPlaca et al., 2001). PARP-1-mediated neuronal cell death plays a primary role in glutamate excitotoxicity through *N*-methyl-D-aspartate (NMDA) glutamate-receptor activation, as mice lacking PARP-1 are highly resistant to excitotoxicity induced by NMDA, but are equally susceptible to AMPA excitotoxicity as wild-type (WT) mice (Mandir et al., 2000). Recent observations reveal that PARP-1-mediated cell death is linked to the translocation of the cell death effector, apoptosis inducing factor (AIF), from the mitochondria to the nucleus (Yu et al., 2002; Du et al., 2003; Hong et al., 2004) through the actions of PAR (Andrabi et al., 2006; Yu et al., 2006). Further, AIF translocation occurs during excitotoxic neuronal injury *in vivo* following NMDA receptor stimulation, suggesting AIF can substitute as a caspase executioner in PARP-1-dependent cell death (Wang et al., 2004). Therefore, PARP-1 mediates cell death in the nervous system at least in part through AIF, with other apoptotic or necrotic mechanisms occurring downstream of AIF translocation.

Following PARP-1 activation, the appearance of PAR is transient due to its rapid degradation by poly(ADP-ribose) glycohydrolase (PARG) into free ADP-ribose residues (Jonsson et al., 1988a; Brochu et al., 1994a; Davidovic et al., 2001). While there exists a family of PARP homologs capable of synthesizing PAR, to date only one PARG has been shown to catabolize PAR *in vivo* in mammals. Oka et al. (2006) suggest that there may be an additional PARG gene. However the specific PARG activity was quite low and no knockdown or overexpression studies were performed to confirm the hypothesized function of this gene. Isolation and characterization of the PARG cDNA from several species demonstrated only one mRNA transcript which encodes a 110–111 kDa protein (Lin et al., 1997; Shimokawa et al., 1999). However, recent studies revealed the existence of multiple splice variants of PARG, with full-length PARG encoding a protein of 111 kDa and two shorter forms of 102 and 99 kDa (Meyer-Ficca et al., 2004). PARG has been purified to homogeneity from different tissues of different species revealing important differences in molecular weight (ranging from 50 to 110 kDa) and catalytic activity (Tavassoli et al., 1983; Hatakeyama et al., 1986; Tanuma and Endo, 1990; Maruta et al., 1991; Uchida et al., 1993; Abe and Tanuma, 1996). Since there has not been any molecular evidence of shorter forms of PARG, it is likely that the previous reports describing shorter forms of purified PARG were probably descriptions of degradation fragments. Indeed, PARG degradation fragments (two C-terminal fragments of 85 and 74 kDa) are generated by caspase-3 during apoptosis (Affar et al., 2001), suggesting the possible generation of proteolytic PARG fragments *in vivo* or during tissue preparation.

The emerging role of PARG is to facilitate cell survival (Koh et al., 2005). Previous reports demonstrating a role for PARG in facilitating cell death by the prevention or re-activation of automodified PARP-1 (Ying and Swanson, 2000; Ying et al., 2001) proved to be inconclusive, since the PARG inhibitors utilized in these studies were later demonstrated to be non-specific and non-selective (Falsig et al., 2004). Characterization of the complete absence of functional PARG protein in mice via disruption of the *Parg* gene demonstrated that PARG is required for the proper cellular response to DNA damage, since PARG null trophoblast stem (TS) cells derived from these mice were hypersensitive to sublethal doses of DNA damaging agents (Koh et al., 2004). Further, PARG was shown to be essential for normal embryonic development and normal homeostatic cellular functions, since PARG null embryos did not develop past embryonic day 3.5 (E3.5) and PARG null TS cells did not remain viable in the absence of stress, respectively (Koh et al., 2004). Although other studies regarding the disruption of the *Parg* gene report the survival of PARG knockout (KO) animals, these mice are actually hypomorphs expressing functional PARG protein (Cortes et al., 2004). Thus, the viability of these mice confirms the critical role of PARG to the organism. Together with other reports demonstrating a role for PARG in development (Hanai et al., 2004), normal circadian function (Panda et al., 2002), and the response to DNA damage (Cortes et al., 2004), PARG appears to have a protective role and its activity therefore leads to viability.

In vitro studies demonstrate a predominantly cytoplasmic localization of PARG (Meyer-Ficca et al., 2004), while most PARPs have a nuclear localization. However, very little is known about the anatomical or subcellular distribution of PARG in the brain, and nothing is yet known regarding its spatial and functional relationship to PARP-1. The message for PARG is present in the brain (Shimokawa et al., 1999) and active PARG is present in cultured neurons and astrocytes (Sevigny et al., 2003). The purification of PARG from nuclear and post-nuclear extracts suggests that PARG could be localized both in the cytoplasmic and the nuclear compartments. Overexpressed PARG (Winstall et al., 1999) and the 102 and 99 kDa PARG splice variants display a cytoplasmic localization *in vitro*, while the 111 kDa PARG splice variant displays a nuclear localization (Meyer-Ficca et al., 2004). Other observations demonstrated that green fluorescent protein–PARG fusion protein (GFP-PARG) overexpressed in NIH3T3 cells is exclusively expressed in the nucleus during interphase and that GFP-PARG shuttles between nucleus and cytoplasm during the cell cycle (Ohashi et al., 2003). Therefore, the subcellular localization of PARG and its redistribution throughout the cell are likely to play an important role in the regulation of regional poly(ADP-ribose) metabolism. The subcellular localization of PARG could then possibly depend on many factors, including the splice variant expressed, the tissues or cells studied, and/or the phase of the cell cycle. We report here for the first time the cellular immunolocalization of endogenous PARG in the brain. Using immunocytochemical techniques and subcellular fractionation, we show that PARG is co-localized with PARP-1 throughout the brain, but in different subcellular compartments. In the brain, PARP-1 is primarily enriched in the nuclear fraction and PARG is primarily enriched in the mitochondrial fraction, suggesting that their subcellular segregation would necessitate PARG redistribution for their functional interaction. We also show that