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Research paper

SodC modulates ras and PKB signaling in *Dictyostelium*

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ABSTRACT

We have previously reported that the basal RasG activity is aberrantly high in cells lacking Superoxide dismutase C (SodC). Here we report that other Ras proteins such as RasC and RasD activities are not affected in *sodC*$^-$ cells and mutagenesis studies showed that the presence of the Cys^{118} in the Ras proteins is essential for the superoxide-mediated activation of Ras proteins in *Dictyostelium*. In addition to the loss of SodC, lack of extracellular magnesium ions increased the level of intracellular superoxide and active RasG proteins. Aberrantly active Ras proteins in *sodC*$^-$ cells persistently localized at the plasma membrane, but those in wild type cells under magnesium deficient medium exhibited intracellular vesicular localization. Interestingly, the aberrantly activated Ras proteins in wild type cells were largely insulated from their normal downstream events such as Phosphatidylinositol-3,4,5-P_3 (PIP3) accumulation, Protein Kinase B (PKB) activation, and PKBs substrates phosphorylation. Intriguingly, however, aberrantly activated Ras proteins in *sodC*$^-$ cells were still engaged in signaling to their downstream targets, and thus excessive PKBs substrates phosphorylation persisted. In summary, we suggest that SodC and RasG proteins are essential part of a novel inhibitory mechanism that discourages oxidatively stressed cells from chemotaxis and thus inhibits the delivery of potentially damaged genome to the next generation.

1. Introduction

Regulation of complex cell behaviors such as cell motility and phagocytosis has been traditionally envisioned as a process that is directed by membrane receptor dependent signaling events. Recently, a series of elegant experiments and modeling studies provided a revised view that the external information becomes available as a signaling network with which the internal excitable network could interact (Iglesias and Devreotes, 2012; Shi et al., 2013; Artemenko et al., 2014). Cells then modify their motility behavior as a consequence of the interaction of these two signaling networks. The excitable network is modeled to contain signaling components equipped with positive and negative feedback loops. Potential components that fit to the model are suggested to include Ras and PKBs. Biochemical evidence for such feedback loops include F-Actin mediated Ras activation and PKB mediated inhibition of Ras (Shi et al., 2013).

The current paradigm of Ras regulation is largely dependent on the regulation of RasGEF and RasGAP proteins, which are the targets of signaling cascades initiated from chemoattractant engaged G protein coupled receptors (GPCR). Active heterotrimeric G proteins Gα and Gβγ interact and activate these Ras regulatory proteins (Wu et al., 1995; Khanna et al., 2016). Alternatively, some of the Ras proteins display GPCR independent autonomous activation, of which mechanism is largely to be determined (Sasaki et al., 2007; Iglesias and Devreotes, 2012; Shi et al., 2013; Artemenko et al., 2014). In addition to these two modes of Ras activation, here we report that one of the *Dictyostelium* Ras proteins, RasG, was constitutively activated in the absence of apparent GPCR receptor activation. It seems that aberrantly increased level of intracellular superoxide radicals persistently activates RasG in *Dictyostelium* cells under dysregulated superoxide metabolism.

During chemotaxis the GPCR activated Ras proteins activate Target of Rapamycin (Tor) complex and Phosphoinositide-Dependent Protein Kinase (PDK), which in turn activate PKBA and PKBR1. These two members of the PKB kinase family are largely responsible for orchestrating dynamic F-Actin remodeling in response to chemoattractant gradient (Lee et al., 2005; Kamimura et al., 2008; Cai et al., 2010; Liao et al., 2010).

Previous studies demonstrated that the production of superoxide anion is an electrogenic process and thus should be coupled with another counteracting electrogenic process, such as proton efflux, to continue the production (Lamb et al., 2009; Liu et al., 2013). Cells in a multicellular organism under homeostatic conditions would be

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best suited to properly operate this process, but cells of which natural habitats are deficient of such protective mechanisms are likely to experience significant dysregulation of electrogenic superoxide processes. For example, *Dictyostelium discoideum* cells dwell in soil and manure, of which levels of ionic components would fluctuate significantly from their average values depending on the weather situation.

Several studies using mammalian systems also reported that superoxide levels are susceptible to the presence of specific ions such as magnesium. Superoxide production of macrophages and polymorphonuclear neutrophils (PMN) were attenuated by the presence of magnesium ions (Cairns and Kraft, 1996) and mouse on magnesium deficient diet exhibited persistent or facilitated superoxide generation in their neutrophils and macrophages, indicating that hypomagnesia is linked with inflammatory responses in animals (Mazur et al., 2007; Weglicki, 2012).

Here we provide evidence that extracellular magnesium ions affect the basal level of RasG in a superoxide radical dependent manner in *Dictyostelium* and thus modulate cell motility. Specifically, under conditions lacking magnesium, RasG signaling was insulated from its normal downstream PKB activation, suggesting that the redox regulation of RasG/PKB signaling functions as an inhibitory component that discourages oxidatively stressed cells from chemotaxis and thus impedes the delivery of a potentially damaged genome to the next generation.

We previously published that *Dictyostelium* extracellular superoxide dismutase (SodC) regulates RasG activity (Veeranki et al., 2008). Extracellular superoxide dismutases present from bacteria to human, underscoring their critical roles in protecting the organism form oxidative stresses. Bacterial cells expressing periplasmic Sod often pathogenic, resistant to oxidative burst (De Groote et al., 1997). Extracellular Sod proteins in mammalian cells were repeatedly shown to protect cells from various oxidative stresses. In mammalian systems, the endothelial cells are the main source of extracellular Sod, but neutrophils were also equipped with the extracellular Sod protein through endocytosis of extracellular Sod proteins (Iversen et al., 2016). The extracellular Sod proteins in immune cells are likely to protect the tissue under inflammation. Several studies also uncovered that extracellular Sod proteins not only protect extracellular environments, but also induce intracellular responses such as suppression of pro-inflammatory genes and suppression of cell cycle (Laurila et al., 2009; Break et al., 2012; Jeon et al., 2012; Lee et al., 2013) and loss of extracellular Sod was shown to promote certain types of cancer progression (Teoh-Fitzgerald et al., 2012; Teoh-Fitzgerald et al., 2014; O'Leary et al., 2015). Interestingly, extracellular Sod proteins suppressed cell proliferation by inhibiting multiple proliferative signaling including Ras (Laukkanen et al., 2015).

In this report, we uncovered that cells lacking Superoxide Dismutase C (SodC) not only exhibited higher level of RasG activity, but also displayed persistent PKBs activation. Previous studies using superoxide radical scavengers or *Dictyostelium* cells lacking Superoxide dismutase C (SodC) demonstrated that the proper regulation of superoxide metabolism is essential for chemotaxis, but the signaling pathway that affects the metabolism of superoxide is yet to be fully defined (Veeranki et al., 2008; Bloomfield and Pears, 2003).

Currently, the mechanism of superoxide-mediated Ras activation is not completely uncovered. The small GTPase Ras proteins bind to Guanine nucleotide through multiple regions. One of the regions that mediates interaction with the guanine base is the NKxD motif. Some of the Ras proteins contain Cysteine residue at the variable site of the NKxD motif and it is often the target of nitric oxide mediated activation of Ras proteins (Lander et al., 1996; Lander et al., 1997). Other independent *in vitro* studies also shown that superoxide anions can also activate Ras proteins by targeting Cysteine 118 (Heo and Campbell, 2005). In this study, we further determined that the Cys^{118} residue of the NKCD motif of Ras proteins is essential for the Ras activating function of superoxide *in vivo*. RasG, but neither RasC nor RasD, contains the Cys^{118} and was aberrantly activated in wild type cells under oxidative stress and constitutively in *sodC*$^-$ cells. Substitution mutant RasG($C^{118}A$) became redox insensitive, but RasD($A^{118}C$) mutant displayed redox sensitivity in *sodC*$^-$ cells.

The initial view on the interaction of reactive oxygen species and the small GTPase Ras was that Ras is stimulating the production of reactive oxygen species (ROS). For example, oncogenic H-Ras (V12) transformed mouse fibroblasts produced more ROS (Irani et al., 1997). Follow-up studies revised that cell transformation requires both oncogenic Ras mutation and ROS production (Suh et al., 1999; Lambeth, 2004; Wu and Terada, 2009). Interestingly, other studies uncovered that Ras can function as a ROS downstream target. For example, lactosylceramide, a mitogenic lipid, activated both Ras and Nox, but cells treated with Nox inhibitor failed to show Ras activation in response to this mitogen (Bhunia et al., 1997). Furthermore, it was shown that the expression of Nox1 was essential for PDGF and Angiotensin II (Ang II) mediated production of ROS and for the activation of redox sensitive signaling such as p38 mitogen-activated protein kinase and Akt in response to Ang II (Lassegue et al., 2001).

F-Actin remodeling is the main spatio-temporal Ras signaling target in chemotaxing *Dictyostelium* cells, and cortical membrane remodeling such as membrane ruffles, macropinosomes, and lamellipodia are also reported to be downstream targets of active Ras in various mammalian cells in addition to the classical cell cycle machinery (Bar-Sagi and Feramisco, 1986; Wozniak et al., 2005; Ada-Nguema et al., 2006; Gao et al., 2009; Conklin et al., 2010; Welliver and Swanson, 2012). Furthermore, a proper spatiotemporal regulation of PI3 K was shown to be essential for Ras mediated membrane ruffle formation (Welliver and Swanson, 2012; Gao et al., 2009). Thus dysregulation of spatio-temporal Ras/PI3 K/PKB signaling induced by loss of SodC or disturbance of extracellular magnesium level could affect F-Actin remodeling driven events in large varieties of cell types that include *Dictyostelium* and human.

2. Results

2.1. The RasG-Cys^{118} is essential for redox regulation of RasG in vivo

We have previously showed that RasG activity is aberrantly high in *sodC*$^-$ cells (Veeranki et al., 2008). To further uncover the mechanism of Ras dysregulation in *sodC*$^-$ cells, three Ras proteins, RasG, RasC, and RasD, were flag tagged and introduced into wild type and *sodC*$^-$ cells. RasG contains Cys^{118}, but RasC and RasD contain Ala^{118}. Previous *in vitro* studies (reviewed by Heo, 2011) suggested that superoxide radicals could facilitate GDP exchange by targeting the cysteine residue of the $NKC^{118}D$ motif. The levels of basal activities of RasG, RasC, and RasD proteins in wild type and *sodC*$^-$ cells pretreated with pulsatile cAMP for 4 h were determined by GST-RBD binding assay: RasG proteins displayed consistent a two-fold higher basal Ras activity in *sodC*$^-$ cells compared to that of wild type cells, whereas RasC and RasD displayed no such misregulation in *sodC*$^-$ cells (Fig. 1A).

To further determine the role of cysteine 118 of the NKCD motif, RasD($A^{118}C$) mutant was generated and introduced into wild type and *sodC*$^-$ cells. Consistent with the possibility that the cysteine 118 is the *in vivo* redox target, RasD($A^{118}C$) showed two fold increase in the basal activity (Fig. 1B). Conversely, RasG($C^{118}A$) mutant was generated and introduced into wild type and *sodC*$^-$ cells. Compared to the wild type RasG, RasG($C^{118}A$) mutant displayed significantly lower basal activity in *sodC*$^-$ cells (Fig. 1B).