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The antagonistic roles of PDGF and integrin αvβ3 in regulating ROS production at focal adhesions

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ABSTRACT

Reactive oxygen species (ROS) have been shown to play crucial roles in regulating various cellular functions, e.g. focal adhesion (FA) dynamics and cell migration upon growth factor stimulation. However, it is not clear how ROS are regulated at subcellular FA sites to impact cell migration. We have developed a biosensor capable of monitoring ROS production at FA sites in live cells with high sensitivity and specificity, utilizing fluorescence resonance energy transfer (FRET). The results revealed that platelet derived growth factor (PDGF) can induce ROS production at FA sites, which is mediated by Rac1 activation. In contrast, integrins, specifically integrin αvβ3, inhibits this local ROS production. The RhoA activity can mediate this inhibitory role of integrins in regulating ROS production. Therefore, PDGF and integrin αvβ3 coordinate to have an antagonistic effect in the ROS production at FA sites to regulate cell adhesion and migration.

1. Introduction

Living cells are continuously exposed to environmental cues. It becomes clear that the changes in microenvironments play crucial roles in regulating normal cellular functions as well as the progression of diseases [1–3]. Recent papers revealed that matrix mechanics controls the cell fate by modulating the density and types of the bonds between membrane receptor integrins and extracellular matrix (ECM) proteins coupled to the materials [4,5]. However, it remains elusive on how specific ECM proteins interact with integrins to mediate the transmission of the environmental cues. Reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion are crucial secondary messengers in cellular adhesion [6], spreading [7], and migration [8]. It has been shown that ROS can regulate these processes via focal adhesions (FA) and focal complexes mediated by the ligation of integrins [8], heterodimeric transmembrane glycoprotein receptors composed of noncovalently linked α and β subunits. ROS induction has also been shown to significantly reduce the surface expression of the αv and β3 integrins [9,10]. Conversely, the overexpression of integrin αvβ3 can increase glutathione (GSH), indicating an inhibitory role of integrin αvβ3 in ROS production [9].

Platelet derived growth factor (PDGF) was shown to cause an ROS increase in smooth muscle cells [11] and induce tyrosine phosphorylation events by inhibiting protein tyrosine phosphatase (PTP) [12]. Consistently, the inhibition of intracellular ROS by scavengers can block the PDGF-stimulated signal transduction [11]. These PDGF effects on ROS can be regulated by integrins and their mediated cell adhesion on extracellular matrix (ECM) proteins [13]. In fact, integrins have been shown to directly regulate growth factor receptors by changing their expressions or localizations/functions at focal contacts, subcellular regions where integrins co-localize

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Abbreviations

5-LOX	5-lipoxygenase
Cys	cysteine
DTT	dithiothreitol
ECFP	enhanced cyan fluorescent protein
ECM	extracellular matrix
FBS	fetal bovine serum
FN	fibronectin
FA	focal adhesion
FRET	fluorescence resonance energy transfer
GSH	glutathione
HEK	human embryonic kidney
IPTG	isopropyl-β-d-thiogalactopyranoside
LMW-PTP	low molecular weight- protein tyrosine phosphatase
MEF	mouse embryonic fibroblast
NAC	N-acetyl cysteine
PDGF	platelet derived growth factor
PDGFR	PDGF-receptor
ROS	reactive oxygen species
Redox	reduction–oxidation
His6	six histidines
Ypet	yellow fluorescent protein

with signaling and cytoskeletal molecules [13–16]. For example, cell adhesion on ECM protein fibronectin can enhance the PDGFR-β auto-phosphorylation and protein levels [17]. Integrin αvβ3 and PDGFR-β were also shown to interact physically for the regulation of cell migration [18]. Consistently, PDGFR-β activation increases endothelial cell migration on vitronectin, a ligand for integrin αvβ3 [18]. Migration of mesenchymal stem cells is also promoted by the crosstalk between PDGFR-β and integrin α5β1 in the presence of fibronectin [19].

Rac1 and RhoA can act downstream to integrin and PDGFR signaling [20], and trigger a variety of opposite responses [21–23]. For example, high concentration of fibronectin can downregulate Rac1 but upregulate RhoA, and subsequently inhibit migration [20]. ROS has also emerged as a crucial mediator of Rac1 and RhoA signaling by covalently modifying specific cysteine residues. Although both Rac1 and RhoA contain the GXXXXGK(S/T)C motif that can respond to ROS, RhoA has an extra cysteine (Cys20) in the redox motif, which can be oxidized by ROS to promote the formation of an intramolecular disulfide bond and cause RhoA inactivation [24]. The activation of Rac1 can also induce the downregulation of RhoA via the ROS-dependent inactivation of LMW-PTP (Low molecular weight-protein tyrosine phosphatase) [25]. As such, ROS can mediate the antagonistic crosstalk between Rac1 and RhoA [26–29].

We have developed a ROS sensor based on fluorescence resonance energy transfer (FRET) that allows the real-time assessment of intracellular reduction–oxidation (redox) conditions at subcellular focal adhesion (FA) sites. Herein, we report the design, generation, and investigation of this FRET-based redox sensor. The FRET sensor displayed increased redox sensitivity upon integration of paxillin, which acts to localize the sensor specifically to FA sites. In addition to the detection of ROS at FA sites in response to exposure to oxidants such as diamide or hydrogen peroxide, we can monitor intracellular activities of signaling pathways involving Rac1 and RhoA. The ROS production at FA sites of mouse embryonic fibroblasts was investigated upon stimulation by PDGF of cells cultured on different concentrations of extracellular matrix protein (fibronectin). Finally, we examined the relationship between PDGF and integrins (specifically αvβ3 or α5β1) in regulating ROS production as mediated by Rac1 and RhoA signaling at FA sites.

2. Materials and methods

2.1. Gene construction and plasmids

The gene encoding the cytosolic ROS sensor includes a central fragment consisting of the peptide sequence **C**EGGSTSGSGKPGSGEGSTKG**C**EG flanked by a SphI site at the N terminus and a SacI site at the C terminus, which was further fused in between an N-terminal ECFP and a C-terminal YPet (Table 1). This cytosolic ROS sensor was cloned into pRsetB (Invitrogen), and the insert was sequenced (W. M. Keck Center for Functional and Comparative Genomics, University of Illinois at Urbana-Champaign) to verify the integrity of the coding sequence. The plasmid has the coding sequence in-frame with six histidines (His_6-tag), and hence when expressed, the product carried an N-terminal His_6-tag to allow Ni column purification. The DNA insert was then digested by BamHI/EcoRI and ligated into a pcDNA3 vector (Invitrogen) to create the resultant ROS sensor for mammalian cell expression (Fig. 1). The ROS sensor mutant was generated by site-specific mutations of cysteine to serine on both CEG regions, thus resulting in its resistance to oxidative modification. The ROS-paxillin sensor was constructed by adding the paxillin gene to the C-terminus of the cytosolic ROS sensor. Recombinant plasmids pRsetB and pcDNA3.1 were from Invitrogen. Dominant negative forms of Rac1 (RacN17) and RhoA (RhoN19), and the constitutively active forms of Rac1 (RacV12) and RhoA (RhoV14) were gifts from Dr. Alan Hall (Memorial Sloan–Kettering Cancer Center, NY, USA).

2.2. Materials and reagents

Phusion DNA polymerase and enzymes for DNA digestion were from NEB (MA, USA). Ni-NTA agarose, QIAprep spin miniprep and QIAquick gel purification kits were from Qiagen (CA, USA). The Bradford protein assay kit was from BioRad (CA, USA). Oligonucleotides were obtained from Invitrogen (CA, USA). Dithiothreitol (DTT), an anti-oxidant used to stabilize proteins containing sulfhydryl groups, was purchased from Promega (WI, USA). Diamide as a thiol oxidizing agent and N-acetyl cysteine (NAC) as a ROS scavenger were purchased from Sigma (MO, USA). Antibodies for blocking integrins αvβ3 (clone LM609) and α5β1 (Clone BMB5) were from Millipore (MA, USA).

2.3. Protein purification

The ROS sensors were expressed in *Escherichia coli* (BL21 strain) as fusion proteins with an N-terminal His_6-tag and purified by nickel chelation chromatography. In brief, BL21 cells expressing the ROS sensor in the pRsetB vector were grown in LB medium containing ampicillin (100 mg/L) at 37 °C until OD600 measured around 0.2. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM, and the culture was incubated for another 16 h at 25 °C. Cells were collected by centrifugation, and re-suspended in 10 ml binding buffer (50 mM Tris·HCl, 200 mM NaCl, 10 mM imidazole, pH 7.4) and lysed by B-PER protein extraction reagents (Thermo Scientific). The cell lysate was clarified by centrifugation and subjected to the incubation with nickel-NTA beads. The protein-coated beads were washed with the binding buffer and the proteins were then eluted with 5 ml elution buffer (50 mM Tris, 200 mM NaCl, 200 mM imidazole, pH 7.4).

Table 1
Description of ROS sensor constructs.

Construct name	Construct features
Cytosolic ROS sensor	His_6 tag-ECFP-Cys-EGGSTSGSGKPGSGEGSTKG-Cys-EG-YPet
Cytosolic ROS mutant	His_6 tag-ECFP-Ser-EGGSTSGSGKPGSGEGSTKG-Ser-EG-YPet
ROS-paxillin sensor	His_6 tag-ECFP-Cys-EGGSTSGSGKPGSGEGSTKG-Cys-EG-YPet-Paxillin
ROS-paxillin mutant	His_6 tag-ECFP-Ser-EGGSTSGSGKPGSGEGSTKG-Ser-EG-YPet-Paxillin