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Direct interaction between Tks proteins and the N-terminal Proline Rich Region (PRR) of NoxA1 mediates Nox1-dependent ROS generation

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Abstract

NADPH oxidase (Nox) family enzymes are one of the main sources of cellular reactive oxygen species (ROS), which have been implicated in several physiological and pathophysiological processes. To date 7 members of this family have been reported, including Nox1–5 and Duox1 and 2. With the exception of Nox2, the regulation of the Nox enzymes is still poorly understood. Nox1 is highly expressed in the colon, and requires two cytosolic regulators, the organizer subunit NoxO1 and the activator subunit NoxA1, as well as the binding of Rac1 GTPase, for its activity. Recently, we identified the c-Src substrate proteins Tks4 and Tks5 as functional members of a p47^{phox}-related organizer superfamily. As a functional consequence of this interaction, Nox1 localizes to invadopodia, actin-rich membrane protrusions of cancer cells which facilitate pericellular proteolysis and invasive behavior.

Here, we report that Tks4 and Tks5 directly bind to NoxA1. Moreover, the integrity of the N-terminal PRR of NoxA1 is essential for this direct interaction with the Tks proteins. When the PRR in NoxA1 is disrupted, Tks proteins cannot bind NoxA1 and lose their ability to support Nox1-dependent ROS generation. Consistent with this, Tks4 and Tks5 are unable to act as organizers for Nox2 because of their inability to interact with p67^{phox}, which lacks the N-terminal PRR, thus conferring a unique specificity to Tks4 and 5.

Taken together, these results clarify the molecular basis for the interaction between NoxA1 and the Tks proteins and may provide new insights into the pharmacological design of a more effective anti-metastatic strategy.

Keywords

NADPH oxidase; Nox1; NoxA1; p67^{phox}; Reactive Oxygen Species (ROS); Tks proteins; Invadopodia; Cancer

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Introduction

The NADPH oxidase (Nox) family members are transmembrane proteins that catalyze the NADPH-dependent one-electron reduction of oxygen to form superoxide (Bedard and Krause, 2007). To date, 7 members of this family have been described: Nox1–5 and Duox (dual oxidase) 1 and 2 (Bokoch and Knaus, 2003; Lambeth, 2004). These homologs of the phagocyte gp91 cytochrome b subunit (Nox2) are found in virtually all tissues, and have been implicated in such biological processes as inflammation, innate immunity, apoptosis, cancer, cell signaling, motility, and transcription. Reactive Oxygen Species (ROS) generated via Nox also contribute to a growing number of diseases, including atherosclerosis, hypertension, arthritis, Alzheimer's disease and other neurological disorders, stroke, respiratory syndromes, cancer, and inflammation (Lambeth, 2007; Lambeth *et al.*, 2008). However, very little is known about the signals and the molecular machinery regulating the formation of ROS by Nox proteins.

Nox2, expressed by phagocytic leukocytes, is currently the only Nox enzyme whose regulation is reasonably well understood (Bokoch and Zhao, 2006). Microorganisms and inflammatory mediators trigger leukocyte signaling cascades that induce the assembly of four cytosolic regulatory proteins ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac2-GTPase) with the Nox2 core enzyme to stimulate superoxide formation (Bokoch, 2005). This membrane-associated enzyme is a cytochrome b heterodimer consisting of $gp91^{phox}$ and p22 subunits.

The regulation of other Nox enzymes appears to differ significantly from Nox2, and their regulatory mechanisms are still poorly defined (Bokoch and Knaus, 2003; Lambeth *et al.*, 2007). As Nox1 is structurally most closely related to Nox2, there are certain similarities in their requirements for activity. First, homologs of the $p47^{phox}$ and $p67^{phox}$ cytosolic regulatory proteins have been identified in colon epithelial cells, giving rise to two families of functionally different cytosolic regulators (Banfi *et al.*, 2003; Geiszt *et al.*, 2003; Takeya *et al.*, 2003). NoxO1 is homologous to $p47^{phox}$ and $p40^{phox}$, composing the $p47^{phox}$ organizer superfamily, while NoxA1 is homologous to $p67^{phox}$ making up the activator superfamily. Second, $p22^{phox}$, an essential component of the Nox2 heterodimer, is also required for the function of Nox1, Nox3, and Nox4 (Kawahara *et al.*, 2005; Martyn *et al.*, 2006). Finally, the small GTPase Rac1 is also required for complete Nox1 activity (Cheng *et al.*, 2006).

Although $p67^{phox}$ and NoxA1 share only 28% amino acid identity, as illustrated in Figure 4A, their overall domain structure is similar. Both contain *1*) an N-terminal tetratricopeptide repeat (TPR), *2*) highly conserved Proline-Rich regions (PRRs), *3*) a less conserved “Phox and Bem 1” (PB1) domain, and *4*) a C-terminal Src Homology-3 (SH3) domain. Members of this family function as molecular bridges between different components of the oxidase complex thus catalyzing superoxide formation (Bedard and Krause, 2007). $p67^{phox}$ and NoxA1 interact through their C-terminal SH3 domain with the PRR of the organizer subunits, whereas their N-terminal TPR domains interact with Rac (Lambeth, 2004). Thus, the overall structure and protein-protein interactions of the two homologous subunits are highly similar. However, there are some notable differences. For instance, NoxA1 lacks the central SH3 domain found in $p67^{phox}$, while $p67^{phox}$ lacks the N-terminal PRR found in NoxA1. The functional outcome of these structural differences, however, is not known.

Members of a $p47^{phox}$ organizer superfamily also have similar domain architectures (Kawahara and Lambeth, 2007). They possess 1) an N-terminal ‘*phox*’ domain (PX) responsible for binding to various phosphoinositol lipids, 2) two SH3 domains (tandem SH3) involved in the binding to $p22^{phox}$ and 3) PRRs important for the interaction with activator subunits (DeLeo and Quinn, 1996; Vignais, 2002) (absent in $p40^{phox}$).

Functionally, it has been proposed that these organizers serve as regulatory response elements for the correct NADPH oxidase assembly at specific subcellular compartments (Bissonnette *et al.*, 2008; Tian *et al.*, 2008).

The protein Tks5, originally known as 'Fish' (for five-SH3 domains), was identified in a cDNA library screen for c-Src substrates (Lock *et al.*, 1998). Subsequently, a closely related homolog with four SH3 domains, termed Tks4, was also described (Courtneidge, 2003; Courtneidge *et al.*, 2005). Examination of subcellular localization in Src-transformed cells revealed that Tks4 and Tks5 localize to invadopodia (Abram *et al.*, 2003; Buschman *et al.*, 2009). These are dynamic phosphotyrosine-rich structures with an actin core and abundant actin regulatory proteins (e.g. cortactin) found in macrophages, osteoclasts, and cancer cells (Linder, 2007; Gimona *et al.*, 2008). Invadopodia contain proteases capable of degrading the extracellular matrix (ECM) and they have been shown to play an important role in invasive cell motility and tumor metastasis. The Tks proteins are widely distributed in mouse and human tissues, with the notable exception of low abundance in leukocytes. Tks4 and Tks5 show a similar domain architecture and composition with other members of the p47 organizer superfamily (Kawahara and Lambeth, 2007). In addition to containing the N-terminal tandem SH3 domains structurally similar to those of $p47^{phox}$ (up to 47% identity) and arranged in the same orientation, Tks proteins also contain a highly conserved PX domain, multiple PRRs, and several Src phosphorylation sites. A schematic representation summarizing domain composition and molecular interactions between the known organizer and activator subunits is illustrated in Supplementary Table 1.

Our group has recently shown that Tks proteins are novel functional members of the $p47^{phox}$ organizer superfamily as they can support Nox1- and Nox3-dependent ROS generation (Gianni *et al.*, 2009). Similar to previously identified members of the organizer superfamily, Tks proteins mediate Nox1-dependent ROS generation by binding the activator subunit NoxA1. Interestingly, as a functional consequence of the endogenous interaction between Tks4 and NoxA1 in human DLD1 colon cancer cells, Nox1 is localized to ECM-degrading invadopodia, confirming that organizer subunits play a pivotal role in differential recruitment of the oxidase complex to specific subcellular compartments. However, functional differences between Tks proteins and the previously identified members of the organizer superfamily exist. Tks proteins can only mediate Nox1- and Nox3-dependent ROS generation, while they are unable to support ROS production by Nox2. The former may represent an inability of Tks proteins to functionally recruit $p67^{phox}$. Importantly, we have shown that the interaction between NoxA1 and Tks adaptors requires the functional integrity of all five Tks5 SH3 domains. This indicates that in this case the recruitment of the activator protein does not involve the PRR of the organizer subunits, but instead it may take place between the SH3 domains of the Tks proteins and the PRR of the activator subunits.

Here we clarify the molecular basis of the interaction between NoxA1 and Tks proteins. We report that Tks4 and Tks5 directly bind to NoxA1. Moreover, the integrity of the N-terminal PRR of NoxA1 is essential for its direct interaction with Tks proteins. When the PRR in NoxA1 is disrupted, Tks proteins cannot bind NoxA1 losing their ability to support Nox1-dependent ROS generation. Consistent with this, Tks4 and Tks5 are unable to act as organizers for Nox2 because of their inability to interact with $p67^{phox}$, in which the N-terminal PRR is not conserved.

Taken together, these results provide novel insights into the molecular basis of the interaction between NoxA1 and Tks proteins and could be employed in the pharmacological design of a more effective anti-metastatic strategy.

Material and methods

Reagents

Cell culture medium, fetal bovine serum, supplements and Hanks' balanced salt solution (HBSS; catalog no. 24020-117) were from Invitrogen (Carlsbad, CA). Plasmids for transfection were purified using the Qiafilter system (QIAGEN, CA). The following reagents were obtained as indicated: horseradish peroxidase (HRP; 77330) and luminol (09253) were purchased from Sigma (St. Louis), 9E10 anti-myc antibody was prepared in-house; polyclonal anti-NoxA1 antibody was generated in house as part of the Centers for Disease Control Program PO1 CI000095; polyclonal anti- $p67^{phox}$ antibody (number 3958) was a kind gift of Dr. Sergio Catz (The Scripps Research Institute). Rabbit polyclonal Tks4 and Tks5 antibodies were obtained from Dr. Sara Courtneidge (The Burnahm Institute for Biomedical Research) and were previously described in (Lock *et al.*, 1998; Buschman *et al.*, 2009). Protein G-Sepharose and Glutathione beads were from Amersham Biosciences. Talon magnetic beads (635636) were purchased from Clontech.

DNA plasmids

Human Myc-tagged NoxO1 and NoxA1 were originally obtained from Tom Leto (National Institutes of Health). Myc-tagged $p47^{phox}$ expression vector was generated by subcloning $p47^{phox}$ cDNA in pRK5m vector. $p67^{phox}$ expression plasmid was obtained by subcloning $p67^{phox}$ cDNA into pCDNA3.1 vector. Myc- and Flag-tagged Tks4 and Tks5 expression vectors were described in (Gianni *et al.*, 2009). GST-tagged NoxO1, NoxA1, Tks4 and Tks5 were generated by subcloning the respective Myc-tagged expression vector in pGEX-4T1. His-tagged NoxA1 vector was created by inserting NoxA1 cDNA into pTrcHIS (A) vector (InVitrogen). All Myc- and GST-tagged deletion mutants of NoxA1 as well as the NoxA1 PRR mutants were created using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The sequences of all constructs were confirmed by DNA sequencing.

Cell Culture and Transfection

Human embryonic kidney HEK293 and human DLD1 colonic adenocarcinoma cells were maintained in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin) at 37°C in 5% CO_2. For transfection, HEK293 cells were plated in either six-well plates or in 10 cm-diameter plates (Falcon) at appropriate density, grown overnight, and then transfected by using Lipofectamine 2000 (InVitrogen) following the manufacturer's instructions. 16 hrs after transfection, cells were processed accordingly.

Western blot and Immunoprecipitation

For the preparation of total cell extracts, monolayer cultures were washed in cold PBS and lysed in appropriate amount of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1%, Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM EDTA) supplemented with 1 mM leupeptin, 1 mM aprotinin, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were clarified by centrifugation at 16,000 × *g* at 4 °C, and the protein concentration was estimated using the Bio-Rad assay according to the manufacturer's instructions. For immunoprecipitations, 1–2 μl of specific antibodies were incubated with 1 mg of protein lysates for 2 h at 4 °C, followed by 30 min incubation with 20 μl of Protein G–Sepharose (Amersham). The samples were incubated for an additional 30min after adding 20 μl of protein G plus-Sepharose. Immunoprecipitates were washed three times in lysis buffer and proteins released by boiling in Laemmli SDS sample buffer, and samples were resolved by 10% SDS-PAGE. Gels were transferred onto nitrocellulose membranes

using the electrophoretic transfer cell (Bio-Rad) at 100 V for 1 h. After blocking with nonfat dry milk (5%), proteins were probed overnight using antibodies at appropriate dilution. Anti-Myc dilution was (1:1000), anti-GST was used at a dilution of (1:10000). Rabbit polyclonal antibodies against NoxA1, $p67^{phox}$, Tks4 and Tks5 were used at a dilution of (1:5000). The excess antibody was removed by sequential washing of the membranes in Tween-PBS, and then a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Pierce Chemical) was added to the filters followed by incubation for 1 h at room temperature. After sequential washing of the membranes in T-PBS to remove excess secondary antibody, the signals were detected by chemiluminescence using the ECL system (Pierce Chemical). Blots were stripped and re-probed as necessary.

Expression of recombinant proteins and GST-pulldowns

The recombinant NoxA1, NoxO1, Tks4 and Tks5 as well as NoxA1 deletion mutants were expressed and purified as GST- or His-fusion proteins by isopropyl-thiogalactoside (IPTG) induction of *Escherichia coli* cultures harboring the corresponding pGEX-4T1 or pTrcHis (A) vectors. Affinity purification of the GST-and His-fusion proteins was performed on Glutathione-Sepharose resin or Talon magnetic beads using standard isolation protocols. For the experiment shown in Fig.1A, NoxA1 recombinant protein was also expressed and purified as GST fusion protein in *E. coli*, but the GST tag was cleaved by thrombin digestion prior to use following manufacturer's instructions.

For the pulldown experiments, equal amounts of fusion proteins (10 μg) were bound to glutathione-Sepharose beads (10 ul) and challenged with appropriate amounts of His-tagged or untagged NoxA1 recombinant protein, or with 10 μg of DLD1 cell lysate. Unbound proteins were removed by washing the beads three times with RIPA buffer, whereas retained proteins were resolved by SDS-PAGE and analyzed by Western blot. Loading of the GST proteins was checked by staining the filters with Ponceau S (Sigma).

Measurement of ROS

ROS were measured using a luminol-based chemiluminescence assay (CL-assay), essentially as described previously (Gianni *et al.*, 2008). Briefly, 16 hrs after transfection HEK293 or DLD1 cells were harvested and re-suspended in HBSS containing calcium and magnesium. Approximately 5×10^5 cells per assay were dispensed in white 96-well plate (Berthold Technologies, Germany) and mixed with 250 μM luminol and 1 U of HRP (final concentration) in 200 μl total final volume in each well. Chemiluminescence was recorded using 96-well plate luminometer (Berthold) 5 min after the addition of HRP/luminol mixture for 30 min at room temperature without any stimulation. The data output consisting of the emission intensities for each well was imported onto a spreadsheet program (such as Excel; Microsoft, WA) for further processing.

Statistical Analysis

In this study, representative experiments from three independent experiments are shown. Results for chemiluminescence experiment are given as mean of triplicates ± SD. Statistically significant differences between sample groups are determined using *t* tests (Excel, Microsoft). A p value of <0.01 was considered significant.

Results and Discussion

Tks proteins interact directly with NoxA1

In our previous work, we have described that the c-Src substrate proteins Tks4 and Tks5 are functional members of a $p47^{phox}$-related organizer superfamily that selectively sustain Nox1- and Nox3-dependent ROS generation (Gianni *et al.*, 2009). The Tks-mediated

support of ROS production by Nox1 and Nox3 is Rac-dependent and it is due to their ability to bind the activator protein NoxA1. Moreover, such an interaction is not affected by the GTP-binding state of Rac1. However, these experiments do not reveal the nature of the interaction between Tks proteins and NoxA1 nor even whether this interaction is direct or indirect.

To test whether the interaction between NoxA1 and Tks proteins is direct, we performed *in vitro* GST-pulldown experiments using GST-fused organizer subunit proteins (Tks4, Tks5 or NoxO1) and recombinant NoxA1 protein. As shown in Fig. 1A, recombinant NoxA1 protein was able to directly interact with Tks4 and Tks5 as well as NoxO1 protein included as a positive control, while no binding was detected using GST protein alone. Similarly, we performed a reverse pulldown using a recombinant His-tagged NoxA1 protein incubated in the presence of either GST-fused Tks4, Tks5, NoxO1 proteins or GST protein as a control. As shown in Fig. 1B (upper panel), we observed a specific binding between GST-fused Tks4 and Tks5 and His-tagged NoxA1 protein, which indicates that Tks proteins directly bind to NoxA1. Of note, using this approach we again detected an interaction with GST-fused NoxO1 protein used as a positive control, while no binding was found using GST protein alone (lower panel).

The N-terminal region of NoxA1 is necessary for the interaction with Tks proteins

We have previously shown that the interaction between NoxA1 and Tks proteins as well as Tks-mediated ROS generation can be prevented by the disruption of all five Tks SH3 domains (Gianni *et al.*, 2009). The latter have been reported to preferentially bind PRRs, especially those containing a PXXP motif. This suggests the possibility that the PRRs on NoxA1 might be responsible for the interaction with Tks proteins, possibly representing a difference compared with the binding of the other members of the $p47^{phox}$ organizer superfamily, which instead occurs between the C-terminal PRR of the organizer and the SH3 domains of the activator subunit.

To verify this hypothesis, we used bioinformatics tools available online (http://scansite.mit.edu) and scanned human NoxA1 protein (accession number NM_006647) for SH3-binding domains. As indicated in Fig. 4A, we found four PRRs containing PXXP motifs in the first N-terminal 300 aa of NoxA1 surrounding the TPR domains, while no SH3-binding sites were found in the C-terminal region (from aa 301 to 477). Therefore, to understand which region of NoxA1 was necessary for the binding of Tks proteins, we generated a battery of Myc- and GST-tagged NoxA1 deletion mutants. A comprehensive table summarizing all the mutant constructs is shown in Fig. 2A (for Myc-tagged NoxA1 constructs) and in Fig. 2B (for GST-tagged NoxA1 mutants). Their expression was confirmed by Western blot analysis using Myc antibody or by affinity purification followed by SDS-PAGE respectively, as indicated in Fig. 2C and 2D.

To assess whether the four PRRs present in the first 300 aa of NoxA1 were involved in the binding with Tks5, co-immunoprecipitation experiments were performed in HEK293 cells co-transfected with Flag-tagged Tks5 and with the Myc-tagged NoxA1 deletion mutants, as shown in Fig. 3A. The NoxA1 (1–300) deletion mutant bound Tks5 even stronger than the NoxA1 wild-type construct, while almost no binding was detected using the C-terminal region of NoxA1 (301–477) (upper panel). These data indicate that the N-terminal region of NoxA1 is responsible for the interaction with Tks5 and are also suggestive of an intramolecular auto-inhibition mechanism by the C-terminal part of NoxA1. Consistent with this, the C-terminal region of NoxA1 contains an SH3 domain, which has been described to interact with PRR of NoxO1 and $p47^{phox}$ (Takeya *et al.*, 2003), but which it might also bind to the multiple N-terminal NoxA1 PRRs, acting as an intramolecular auto-inhibitory domain for NoxA1 function. Of note, our observations are in agreement with a similar model

proposed by (Valente *et al.*, 2007). Furthermore, as shown in the lower panel, the interaction with Tks5 was not lost even when the NoxA1 (1–211) and (1–155) deletion mutants were tested, while very little binding was observed using the NoxA1 (150–250) and (251–300) constructs. This result strengthens the hypothesis that the three PRRs present in the central region of NoxA1 (from 174 to 286 aa, see Fig. 4A) are probably not involved in the interaction with Tks5. All six Myc-tagged NoxA1 constructs exhibited strong levels of expression as shown by Western blot in Fig. 3B.

To ascertain whether the N-terminal region of NoxA1 is also responsible for the binding with Tks4, we pursued an alternative biochemical approach, performing a pulldown using the recombinant GST-tagged NoxA1 truncated proteins incubated with human DLD1 colon cancer cell lysates, which we had previously demonstrated to endogenously express Tks4. As shown in Fig. 3C, we observed that Tks4 strongly bound the GST-NoxA1 (1–211) protein as well as the full length protein used as a positive control, while almost no binding was detected using either GST-NoxA1 (301–477) and GST protein alone. The Ponceau S stain of the nitrocellulose membrane in Fig. 3D shows that the GST-fusion proteins were present at similar levels in the GST-pulldown analysis. These experiments strengthen the findings illustrated in Fig. 3A and support the hypothesis that the N-terminal region of NoxA1 is responsible for the interaction with the Tks proteins.

The integrity of PRR (34–37) of NoxA1 is necessary for the interaction with Tks5 and for Tks5-mediated ROS generation by Nox1

In our previous work, we had reported that Tks proteins can only mediate Nox1- and Nox3-dependent ROS generation, while they were unable to support ROS production by Nox2, probably reflecting their inability to functionally recruit $p67^{phox}$ (Gianni *et al.*, 2009). Here, our analysis of NoxA1 deletion mutants suggests that the PRR present at the N-terminal region of NoxA1 (34–37) is responsible for the binding of Tks proteins.

To investigate the structural differences between NoxA1 and $p67^{phox}$, we used bioinformatics tools (http://smart.embl-heidelberg.de/ and www.ebi.ac.uk/clustalw/) available online and compared their domain and amino acid composition. As indicated in Fig. 4A (upper panel), although $p67^{phox}$ and NoxA1 share only 28% amino acid identity, their overall domain structure is similar. Importantly, we observed that the PRRs present in the central part of both proteins are conserved, while the most N-terminal PRR of NoxA1 (34–37) is lost in $p67^{phox}$, as indicated in more detail in the sequence alignment shown in the lower panel.

To assess whether Tks proteins are unable to recruit $p67^{phox}$, we performed co-immunoprecipitation experiments in HEK293 cells co-transfected with the expression vector for $p67^{phox}$ and either Myc-tagged Tks5, Tks4 or $p47^{phox}$. As indicated in Fig. 4B (upper panel), no interaction was detected between Myc-tagged Tks proteins and $p67^{phox}$, while a specific binding was observed between $p67^{phox}$ and $p47^{phox}$ as expected. The lower panel shows that the Myc-tagged proteins were present at similar levels in the lysates and in the immunoprecipitates. This experiment suggests that the Tks proteins cannot support Nox2-dependent ROS generation because they fail to interact with the Nox2 activator protein $p67^{phox}$. Moreover, since the PRR (34–37) of NoxA1 is not conserved in $p67^{phox}$, this result strongly pinpoints the pivotal role of this PRR in the ability of NoxA1 to recruit Tks proteins.

To examine the importance of PRR (34–37) in the capacity of NoxA1 to bind Tks proteins, we generated mutant constructs (called NoxA1 AXXA), in which each of the PRRs of NoxA1 was disrupted by the insertion of point mutations changing the proline residue into alanine. Of note, when such a mutation was introduced in PRR (220–226) and (280–286) of

NoxA1, it completely abolished the expression of the protein. The NoxA1 AXXA mutant constructs were tested in co-immunoprecipitation experiments in HEK293 cells for their ability to bind Flag-tagged Tks5. As shown in Fig 4C, we detected an interaction between Tks5 and the NoxA1 mutant of PRR (174–179) as well as NoxA1 wild-type used as a positive control. As previously stated, the NoxA1 mutant of PRR (220–226) was not present in the lysate and therefore not viable for binding to Tks5. Importantly, we noticed that although the NoxA1 mutant of PRR (34–37) was expressed at comparable levels to both NoxA1 wild-type and PRR (174–179) constructs, it lost its ability to recruit Tks5, confirming that the integrity of this motif is essential for this interaction. This data nicely fits with the co-immunoprecipitation analysis illustrated in Fig. 3A (lower panel), in which we have shown that the NoxA1 (1–155) deletion mutant that only contains PRR (34–37) is still able to bind Tks5. Notably, all other NoxA1 deletion mutants which do not contain the PRR (34–37), such as NoxA1 (301–477) or NoxA1 (150–250), lost their ability to bind Tks5.

To investigate whether the binding of NoxA1 to Tks5 mediated by the NoxA1 N-terminal PRR (34–37) has an effect on Tks5-mediated ROS generation by Nox1, we performed a luminol-based chemiluminescence assay in HEK293 cells co-expressing NoxA1 AXXA (34–37) mutant, Nox1, Tks5 and constitutive active Rac1 (Rac1-Q61L). As shown in Fig. 4D, the presence of NoxA1 AXXA (34–37) mutant led to a 2-fold decrease in Nox1-dependent ROS formation compared with NoxA1 wild-type. This confirms that the integrity of the PRR (34–37) of NoxA1 is essential for Tks5 binding and has a functional impact as it is necessary for Tks-induced ROS generation by Nox1. This interesting result represents a difference compared with the binding of the other members of the $p47^{phox}$ organizer superfamily, which occurs between the C-terminal PRR of the organizer and the SH3 domains of the activator subunit (Banfi *et al.*, 2003;Geiszt *et al.*, 2003;Takeya *et al.*, 2003).

Taken together, these results shed new light on the molecular basis of the interaction between NoxA1 and the Tks proteins, providing novel insights into the mechanisms regulating Tks-mediated ROS generation by Nox1. These findings could represent a starting point for the development of new, more effective pharmacologic approaches for the therapeutic treatment of various redox stress-dependent diseases and/or cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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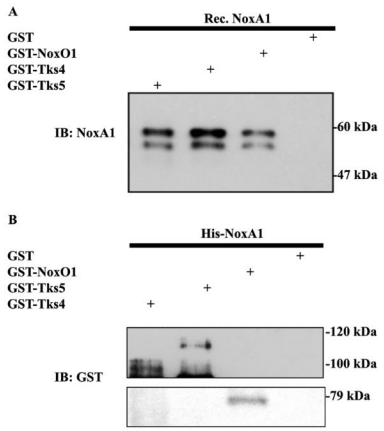

Fig. 1.

The interaction between Tks proteins and NoxA1 is direct. (A) GST-fused organizer subunit proteins Tks4, Tks5 and NoxO1 were bound to glutathione-Sepharose beads and challenged with appropriate amounts of GST-cleaved recombinant NoxA1 protein. GST-pulldown was performed as described in Materials and methods. Bound proteins were resolved by SDS-PAGE and probed with NoxA1 antibody. One representative experiment from three separate experiments is shown. (B) The reverse pulldown approach confirms that the interaction between Tks proteins and NoxA1 is direct. GST-fused organizer subunit proteins Tks4, Tks5 and NoxO1 were incubated with appropriate amounts of His-tagged NoxA1 protein. The pulldown was performed using Talon magnetic beads (see Material and methods). Bound proteins were resolved by SDS-PAGE and probed with GST antibody. One representative experiment from three separate experiments is shown.

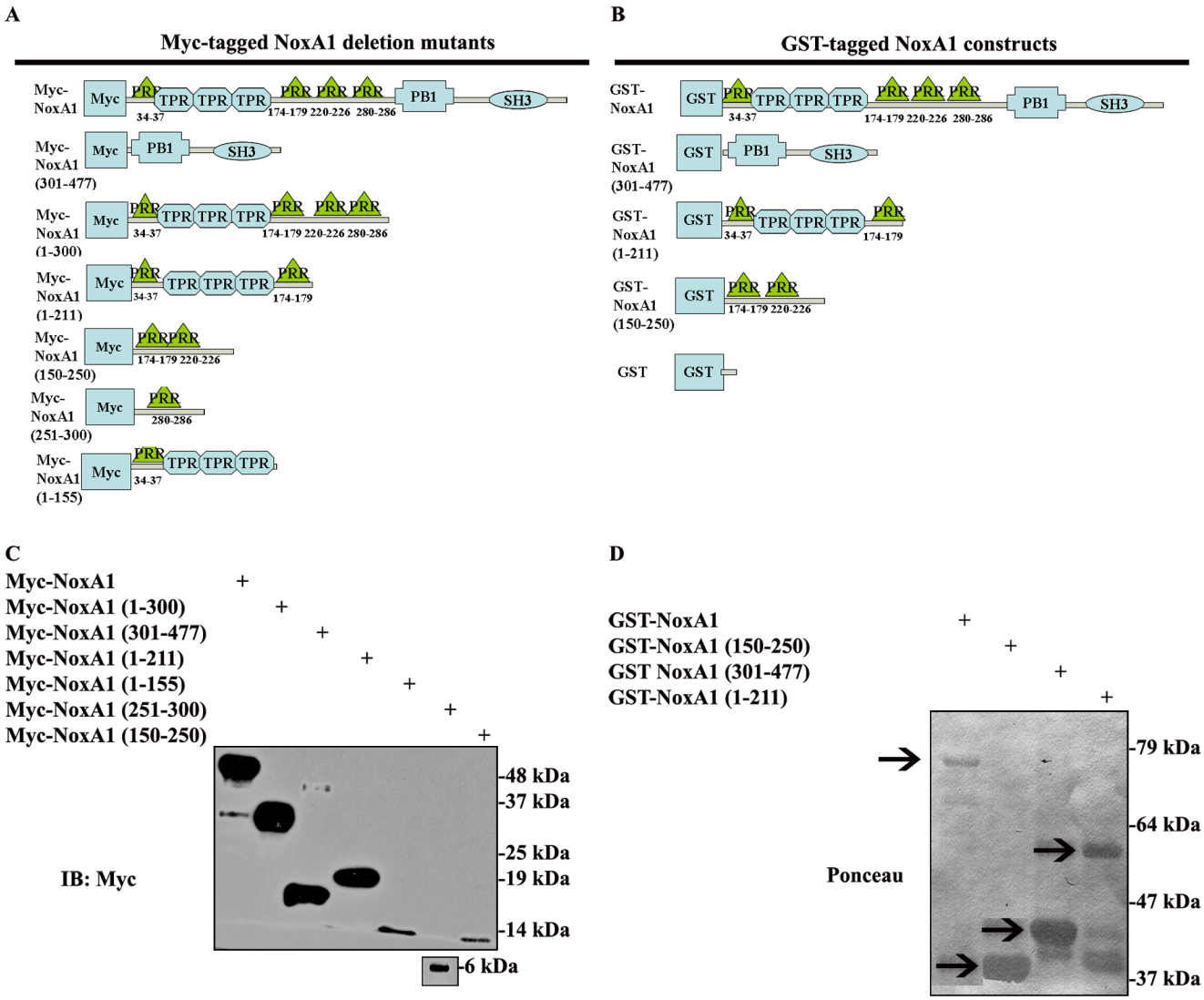

Fig. 2.

Schematic representation and expression of Myc- and GST-tagged NoxA1 deletion mutants. (A, B) Schematic representation of Myc-tagged (A) and GST-tagged (B) NoxA1 deletion mutants which we have used to identify the NoxA1 region responsible for the interaction with Tks proteins. Rectangles indicate N-terminal Myc (A) or GST (B) tag. Triangles indicate Proline-Rich Regions (PRRs). Octagons indicate tetratricopeptide (TPR) domains. Crosses indicate “Phox and Bem 1” (PB1) domain, while ovals indicate Src Homolgy-3 (SH3) domains. (C) Myc-tagged NoxA1 deletion mutants produce a protein of the expected molecular size when transfected into HEK293 cells. HEK293 cells were transfected as indicated with Myc-tagged NoxA1 deletion mutants and after 24 hrs their expression was determined by Western blot as described in Materials and methods using Myc antibody. (D) GST-tagged NoxA1 deletion mutants exhibit the expected molecular size when expressed and purified in *E.Coli* as described in Materials and methods. Purified proteins were then resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and the molecular size of the purified truncated and wild-type proteins (indicated by black arrows) was checked by staining the membrane with S-Ponceau.

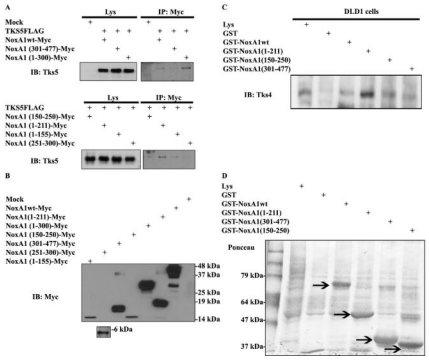

Fig. 3.

The N-terminal region of NoxA1 is responsible for the interaction with Tks proteins. (A, B) Co-immunoprecipitation analysis of Myc-tagged NoxA1 deletion mutants indicates that the first 155 amino acid residues of NoxA1 are involved in the interaction with Tks5. One representative experiment from three separate experiments is shown for each. (A) HEK293 cells were transfected as indicated with Myc-tagged NoxA1 deletion mutants and with Flag-tagged Tks5. After 24 hrs, cells were lysed and immunoprecipitation (IP) was carried out (see Material and methods) using Myc antibody. The interaction between Myc-tagged NoxA1 deletion mutants and Tks5 was tested by immunoblot (IB) using Tks5 antibody. Lys indicates lysate before IP was performed. (B) The expression level of all transfected Myc-tagged NoxA1 deletion mutants was analyzed by Western blot using Myc antibody. (C, D) GST-pulldown analysis confirms that the N-terminal region of NoxA1 is involved in the binding with Tks4. One representative experiment from three separate experiments is shown for each. (C) Cell lysates from human DLD1 colon cancer cells were used as the source of Tks4 protein and incubated as indicated with equal amounts of GST alone or GST-fusion NoxA1 truncated or full length proteins, which were pre-bound to glutathione-Sepharose beads. GST-pulldown was performed as described in Material and methods and the interaction between GST-fusion NoxA1 proteins and endogenous Tks4 was tested using the Tks4 antibody. (D) Ponceau S stain of nitrocellulose membrane from GST-pulldown experiment performed in C shows that the GST-fusion proteins (indicated by black arrows) were present at similar levels in the GST-pulldown analysis.

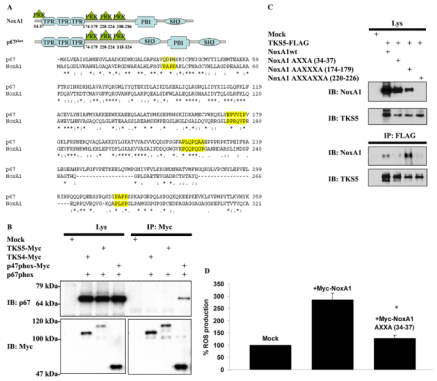

Fig. 4.

The integrity of PRR (34–37) of NoxA1 is necessary for the interaction with Tks5 and for Tks5-mediated ROS generation by Nox1. (A) Schematic representation of domain composition (upper panel) and amino acid sequence alignment (lower panel) of activator subunits NoxA1 and p67^{phox}. The highlighted light gray sequences indicate the conserved PRR between NoxA1 and p67^{phox}, while the highlighted dark gray sequence indicates the N-terminal non-conserved PRR lost in p67^{phox}. (B) Co-immunoprecipitation analysis reveals that Tks proteins cannot bind p67^{phox}. HEK293 cells were transfected as indicated with p67^{phox} and either Myc-tagged Tks4, Tks5 or p47^{phox}. After 24 hrs, cells were lysed and immunoprecipitation (IP) was carried out using Myc antibody. The interaction between Myc-tagged organizer subunits and p67^{phox} was tested by immunoblot (IB) using p67 antibody (upper panel), while comparable expression levels of transfected Myc-tagged organizers in cell lysates and immunoprecipitation efficiency was assessed by re-blotting the membrane with Myc antibody (lower panel). One representative experiment from three separate experiments is shown. (C) Co-immunoprecipitation analysis indicates that the PRR (34–37) of NoxA1 is involved in its interaction with Tks5. HEK293 cells were transfected as indicated with NoxA1 AXXA mutants, NoxA1 wild type and with Flag-tagged Tks5. After 24 hrs, cells were lysed and immunoprecipitation was carried out using Flag antibody. Similar expression levels of Flag-tagged Tks5 and NoxA1 AXXA mutants in the lysates was tested using Tks5 and NoxA1 antibody respectively (upper panels). The interaction between NoxA1 AXXA mutants and Tks5 was tested using NoxA1 antibody (lower panel, top), while comparable immunoprecipitation efficiency was controlled by re-blotting the membranes with Tks5 antibody (lower panel, bottom). One representative experiment from three separate experiments is shown. (D) The NoxA1 AXXA (34–37) blocks Tks5-mediated, Nox1-dependent ROS generation compared with wild-type NoxA1. HEK293 cells were co-transfected as indicated with empty vector or with Nox1, Rac1-Q61L and Tks5 expression plasmids along with either empty vector, wild type NoxA1 or NoxA1 AXXA (34–37). After 24 hrs, ROS production was determined using the luminol-based chemiluminescence assay. One representative experiment from three separate experiments is shown, and data are given as mean of triplicates +/− S.D. * p<0.01 compared to the condition with wild type NoxA1.