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Ras and Nox: linked signaling networks?

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Abstract

Both Ras and Nox represent ancient gene families which control a broad range of cellular responses. Both families mediate signals governing motility, differentiation, and proliferation, and both inhabit overlapping subcellular microdomains. Yet little is known of the precise functional relationship between these two ubiquitous families. In this review, we examine the interface where these two large fields meet.

Keywords

Reactive oxidant species (ROS); signal transduction; NADPH oxidase; transformation; apoptosis; MAPK

On the surface, the downstream consequences of Ras signaling broadly overlap with a subset of cellular events now ascribed to oxidants in general and the Nox family in particular. Ras is known to exert proliferative effects, cause cell shape change, and increase cell motility, all basic cell functions in which various Nox proteins have also been implicated. However, these signaling pathways and their various control points are intricate; in addition, there are seven known Nox/Duox mammalian paralogs and 36 human Ras family genes. At this level of complexity, then, simple mechanistic paradigms are not likely to explain the relationship between Ras and Nox-dependent signaling pathways. Still, Ras and Nox appear to interface recurrently in specific pathways throughout evolution, suggesting that cooperative functional interactions exist between the two protein families. Where and how do these families intersect?

Nox and oncogenic Ras signaling

H- and K-Ras were discovered as the cellular homologues of the transforming proteins of Harvey and Kirsten murine sarcoma retroviruses in 1981 [1,2], setting the stage for much of the subsequent work on Ras GTPases. Abundant work has linked *RAS* mutations with both experimental and naturally-occurring malignancies, and roughly 15–30% of human tumors are now known to possess activating *RAS* mutations. Such mutations are especially common in gastrointestinal cancers, with as many as 60% of pancreatic carcinomas expressing activated forms of K-Ras [3]. Three of the principal Ras effectors—Raf, p110 (the catalytic subunit of PI3K), and RalGDS—have all been shown to facilitate cellular transformation through both proliferative and survival effects. Endogenous Ras was also found to act proximally in growth

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factor-receptor tyrosine kinase pathways through recruitment of its adaptor Grb2 to tyrosine phosphorylated forms of either Shc or the intracellular part of the receptor itself, placing Ras in its physiologic context.

In early landmark studies, rapid increases in oxidant production were found to transduce signals from a variety of extracellular agonists, including TGF β , IL-1, TNF, LPS, and lectin [4–6]. Subsequent studies documented a similar role for oxidants in the initiation of mitogenic signals by the growth factors bFGF and PDGF [7,8], suggesting a possible link between oxidants and Ras. This link was more firmly established with the demonstration that oncogenic H-Ras(V12) increases oxidant production by NIH 3T3 cells, thereby causing cellular transformation, directly linking Ras-dependent mitogenic signaling and oxidant production [9]. This relationship was further strengthened by the observation that nontransforming effects of Ras (V12), such as the senescence induced in primary, nonimmortalized cells, were also blocked by oxidant scavengers [10].

The enzymatic source of oxidants downstream of Ras(V12) has variably been assigned to either a Nox or to mitochondria, possibilities which are not mutually exclusive. A link between Ras and Nox, however, was established when K-Ras(V12) was shown to strongly induce Nox1 expression in a rat fibroblast cell line [11]. EGF and serum also induced Nox1 expression, suggesting its induction by physiologic Ras-dependent pathways as well. In this study, knockdown of Nox1 blocked Ras(V12)-dependent anchorage independence, cell rounding, and tumor formation *in vivo*, confirming the role of Nox1 downstream of Ras(V12)-dependent oncogenic effects in this system. An early report that ectopically expressed Nox1 caused cell transformation [12] appears to have been due to the presence of human Ras(V12) [13]. Nox1 may therefore be necessary but insufficient to cause Ras(V12) transformation.

Less is known about up and downstream events in Nox1 oncogenic signaling. In CaCo-2 cells, GATA-6 appears to transactivate the Nox1 promoter in a MEK-ERK dependent fashion, and H-RasV12 requires an intact GATA binding site in order to activate a Nox1 promoter-luciferase reporter, suggesting phosphorylation of GATA by ERK downstream of H-Ras [14]. These findings suggest that oncogenic forms of either H- or K-Ras may induce Nox1, although when overexpressed in NIH 3T3 cells, wild type H-Ras increases oxidant production whereas wild type K-Ras decreases oxidant levels [15]. Presumably, differences in wild type versus active Ras mutants or perhaps levels of expression may explain such divergent observations. Downstream of Nox1, the VEGF promoter is activated through an ERK/Sp1 pathway [16], correlating with the enhanced tumorigenicity of a Nox1-overexpressing prostate cell line [17]. Another specific oxidant target of the K-Ras-Nox1 pathway is LMW-PTP, whose oxidative inactivation increases p190RhoGAP activity, diminishing RhoA activity and altering cell morphology [18] (Figure 1).

The extent of Nox1's involvement in the pathogenesis of human cancers, which more commonly involve mutations in K-Ras than H-Ras, is unclear at this point. Nox1 is most heavily expressed in the gastrointestinal tract, a site which gives rise to a relatively high incidence of tumors bearing activating K-Ras mutations. Nox1, however, does not appear to be overexpressed in carcinomas from a variety of organs (breast, kidney, ovary, thyroid, stomach, uterus, lung) with varying overall rates of Ras mutations, including colorectal carcinomas and adenomas [19]. In a recent study, however, Nox1 expression was correlated with K-Ras mutations in individual colorectal tumor specimens [20]. Of 12 tumors with activating K-Ras mutations, seven displayed a >5-fold elevation in Nox1 expression compared to normal adjacent tissue, compared with only one of 11 tumors without such mutations. It is possible that Nox1 expression is regionally heterogeneous or stage-dependent, accounting for some of these discrepancies. It is also possible that Nox1 is controlled by other factors besides Ras. In a different study, for instance, 80% of prostate tumors showed markedly increased Nox1

protein levels [21], despite the relatively low prevalence of activating K-Ras mutations in prostate carcinoma.

Likewise, other Nox proteins may contribute to Ras transformation. H-Ras(V12)-dependent transformation of mouse embryonic fibroblasts, for instance, is suppressed by p38a MAPK. Thus, transformation by Ras(V12) is enhanced in p38a^{-/-} cells; in these cells both Nox1 and Nox4 are induced [22]. Indeed, Akt, downstream from Ras, induces Nox4 in melanoma cells and confers an aggressive phenotype to melanoma tumors *in vivo*, whereas Nox4 antisense reduces melanoma cell proliferation [23,24].

Oxidants mediate Ras activation

While the above studies strongly implicate Nox gene induction downstream of Ras, other studies clearly suggest Ras activation concomitant with or as a consequence of oxidant production. Growth factor stimulation, for instance, typically causes both Ras activation and oxidant production within minutes, too short a time for protein synthesis. As both Ras and Nox proteins are membrane targeted, one might expect rapid and coordinated activation of these proteins shortly after receptor engagement. The mitogenic lipid lactosylceramide, as an example, causes oxidant production within one minute, with robust increases in Ras GTP loading and plasma membrane NADPH oxidase activity at the same time point [25]. The flavoprotein inhibitor DPI and N-acetyl cysteine both block this rapid activation of Ras, placing the unidentified Nox upstream of Ras. In more chronic signaling scenarios, there is also the suggestion that oxidants may act upstream of Ras. Nox1 overexpression in NIH 3T3 cells, for instance, causes transactivation of the antioxidant response element ARE4 through ERK and JNK [26]. In this case, ERK, JNK, and ARE4 activation by Nox1 are all suppressed by dominant negative H-Ras(N17), placing Ras downstream of Nox1 but upstream of the MAPKs. Consistent with these observations, PDGF, known to activate Ras and thus PI3K, causes an oxidant burst and Akt phosphorylation within 30 min which are both blocked by antisense RNA against Nox1 [27]. This scenario also suggests the possibility of a positive feedback loop between Ras activation and Nox1 induction, although such a loop has not been studied.

The clearest effect of oxidants on Ras is the direct modification of H-Ras Cys118 [28], which resides on a loop vicinal to the guanine nucleotide and is thus potentially able to influence GTP/GDP exchange. Although initially described with NO₂, a variety of exogenous oxidants including H₂O₂ were subsequently found to increase Ras GTP loading [29,30]. In the case of H₂O₂, millimolar concentrations are required to activate Ras in intact cells, whereas only nanomolar levels activate purified Ras. This scenario presumably reflects the efficient cellular antioxidant system and suggests that any physiologic oxidant source would likely need to be colocalized with the membrane-targeted Ras signaling complex.

Oxidative modification of Ras has best been described in simple *in vitro* systems [28–30], although in several cases, physiologic stimuli also appear to be capable of activating Ras through this mechanism. Advanced glycation end products and cyclic mechanical strain both cause oxidative modification and activation of Ras [31,32]. In the latter instance, cardiac myocyte cyclic strain results in catalase-inhibitable S-glutathiolation of Ras Cys118, implicating H₂O₂ as a proximal oxidant. Notably, the C118S Ras mutant blocked Ras and ERK activation, suggesting a role for Ras oxidative modification in cyclic strain-dependent myocyte hypertrophy.

In physiologic signaling, the source of oxidants upstream of Ras has not been well studied but at least some investigations suggest involvement of Nox protein(s). Besides lactosylceramide (see above), another mitogen thought to stimulate a Nox upstream of Ras is angiotensin II (Ang II). Ang II causes both a rapid, transient burst of oxidants as well as a delayed and sustained phase of oxidant production; both phases are thought to arise from Nox proteins [27,33].

Notably, the Nox2 adapter p47^{phox} is required for Ang II-dependent oxidant production [34], and p47^{phox} is phosphorylated and translocates to the membrane with p67^{phox} within 15 min of Ang II stimulation [35]. Nox2 and Nox1 expression by vascular smooth muscle appears to vary with species and vascular bed [35]; however, p47^{phox} may functionally participate in both Nox1 and Nox2 activation [36]. Accordingly, Ang II activates and causes S-glutathiolation of Ras within 15 min, an effect blocked by either catalase or a nonphosphorylatable p47^{phox} mutant, again implicating either Nox1 or Nox2 upstream of Ras [37]. Interestingly, the Ras (C118S) mutant prevents phosphorylation of p38 MAPK and Akt but not ERK, suggesting that glutathiolated Ras may selectively activate some pathways but not others (Figure 2A). Whether this is a steric effect selectively hindering Ras interactions with specific effectors, or whether this is due to differential activation of certain Ras subpopulations is at this point unknown.

Another clear example of Nox-dependent Ras activation occurs following stimulation of endothelial cells with the angiogenic factor HIV1 Tat. This factor is known to activate an endothelial cell Nox, causing oxidant production, dramatic cytoskeletal rearrangement, and proliferation [38–40]. Again on a rapid time scale (2–5 min), Tat activates both Rho and Ras GTPases, and by 10 minutes, the JNK and ERK MAPKs are simultaneously activated. Of interest, these two MAPK pathways bifurcate beyond Rac1 such that Nox2 specifically activates JNK and causes actin remodeling but has no effect on Ras or ERK activity; in contrast, RhoA and Nox4 are required for Tat-dependent activation of the Ras/MEK/ERK pathway and mitogenic signaling but have no effect on JNK or cytoskeletal rearrangements [40] (Figure 2B). That the two pathways are simultaneously yet independently activated suggests that specific Nox proteins are able to maintain a high level of signaling fidelity, a feature critical for Ras biology. These observations, combined with the fact that Nox1–3 require Rac1 for activation, also suggest an intricate relationship between the small GTPase molecular switches, including Ras, and the Nox family members. Is there evidence that these signaling systems have coevolved to jointly control common cellular processes? The evidence is circumstantial at best, but suggestive.

Evolutionary considerations

Both Ras and Nox ancestral genes arose in eukaryotes, with no convincing evidence for either in prokaryotes [41,42]. Within eukaryotes, *ras* and *nox* genes likely arose separately and, like other genes, perhaps more than once. Nox genes are not present in most unicellular eukaryotes such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Interestingly, however, yeast and plants possess assimilative ferric reductases, which have been likened to Nox proteins as many of these reductases share the six-transmembrane spanning topology and frequently possess NADPH-dependent flavin reductase activity [43]. *S. cerevisiae* in particular expresses a membrane-bound ferric reductase Fre1, a cytochrome *b* with midpoint potential of –250 mV, similar to Nox2; in fact, the production of O₂·⁻ by this reductase has been proposed as an intermediate step to accomplish ferric iron reduction [44]. Red algae and diatoms, primitive unicellular plant organisms, possess what appear to be ancient Nox genes with phylogenetic alignment similar to Nox5 [43]. In plants, the Nox system became well developed by the appearance of eudicotyledons such as *Arabidopsis thaliana*, which expresses ten respiratory burst oxidase homologues (Atrboh) [45]. Phylogenetic analysis of this plant, however, reveals 93 genes that encode small GTPases from the Rab, Rho, Arf, and Ran families, but no Ras GTPases [46]. Despite the lack of Ras proteins, a RhoGDI is necessary to spatially regulate oxidant production by AtrbohC during root hair growth [47]. Thus Nox/Rboh evolution at least in this plant proceeded independently from Ras-dependent functions but not from that of other Ras superfamily GTPases.

Conversely, the yeasts *S. cerevisiae* and *S. pombe* express Ras but no Nox proteins. Studies of Ras in these primitive eukaryotes is instructive, however, and reveal such ancestral Ras proteins

to function at least in part during the differentiation response to nutrient deprivation. Nitrogen starvation triggers filamentous pseudohyphal formation in *S. cerevisiae* which is blocked by deletion of *ras2*, whereas filamentous growth is induced by active Ras2(V19) [48–50]. Similarly, nutrient starvation of *S. pombe* leads to sexual development leading to sporulation, a process controlled by the single *ras1* gene of this yeast, which shares high identity with mammalian H-Ras [51].

Amongst more advanced fungi, Ras continues to control differentiation in response to various stimuli including nutrient deprivation. Filamentous fungi respond to nutrient deprivation by producing aerial hyphae with male reproductive structures at hyphal tips called conidia, which may release spores asexually or fertilize the female ascogonium. Upon fertilization, ascogonia differentiate into fruiting bodies, which contain the sexual spores or ascospores. Notably, acquisition of the capacity to produce fruiting bodies marks the appearance of Nox genes in filamentous fungi [52]. In *Neurospora crassa*, for example, deletion of *nox1* blocks aerial hyphae and conidia formation [53], a phenotype very similar to that resulting from deletion of *ras2* [54]. Loss of Nox1 also blocks development of the fruiting body in *N. crassa*, a developmental program not presently known to be Ras related. *Podospora anserina* Nox1 also controls aerial hyphae formation and development of fruiting bodies [55].

An interesting variant of hyphal growth occurs during the penetration of the rice plant by the filamentous fungus *Magnaporthe grisea* using specialized structures called appressoria. These hyphal structures form following conidial germination from the ends of the germ tube, and require fungal Ras1 and Ras2 upstream of an Mst11-Mst7-Pmk1 MAPK cassette [56]. Recently, the *M. grisea* *nox1* and *nox2* genes have been identified; deletion of either also blocks appressoria formation and thus plant infection [57]. Indeed, NBT and DCF staining of wild type fungi reveal formation of oxidants in conidia and the subsequent appressorium. Thus, in the filamentous fungi, Nox and Ras converge in function at specific stages of differentiation, particularly the early formation of reproductive hyphal structures. Direct links between these two protein families, however, have not been established as of yet.

The amoebazoan *Dictyostelium discoideum* also responds to nutrient deprivation through activation of various Ras pathways. In its amoeboid form, starved individual cells secrete and chemotax toward cAMP to form multicellular slugs. During this migration, Ras activation becomes restricted to the leading edge of the cell through local positive feedback loops with PI3K and exclusion of the RasGAP DdNF1 [58–60]. This form of chemotaxis is also accompanied by a burst of O₂⁻ generated at the plasma membrane and not in mitochondria [61]. Antioxidants, including SOD, therefore suppress Ras-dependent chemotactic migration. Consistent with the involvement of a plasma membrane oxidase in Ras activation, a GPI-anchored plasma membrane SOD (SodC) was recently described in *D. discoideum* [62]. Loss of SodC causes hyperactivation of RasG, one of the Ras forms responsible for Dictyostelial migration [63], with loss of cell polarization and directional migration [62]. While the involvement of a Nox protein seems likely, single knockouts of each of the Dictyostelial Nox genes (*noxA*, *noxB*, *noxC*) do not affect slug formation [64]. It is possible, however, that the Nox forms may overlap in function and compensate for each other; NoxA in particular is found at peak levels in vegetative amoebae, with lower levels of expression of the other two Nox genes [64].

Following Dictyostelial slug formation, a single tip forms in a manner reminiscent of aerial hyphae formation, and differentiates to form the spore-bearing fruiting body. Loss of any of the Nox genes by *D. discoideum* causes abnormal tip formation and thus loss of fruiting body development [64]. Of note, expression of endogenous *rasD* greatly increases during tip formation, and activated mutants of RasD cause multiple-tipped aggregates which do not

develop further [65]. Again, then, Ras and Nox appear to converge at specific developmental stages in this primitive organism, though direct interaction has not been sought.

Spatial coordination of Ras and Nox

A general feature of both Ras and Nox signaling is that signal fidelity is highly dependent upon strict compartmentalization of Ras or Nox into relevant microdomains; thus signaling output depends upon the subcellular location of the complex [66,67]. Cooperative signaling between Ras and Nox would therefore require cohabitation of the two within such subcellular compartments. Given that both are membrane-targeted proteins, is there evidence for such cohabitation?

Raft and caveolar membrane domains clearly house both Ras and Nox components. Roughly half of plasma membrane-associated H-Ras resides within cholesterol-dependent raft microdomains, while K-Ras preferentially localizes to featureless nonraft zones [68,69]. Interestingly, upon activation, H-Ras leaves raft zones by lateral diffusion or internalization; however, raft association is critical for Ras specificity. Ras activation by EGF which occurs within membrane rafts, for instance, preferentially signals through cytosolic PLA₂ through interactions between ERK and the scaffold KSR1, in contradistinction to Ras activated on endomembranes [70]. Further, endocytosis of activated receptor complexes such as EGFR contain Ras within early endosomes which continue to actively signal, hence acting as signalosomes [71,72]. Notably, then, Nox1 and Nox2 also reside in membrane rafts and participate in signaling from this microdomain as well as from early endosomes [73–77]. Both a Nox protein and caveolin-1 are required for Ang II-dependent proliferative signaling, likely through EGFR transactivation [78]. Proliferative signaling also results from abrupt cessation of shear stress through ERK and JNK activation in a process dependent upon both Nox2 and caveolin-1 [79]. While both Ras and Nox activation associated with EGFR phosphorylation are dependent upon raft integrity, however, once again the specific molecular interactions between Ras and Nox within lipid rafts are presently unknown.

Ras is also known to transduce signals from endomembranes which are distinct from those initiated at the plasma membrane. This principal is best demonstrated in *S. pombe*, in which its sole Ras gene controls both morphogenic as well as mating and starvation responses through separate MAPK cassettes. Through differential targeting of *S. pombe* Ras, a recent study clearly demonstrated that Ras1(C215S), which is palmitoylation-deficient and remains restricted to the ER, signals only Cdc42-dependent cellular elongation and not mating or starvation responses [80]. In contrast, replacement of the Ras C-terminal targeting motif with the RitC plasma membrane targeting domain caused failure of cellular elongation but restored the response to starvation and pheromone-induced differentiation.

In mammalian cells as well, both H-Ras and K-Ras are found on ER and Golgi endomembranes, where they initiate specific signaling events. H-Ras restricted to the ER is rapidly activated by growth factors such as EGF, TGF α , and insulin, signaling through ERK and JNK MAPKs but not Akt [81]. This pathway does not appear to require endocytosis but rather activation of ER-resident Ras. Alternatively, K-Ras can be rapidly translocated from plasma membrane to ER, Golgi, and mitochondrial endomembranes and initiate specific signaling pathways [82]. Thus it is of significance that Nox4 appears to be an endomembranous oxidase with most studies reporting strong ER localization [83,84]. Indeed, Nox4 has been reported to mediate the ER stress response [85], a response also triggered by H-Ras(V12) [86]. In addition, following stimulation with HIV1 Tat, ERK is activated in a pathway requiring Nox4-dependent activation of K-Ras [40]. In this situation, Tat causes activation of endogenous Ras on ER membranes where Nox4 is found, and both Ras and Nox4 cooperatively participate in ER signaling (RF Wu and LS Terada, unpublished). Overall, then, both Ras and Nox proteins have been reported

in overlapping subcellular compartments, and their concurrent appearance in membrane rafts, endosomes, and endomembranes suggests functional cooperation in transmitting specific signals.

Conclusions

The Nox and Ras gene families are both relatively ancient, and in primitive organisms they appear to share control of specific pathways responding to basic environmental changes such as nutrient deprivation. As evolution has proceeded, the number of members of each family has increased as has the complexity of their signaling networks. The continued functional cooperation in differentiation, mitogenic, motogenic, ER stress, and perhaps other signaling pathways and the colocalization of Nox and Ras members in relevant microdomains suggest a close molecular interplay between the regulation of focal oxidant production and the dynamic control of the Ras GTPase molecular switches. Future studies in this area are likely to yield new perspectives on how these protein families cooperate in signal transduction events.

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Figure 1.

Role of Nox1 in oncogenic signaling. The active Ras mutant Ras(V12) induces *NOX1* through the GATA-6 transcription factor. Nox1-dependent oxidants appear to inactivate LMW-PTP, thus causing deinhibition of p190-Rho GAP and suppression of RhoA activity, with consequent cytoskeletal changes. Nox1 also causes induction of *VEGF* through ERK and Sp1, increasing angiogenic activity.

Figure 2.

Interaction of Ras and Nox in physiologic agonist signaling. A. Angiotensin II (Ang II) signaling in vascular smooth muscle cells leads to hypertensive changes through activation of Ras through both oxidative modification and by other means. The S-glutathiolation of Ras is thought to be mediated by Nox1 and/or Nox2, downstream of p47^{phox} phosphorylation. B. HIV1 infected cells secrete the pleiotropic factor Tat, which activates both Nox4 and Nox2 downstream of Rac1. The pathways independently activate Ras and ERK (Nox4) or JNK (Nox2) to mediate different physiologic effects on the vascular endothelium.