

# Protection of Epithelial Cells by Keratinocyte Growth Factor Signaling

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Oxidative injury to the lung is associated with widespread injury to the alveolar epithelium, which can be fatal unless the process is controlled and repaired. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, has been shown to protect the lung from a variety of oxidative insults. The mechanism(s) underlying the protective effects of KGF in lung injury is being investigated in many laboratories. Although KGF has potent mitogenic effects on epithelial cells, the proliferative effect of KGF was shown to be abolished in oxygen-breathing animals, but KGF was still able to inhibit alveolar damage. This demonstrates that the protective effect of KGF cannot simply be explained by the ability of KGF to stimulate type II cell proliferation. To identify the mechanisms involved in the protective effects of KGF, we used an inducible lung-specific transgenic approach to overexpress KGF in murine lungs, since constitutive overexpression of KGF in the mouse affects lung development. The transgenic system allowed us to identify the pro-survival Akt pathway as an important mediator of the protective effects of KGF both *in vivo* and *in vitro*. In addition, use of a yeast two-hybrid system led to the identification two proteins p90RSK and PAK4 that associate with the KGF receptor and are important for the protective functions of KGF. Experiments are underway to determine whether the different pathways triggered by KGF all converge on the Akt pathway, or whether they independently induce protective mechanisms that along with Akt are crucial for cell survival.

**Keywords:** Akt; hyperoxia; keratinocyte growth factor; lung; PAK4; p90RSK

The detrimental consequences of hyperoxia on the lung are well recognized; high concentrations of oxygen are toxic for most cellular constituents of the lung and can cause widespread irreversible damage that can be fatal. The damaging effects of oxygen are caused by reactive oxygen species (ROS) such as the superoxide anion ( $O_2^-$ ) and through subsequent enzymatic steps, hydroxyl radicals ( $OH^-$ ) (e.g., in the iron-catalyzed Fenton reaction), which are potent oxidants. The capacity of hyperoxia to induce diffuse alveolar damage has been studied in humans as well as several other species including rats (1), primates (2), rabbits (3), and mice (4, 5). The pathologic sequence of injury and repair are similar across species. The changes seen in the alveolar epithelium in the late stage of hyperoxic injury are similar to those seen in many other forms of alveolar injury. Extensive necrosis of type I epithelial cells, and hyperplasia of cuboidal type II cell occurs. Areas of denuded epithelial basement membrane are present, and hyaline membranes consisting

of fibrin, other plasma proteins, and debris from necrotic cells fill the alveolar space. As in other forms of diffuse alveolar damage, proliferating type II epithelial cells eventually cover the denuded epithelium. In animals that survive beyond this proliferative phase, either the type II cells differentiate into type I cells (6) and alveolar architecture returns to normal, or progressive alveolar fibrosis occurs (7).

Keratinocyte growth factor (KGF), a member of the fibroblast growth factor (FGF) family (also known as FGF-7), is a potent mitogen for epithelial cells (8, 9). The pattern of expression of KGF and its receptor suggest an important role for KGF in mediating mesenchymal-epithelial interactions (10). The receptor for KGF (also called FGFR2-IIIb), which has intrinsic tyrosine kinase activity (11), is expressed specifically on epithelial cells and is diffusely expressed in the Day 11 lung epithelium (12). The expression of KGF in the developing lung needs to be tightly controlled, because transgenic mice transgenic mice constitutively overexpressing KGF display lethal papillary cystadenoma, with marked enlargement of bronchial air spaces (13). However, the need for KGF signaling during development is essential because transgenic mice expressing a dominant-negative mutant of the KGF receptor (KGFR) under the control of the SP-C promoter exhibit embryonic lethality with grossly abnormal lung development, with only two primordial epithelial tubes and no branching morphogenesis (14).

Administration of KGF has been shown to protect lungs of animals from a variety of insults including oxygen (15), radiation and chemotherapy (bleomycin) (5, 16), and acid (17). Thus, intratracheal administration of KGF before oxygen exposure protects rats from hyperoxic injury (15). Intravenous administration of KGF was found to protect mice from oxygen toxicity when administered either before or after oxygen exposure (18). Since KGF mRNA expression has been shown to be induced by cytokines and growth factors such as interleukin (IL)-1 and transforming growth factor (TGF)- $\alpha$  (19), increased production of these molecules from activated alveolar macrophages and other inflammatory cells during hyperoxic injury could potentially stimulate local KGF production, which, in turn, could act on type II cells triggering repair of alveolar damage.

The precise mechanism(s) underlying the protective effects of KGF in lung injury is being investigated in many laboratories. *In vitro* studies reported to date suggest a role in type II cell proliferation (8), inhibition of increased oxidant-induced epithelial cell permeability (20), stimulation of transepithelial Na ion transport (21) and stimulation of DNA repair (22). *In vivo* studies show decreased protein-rich pulmonary edema upon KGF pretreatment both in humans and in animal studies (23, 24). Although KGF induces type II cell proliferation, this proliferative effect of KGF was abolished in oxygen-breathing animals, but KGF was still able to inhibit alveolar damage (18). This demonstrates that the protective effect of KGF within the lung cannot simply be explained by the ability of KGF to stimulate type II cell proliferation. Although *in vitro* studies have shown increased surfactant protein synthesis in the presence of KGF, only modest effects were noted in *in vivo* studies (18). Collectively, while all studies clearly demonstrate a protective role of

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KGF in hyperoxic alveolar damage, the molecular mechanism(s) by which this is achieved has not been adequately addressed. We have used different approaches to investigate the protective effects of KGF in oxidative lung injury. We developed a lung-specific inducible transgenic system to overexpress KGF in lungs of mice in a tightly controlled fashion to study the role of KGF-induced signaling pathways in the protective effects of KGF. In another approach, we have used the yeast two-hybrid system to identify KGFR-associated proteins that are involved in the transduction of pro-survival signals by KGF.

#### IDENTIFICATION OF THE PRO-SURVIVAL AKT PATHWAY IN KGF-MEDIATED PROTECTION USING A CELL-SPECIFIC INDUCIBLE TRANSGENIC SYSTEM

To study the protective effects of KGF *in vivo*, we generated transgenic (Tg) mice that express KGF in an inducible, lung-specific fashion. To minimize leaky expression of the transgene, we used a modified dual repressor-activator tet system to tightly control transgene expression (25). These mice were backcrossed for 10 generations onto the C57BL/6 background. Using these Tg mice in a model of hyperoxic lung injury, we have shown that KGF protects lung epithelial cells from hyperoxia-induced cell death (25). By electron microscopy, we demonstrated preservation of the alveolar epithelial surface in the presence of KGF overexpression (25). However, the endothelium and accessory cells lining the capillaries were not protected in the KGF Tg mice. The epithelial-specific protection by KGF was not surprising, since the KGF receptor is expressed on epithelial and not on endothelial cells. KGF expression in these animals was accompanied by activation of the pro-survival Akt pathway in the lungs of the animals (25). In *in vitro* studies, the protective effect of KGF on oxidant-induced lung epithelial cell death was inhibited by a dominant-negative mutant of Akt (DN Akt), which demonstrated the biological significance of KGF-induced Akt activation (25).

Since KGF-induced Akt activation appeared to be important for the protective effects of KGF, we investigated whether Akt activation *in vivo* would protect cells from oxidant-induced cell death and prolong survival of mice. Toward this end, we used a constitutively active form of Akt (myristoylated Akt; myr-Akt) in which the Src myristoylation signal was fused to the N-terminus of Akt. Myristoylation causes targeting of the enzyme to the cell membrane, which results in its phosphorylation and consequently activation. By intratracheal instillation of Ad-myr-Akt, we have demonstrated inhibition of oxidant-induced injury to the lung and prolongation of survival of mice (26). The protection from hyperoxia correlated with decreased hemorrhage, inflammation, and edema in the lung, and a dramatic suppression of airway cell death (26). Since the adenoviral gene delivery allowed expression of activated Akt in both epithelial and endothelial cells, which could not be achieved by KGF expression alone (because the KGFR is not expressed on endothelial cells), the animals were more resistant to hyperoxia-induced death. Thus, use of a stringently regulated inducible transgenic system for selective expression of KGF in the adult mouse lung allowed us to study the protective effects of KGF on lung epithelial cells. Collectively, our studies show that Akt activation *in vivo* promotes resistance to oxidant-induced cell death (26, 25). Additional recent reports have also shown activation of Akt by KGF in lung epithelial cells (27–29). These results suggest that protection of both epithelial and endothelial cells through activation of pro-survival pathways such as Akt using pharmaceutical agents may provide a better survival advantage during oxidative stress.

#### IDENTIFICATION OF KGFR-ASSOCIATED PROTEINS USING THE YEAST TWO-HYBRID SYSTEM

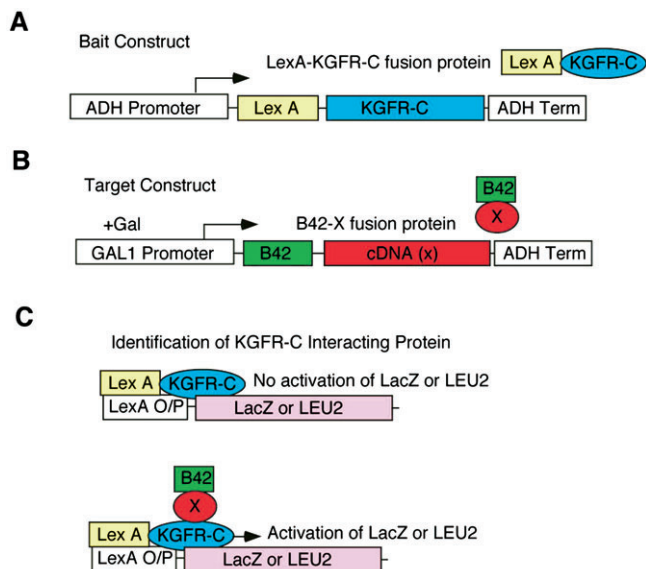
The Akt pathway is induced downstream of multiple growth factor receptors. While dual phosphorylation of Akt on residues Thr<sup>308</sup> and Ser<sup>473</sup> is known to be important for Akt function, the mechanisms involved in Ser<sup>473</sup> phosphorylation and Akt activation are not well understood. Also, although our studies showed Akt activation to be important in KGF-mediated protection, we could not rule out the involvement of additional pathways that may crosstalk with the Akt pathway or may also be important in addition to the Akt pathway in KGF-mediated protection. To identify adaptor proteins that might mediate KGF-induced Akt activation and additional pro-survival mechanisms, we used the yeast two-hybrid system to identify KGFR-interacting proteins. The yeast two-hybrid system has been successfully used to identify interacting proteins for a number of receptor tyrosine kinases (RTKs) including insulin receptor, CSF-1R, Met (HGFR), EGFR, and c-kit (30–32). A key event that triggers interactions between RTKs and other proteins is phosphorylation of the receptor induced by the binding of the cognate ligand. It has been shown that phosphorylation of the receptor is also induced when the receptor is overexpressed in cells. Thus, receptor-interacting proteins have been identified by overexpression of the receptors in yeast cells and many of the interacting proteins identified using this approach have been found to interact in a tyrosine phosphorylation-dependent fashion, demonstrating the validity of this approach. We used the carboxyl terminal domain of FGFR2IIIb (for simplicity called KGFR-C) as bait to identify intracellular proteins that interact with the receptor (Figure 1). We isolated multiple clones that encoded proteins interacting with KGFR-C. We have studied in detail interactions with PAK4 (33) and with p90RSK (34). The following is a brief description of these studies:

##### PAK4

PAK4 is a member of the PAK family of proteins. The p21 Rac/Cdc42-activated kinases (PAKs) are serine/threonine kinases that were identified as downstream targets of the GTPases Rac and Cdc42Hs (35). The PAK members have an N-terminal GTPase (Rac/Cdc42)-binding regulatory domain and a C-terminal kinase domain. PAK4 is the most divergent member of the family in which the kinase domain shares 53% identity with the kinase domains of other PAKs (36). A common property of PAK proteins is their ability to regulate cytoskeletal organization. However, the proteins have been shown to have different roles in the apoptotic response of cells. While PAK2 promote apoptosis (37), PAK1 has been shown to protect cells from apoptosis induced by growth factor withdrawal (38). PAK4 was shown to protect cells from apoptosis induced by multiple stimuli including serum withdrawal, TNF- $\alpha$  treatment, or ultraviolet irradiation (39). Although PAK4 has been shown to be a target for Cdc42, extracellular stimuli that regulate endogenous PAK4 activity have not been identified. Our studies show the ability of activated KGFR to interact with and induce phosphorylation of PAK4 (33). We have also shown that DNP4K4 prevents the ability of KGF to protect small airway epithelial (SAE) cells from hyperoxia-induced cell death (33).

##### p90RSK

The RSK (or p90RSK) family includes three members, RSK1-3, which show 75 to 80% similarity at the amino acid level (40). RSK family members contain two heterologous kinase domains (41). Although originally identified as ribosome S6 protein kinase, S6 protein phosphorylation by RSK family proteins is very restricted and p70<sup>S6K</sup> is the major physiologic S6 kinase (40).



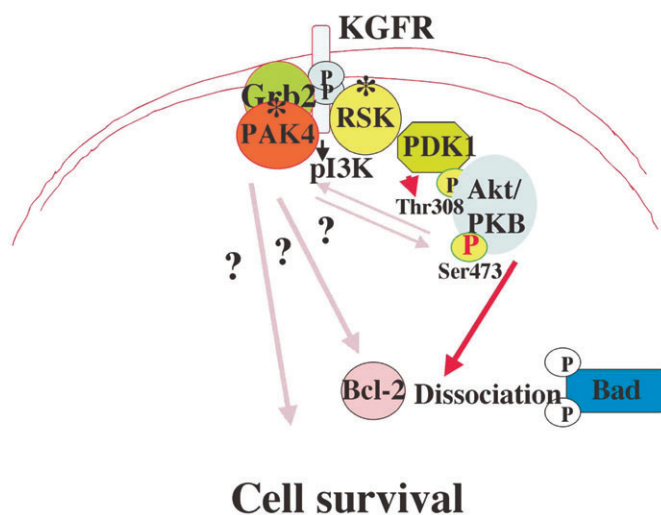
**Figure 1.** Schematic of the strategy used for screening KGFR-interacting proteins using the yeast two-hybrid assay. (A) The KGFR cytoplasmic domain containing the tyrosine kinase domain was cloned into the yeast expression plasmid pEG202-NLS to generate the bait construct. (B) A cDNA expression library constructed from cDNA derived from a 19-d postcoital mouse embryo was fused to the B42 activation domain–HA-tagged expression vector pJG4-5 to generate the target construct. (C) Reporter gene expression was induced only when the KGFR cytoplasmic domain fusion protein (bait) interacted with a target protein. After selecting clones potentially expressing interacting proteins, the specificity of the interaction was confirmed by a yeast mating assay.

RSKs are activated in response to several growth factors and mitogens including EGF (42, 43), insulin, and IGF-I (44, 45). The activated RSKs phosphorylate a number of proteins containing the consensus sequences (R/L)xRxxS and are involved in a wide range of cellular activities (40). Interestingly, the kinase PDK1 has been shown to be recruited by RSK2, resulting in coordinated phosphorylation and activation of PDK1 and RSK2 (46). PDK1 is critical for the activation of Akt and related enzymes, including RSK, that regulate many physiologic processes such as cell survival, proliferation, and gene expression. Since RSK was found to associate with the KGF receptor, it seemed plausible that RSK might actually be involved in Akt activation. Indeed, it has been shown that ERK may be involved in Akt activation under certain circumstances, and the RSK homolog MSK was shown to phosphorylate and activate Akt (47–49).

To test whether RSK1 is involved in Akt activation, we used a mutant of RSK1, RSK1d1, lacking the carboxyl-terminus of RSK1, which includes the ERK binding site of RSK, and checked the Akt kinase activity in cells overexpressing wt or mutant RSK1. KGF stimulation of cells caused a fivefold increase in Akt kinase activity. Overexpression of wtRSK1 caused a three- to fivefold increase in Akt kinase activity independent of KGF stimulation. Overexpression of the mutant RSK1d1, however, caused inhibition of KGF-induced Akt kinase activation (34). These results suggested that RSK proteins are an important component of KGF-induced Akt activation (34).

### RSK1 MUTANT PREVENTS KGF-MEDIATED INHIBITION OF OXIDANT-INDUCED POLY (ADP-RIBOSE) POLYMERASE CLEAVAGE

Since our data suggested an important role for RSK1 in KGF-induced Akt activation, we investigated whether blocking RSK1



**Figure 2.** A model depicting intracellular signaling pathways that have been identified (solid red arrows) downstream of the KGFR with potential interactions shown by patterned lines. The asterisks indicate KGFR-interacting proteins identified by yeast two-hybrid assay.

function would influence anti-apoptotic functions induced by KGF in epithelial cells. For this purpose, we treated cells with  $H_2O_2$ , which has been shown to induce pro-apoptotic pathways in these cells (50). Since apoptosis is associated with caspase-3 activation, a convergence point of different pro-apoptotic pathways, we investigated the activation of both pro-caspase-3 and cleavage of the substrate of caspase-3, poly (ADP-ribose) polymerase (PARP), in the cells under different conditions of treatment. To inhibit the functions of endogenous RSK1, we overexpressed RSK1d1. Treatment of cells with  $H_2O_2$  promoted cleavage of both pro-caspase-3 and the substrate of caspase-3, the PARP protein. Cleavage of both proteins was inhibited in the presence of KGF. However, when the RSK1 mutant was expressed in the cells, KGF was unable to inhibit protein cleavage. Addition of the ERK inhibitor PD98059 inhibited the effects of KGF, suggesting ERK-stimulated RSK activity to be important for KGF-induced effects. These results were similar to our previous observations of the ability of DN Akt and DN PAK4 to block inhibition of PARP cleavage by KGF (25, 33).

### CONCLUSIONS

We have shown that inhibition of any of the three pathways, Akt, PAK4, or RSK1, prevents KGF from exerting its protective effects on epithelial cells. How can we then define a common framework for interactions between these pathways in KGF-induced protective effects? The Akt pathway has been shown to crosstalk with the mitogen-activated protein kinase pathway and promote cell survival. While PAK has been shown to be a downstream target of Ras via PI 3-kinase, one study showed that Akt mediates PAK activation by Ras, and activated PAK (PAK1) promoted cell survival in this study (51). In another study, HIV-1 Nef-associated PI3-kinase and PAK were shown to stimulate Akt-independent Bad phosphorylation to induce cell survival signals (52). Thus, it will be interesting to determine whether the different pathways triggered by PAK4 and RSK1 activation all converge on Akt, or whether they independently induce protective mechanisms that along with Akt are also crucial for cell survival. As illustrated in Figure 2, in the next phase of these studies, we are interested in characterizing the relation-



ships between the different pathways downstream of the KGF receptor that collectively determine the protective functions of KGF.

**Conflict of Interest Statement:** P.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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