Signaling Intricacies Take Center Stage in Cancer Cells

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Abstract

After many years of productive study on the signaling networks, posttranslational regulatory control of effector molecules remains an intensely investigated and continuously evolving field of research to connect signaling with phenotypic changes. In recent years, there have been intriguing results on the interaction of critical molecules to control the growth of cancer cells. This review article will focus on two critical convergence signaling nodules, Akt and p21-activated kinase, two integral components of phenotypic signaling during tumorigenesis. Here we will summarize the recent findings on how these master signaling nodules regulate their targets and alter the subcellular localization of their effectors to control their functionality. Based on the laboratory advances in the Akt and p21-activated kinase signaling pathways, it is conceivable to start defining novel avenues to developed targeted anticancer therapies. (Cancer Res 2005; 65(7): 2511-5)

Introduction

Breast cancer is one of the most common malignancies in the United States, affecting one in eight women. The process of breast cancer metastasis requires, among other steps, changes in signaling pathways, increased directional cell motility, enhanced cell survival, and increased tendency to undergo epithelial-to-mesenchymal transition. The high mortality rate associated with breast cancer is due to a propensity for the tumor to metastasize while the primary tumor is small and undetected; therefore, an understanding of metastasis is urgently needed.

The last decade has witnessed one of the most exciting periods in breast cancer biology. We have begun to learn more about the significance of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases and their critical downstream signaling nodules in the growth control of mammary epithelial cells and pathogenesis of breast cancer. For example, deregulation of the HER2 receptor (also known as ErbB2 or neu) and its effector components such as Akt are frequently associated with aggressive clinical course, decreased disease-free survival, poor prognosis, development of hormone independence, and increased metastasis in human breast cancers (1). Likewise, activation of p21-activated kinase 1 (Pak1), a major target of the small GTPases, growth factors, and lipid signaling, regulates cell motility, hormone action, invasiveness, and survival (2, 3), all of which are required for both tumor development and normal mammary gland development. Interestingly, Akt could also activate Pak1 in a GTPase-independent manner (4) and could potentially influence downstream functions of Pak1. Furthermore, in recent years, cellular localization has emerged as a key mechanism for controlling the functionality of several critical cellular proteins. In this review, we will briefly summarize recent advances, primarily from our laboratories, with a particular focus on Akt and Pak1, their kinase targets, and their subcellular localization in breast cancer cells.

The World of AKT

Akt regulation of cell cycle inhibitors. Overexpression/activation of growth factor receptors such as the HER family activates the phosphatidylinositol 3-kinase/Akt pathway. Akt, a serine/threonine kinase, phosphorylates specific targets to promote cell survival and cell proliferation. On stimulation by growth factors, Akt detaches from the inner surface of the plasma membrane and relocates to the nucleus, suggesting that Akt targets may be also located in the nucleus. In this context, Akt has been shown to influence the cellular localization and functions of p21cip1/WAF1, which induces cell growth arrest by inhibiting the functions of cyclin-dependent kinases in the nucleus (5). In addition to nuclear localization, several studies have indicated that p21cip1/WAF1 can also localize to the cell cytoplasm and this localization was associated with antiapoptotic activity (1). The underlying mechanism of Akt regulation of p21cip1/WAF1 activity includes Akt association with and phosphorylation of p21cip1/WAF1 on a consensus threonine residue (Thr145) in the nuclear localization signal, leading to the cytoplasmic localization of p21cip1/WAF1 (6). Conversely, inhibition of Akt suppresses cell growth in a manner that correlates with the nuclear localization of p21cip1/WAF1. Because activation of growth factor receptors frequently stimulates the kinase activity of Akt, this new signaling pathway provides a novel mechanism for growth factor receptor-mediated cell proliferation by inducing cytoplasmic localization of p21cip1/WAF1 through the serine/threonine kinase Akt. These findings are clinically significant because cytoplasmic localization of p21cip1/WAF1 in primary tumor tissues shows high correlation with overexpression of phospho-Thr145-p21cip1/WAF1 and is associated with worse overall survival in breast cancer patients (7). In brief, these observations signify the emerging need to distinguish the nuclear and cytoplasmic localization of p21cip1/WAF1 when comparing clinical outcomes.

Similarly, Akt can also phosphorylate another cell cycle inhibitor, p27kip1. Phosphorylation of p27kip1 at Thr157 by Akt induces its cytoplasmic localization and also enhances cell proliferation. Cytoplasmic localization of p27kip1 also serves as a marker for poor prognosis in breast cancer patients (8). Thus, cell cycle inhibitors such as p21cip1/WAF1 and p27kip1, thought to be located in the nucleus and to inhibit cell growth, may also be associated with cell survival while in the cytoplasm.

Akt regulation of p53 activity. There are also lines of evidence to suggest that in addition to its cell survival activity, the phosphatidylinositol 3-kinase/Akt pathway can influence apoptosis by delaying the onset of p53-induced apoptosis (9).
The tumor suppressor protein p53 is a transcription factor that can induce either growth arrest or apoptosis and is frequently mutated or deleted in many tumor types (10). The function of p53 is best known to be controlled by a ubiquitin-conjugating E3 ligase, MDM2, which is amplified or overexpressed in a variety of human tumors and can itself function as an oncogene in tissue culture systems (11). In addition to its ubiquitin-related activity, it is known that the nuclear localization signal and the nuclear export signal of MDM2, which are required for MDM2 to shuttle between the nucleus and the cytoplasm (10, 11), are critical for ubiquitin conjugation and subsequent degradation of p53.

In studies of the contribution of Akt to HER2-mediated resistance to DNA-damaging agent, Akt was found to enhance MDM2-mediated ubiquitination and degradation of p53. Akt interacts physically with MDM2 and phosphorylates it at Ser166 and Ser186. The phosphorylation of MDM2 enhances its nuclear localization, increases its interaction with the histone acetyltransferase p300, and inhibits its interaction with p19ARF. All of these molecular events contribute to enhanced p53 degradation. Correlation between the activation of Akt and nuclear localization of MDM2 is also observed in human breast tumor tissues (12). Because p53 expression can be stimulated in response to DNA-damaging chemotherapeutic drugs, this study also shows that blocking the Akt pathway could enhance p53 expression and increase the cytotoxic effect of DNA-damaging drugs in tumor cells in conjunction with wild-type p53. These findings have two important clinical implications: first, degradation of p53 by MDM2 through Akt-mediated MDM2 phosphorylation may contribute to HER2/Akt-induced drug resistance; second, combination therapy of HER2/Akt inhibitors and chemotherapy may have better therapeutic efficacy for tumors with HER2/Akt amplification and wild-type p53.

Akt-IκB kinase-β regulation of forkhead activity. Akt regulates the transcription of a variety of genes through attenuation of the activity of another of its kinase targets, the forkhead FOXO transcription factor, FOXO3a (FKHRL1). Phosphorylation of FOXO3a by Akt results in the relocation of phosphorylated FOXO3a from the nucleus into the cytoplasm, where it binds with the protein 14-3-3. This binding physically masks the FOXO3a nuclear localization signal and prevents its reentry into the nucleus, resulting in cytoplasmic retention and blocked transcriptional regulation (13). Consistent with these observations, a significant correlation exists in human primary tumors between active, phosphorylated Akt and cytoplasmic FOXO3a. However, in some tumor specimens with negative phosphorylated Akt, FOXO3a is still excluded from the nucleus, suggesting that other mechanisms may retain FOXO3a in the cytoplasm of tumor tissues. In the search for additional pathways, IκB kinase-mediated degradation of FOXO3a through phosphorylation of FOXO3a by IκB kinase-α/β was discovered. IκB kinase-α and IκB kinase-β interact with and phosphorylate FOXO3a primarily at Ser644, resulting in cytoplasmic localization and then degradation of FOXO3a. IκB kinase-β was also shown to enhance tumorigenicity in human breast cancer cells through down-regulation of FOXO3a. FOXO3a also possesses tumor suppressor activity when ectopically expressed in breast cancer cells (14). Thus, FOXO3a represents a cellular target for IκB kinase and its fate after phosphorylation by IκB kinase seems to be similar to that of the IκB family members with regards to their cytoplasmic retention, ubiquitination, and degradation. Cancer cells may acquire sustained resistance to apoptosis through an orchestrated inhibition of FOXO3a and IκB by IκB kinase, which can lead to constitutive survival signaling, suggesting that NF-κB-mediated stimulation of cell proliferation and tumorigenesis may require a concurrent inhibition of FOXO3a. In addition, in certain tumor cell lines deficient in PTEN, a tumor suppressor that inhibits phosphatidylinositol 3-kinase/Akt pathway, the forkhead transcriptional factors are located in the cytoplasm and transcriptionally inactive. Thus, forkhead transcriptional factors have also been postulated to be associated with tumor suppressor activity. Indeed, ectopic FOXO3a expression in breast cancer cells inhibits tumorigenicity, and nuclear FOXO3a expression in breast tumor tissues correlates with a better overall survival for breast cancer patients (ref. 14; Fig. 1).

Akt control of epithelial-to-mesenchymal transition via glycogen synthase kinase-3β. In addition to target effector molecules such as p21cip1/waf1, MDM2, and FOXO3a, Akt also regulates other kinases. One example is glycogen synthase kinase-3β. Akt phosphorylates glycogen synthase kinase-3β at Ser9 to make it inactive. Glycogen synthase kinase-3β has recently been found to target a transcriptional factor, Snail, which down-regulates E-cadherin and regulates epithelial-to-mesenchymal transition (15). In epithelial-to-mesenchymal transition, epithelial cells harbor fibroblast-like properties and exhibit reduced intercellular adhesion and increased motility. This process is associated with the functional loss of E-cadherin. In cancer, dominant transcriptional repression is largely responsible for the loss of E-cadherin expression, and this down-regulation of E-cadherin is a critical element towards the invasive phase of carcinoma. Several transcription factors have been implicated in this repression, including zinc-finger proteins of the Snail/Slug family, αE71/ZEB1, SIP1, the basic helix-loop-helix E12/E47 factor (16), and twist (17). Snail in Drosophila was first shown to down-regulate the transcription of shotgun (an E-cadherin orthologue) to control gastrulation (16). Subsequently, Snail was shown to inhibit the expression of E-cadherin and induce epithelial-to-mesenchymal transition in mammalian cells, suggesting that Snail has a fundamental role in epithelial-to-mesenchymal transition through its suppression of E-cadherin (15). However, partly due to short half-life of Snail, how Snail expression was regulated is virtually unknown until a recent study reported that glycogen synthase kinase-3β serves as an upstream regulator of Snail. Glycogen synthase kinase-3β binds to and phosphorylates Snail at two consensus motifs to dually regulate the function of this protein. Phosphorylation of one motif regulates its β-Trcp-mediated ubiquitination, whereas phosphorylation of the other motif controls its subcellular localization. A variant of Snail (Snail-6SA), which possesses mutated glycogen synthase kinase-3β phosphorylation residues, resides exclusively in the nucleus and shows significantly increased protein stability. Expression of this mutant notably induces epithelial-to-mesenchymal transition in cell culture (15). Furthermore, inhibition of glycogen synthase kinase-3β results in the up-regulation of Snail and down-regulation of E-cadherin in vivo. Thus, Snail and glycogen synthase kinase-3β together may function as a molecular switch to regulate epithelial-to-mesenchymal transition. In brief, Akt seems to regulate the functions of its substrates by influencing their subcellular localization.
The World of Pak

**p21-activated kinase 1 regulation of cell motility and invasiveness.** The acquisition of an invasive phenotype by human breast cancer cells has also been attributed to the stimulation of cytoskeleton signaling by growth factors. The Paks, an evolutionarily conserved family of serine/threonine kinases, represent a major convergence node in growth factor and cytoskeleton signaling (2, 3). Because Pak1 was originally thought to be involved in directional cell motility in fibroblasts, and because cell motility is important for productive metastasis, initial studies in this area focused on exploring the role of Pak1 in breast cancer. These studies revealed a definitive role for Pak1 in heregulin-mediated stimulation of breast cancer cell motility and invasiveness (18). Pak1 regulation of cancer cell motility requires cytoskeleton remodeling involving Pak1 phosphorylation of the p41-Arc on Thr21 of the Arp2/3 complex (19) and of filamin A at Ser2152 (20). Activation of HER2 and HER3 receptors by heregulin activates Pak1 and stimulates its relocalization to the leading edges of cells, where it promotes cell migration and invasiveness (18). Heregulin-induced cytoskeletal reorganization and cell invasion of breast cancer cells depend on Pak1 activity and a functional HER2 receptor, as these phenotypic changes are effectively blocked by a pharmacologic inhibitor of phosphatidylinositol 3-kinase (an upstream activator of Pak1), dominant-negative Pak1 mutants, or anti-HER2 antibody Herceptin (18). Interestingly, both phosphatidylinositol 3-kinase and Pak1 are co-hyperactivated in breast tumors (reviewed in ref. 3).

In recent years, Pak1 overexpression and hyperactivation have been linked with the invasiveness of human breast cancer cells and breast tumors (21, 22). In contrast, inhibition of Pak1 in MDA-MB435 cells results in excessive cell spreading, accumulation of focal points, and reduced cell invasiveness (23). Pak1 is also co-overexpressed with cyclin D1 in breast (21). Expression of Pak1 in breast tumor tissue correlates with tumor grade, with higher expression in less differentiated ductal carcinomas of the breast (grade 3 tumors) than in grade 2 and grade 1 tumors (reviewed in ref. 3).

**p21-activated kinase activation and DNA ploidy.** Recent data suggest that Pak1 signaling could potentially contribute to the process of tumorigenesis by promoting chromosomal abnormalities, as Pak1 hyperstimulation has been intimately linked with abnormal organization of mitotic spindles in breast cancer cells (22). Because Pak1 phosphorylates histone H3.3 at Ser10 (24) and because Ser10 modification of histone H3 has been linked with regulation of mitotic chromosomal assembly, congregation at the metaphase plate, and segregation, it is possible that activated Pak1 might lead to incorrect DNA ploidy.
of tumor cells. Another study suggested that this might be also linked with the ability of Pak1 to regulate microtubule reorganization. It was found that Pak1 phosphorylates tubulin cofactor B at Ser65 and Ser128 on newly synthesized microtubules. Further, overexpression of TCoB in several human epithelial cell lines dramatically increased the number of γ-tubulin–containing microtubule-organizing center in a Pak1-dependent manner (25).

**p21-activated kinase 1 regulation of estrogen receptor action.** Clues about the role of Pak1 in the mammary gland come from a recent transgenic model expressing a kinase active Pak1 mutant under a conditional promoter, β-lactoglobulin. Persistent Pak1 activation in murine mammary gland leads to a widespread hyperplasia, lobuloalveolar hyperdevelopment during lactation, and stimulation of the estrogen receptor-α target genes (26). Results from the mechanistic studies reveal that Pak1 regulation of estrogen receptor signaling pathways involves Pak1 phosphorylation of estrogen receptor at Ser305 in the AF2 domain, leading to increased estrogen receptor transactivational activity (26). Accordingly, estrogen receptor Ser305 activation is sufficient to stimulate cyclin D1 expression (3) and to hypersensitize breast cancer cells to hormone response (27). Because growth factor signaling can activate Pak1, these observations imply a role for Pak1 signaling in stimulating the estrogen receptor pathway in estrogen receptor ligand-independent breast cancer phenotypes. However, because the consensus phosphorylation motif in Pak substrates is not restricted to the Pak kinases, in principle estrogen receptor Ser305 could be activated by other kinases. Consistent with such a notion, a recent report showed that protein kinase A also phosphorylates estrogen receptor at Ser305 and that this modification induces tamoxifen resistance (28), as expected from previous literature (26). Thus, estrogen receptor phosphorylation at Ser305 represents an important modification for ligand-dependent and ligand-independent functions of estrogen receptor in breast cancer cells. Because Pak1 can be activated by growth factors (2) and also directly interacts with estrogen receptor (26), it has been proposed that Pak1 may constitute an important point of cross-talk between growth factors and the estrogen receptor. Because the transactivation functions of estrogen receptor are influenced by the dynamic regulatory interactions of estrogen receptor with coactivators or corepressors, which in general are phosphorylated proteins, it is tempting to speculate that Pak signaling or other downstream activators such as HERs may influence the phosphorylation status of estrogen receptor coregulators, and could thus also modulate the outcome of estrogen receptor response.

**p21-activated kinase 1 regulation of cell survival.** One mechanism by which Pak1 signaling might contribute towards cancerous phenotypes is enhanced cell survival, as Paks have been shown to phosphorylate and inhibit the proapoptotic functions of Bad, caspase-3, caspase-8, and FKHR (2). Pak1-phosphorylated Bad exhibits reduced interaction with Bcl-2 and Bcl-xl and increased interaction with the regulatory protein 14-3-3. In addition, Pak1 signaling also confers enhanced cell survival in breast cancer cells by phosphorylating dynein light chain 1, a critical component of the dynein motor complex, on Ser88. Results from mechanistic studies suggest that the antiapoptotic function of Pak1 is derived, in part, from its recognition of DLC1-BimL dimers, which are released on apoptotic signals from the dynein motors to initiate apoptosis. Pak1 phosphorylates both DLC1 and BimL, thus preventing BimL from inactivating Bcl-2 (29).

**p21-activated kinase 1 modulation of corepressor functions.** There is a growing effort within the phenotypic signaling field to link signaling-triggered phosphorylation-dependent modulation of master coregulatory factors with transcriptional outcomes. One example of such influence is Pak1 phosphorylation and functional regulation of the COOH-terminal binding protein (CtBP), a NAD-dependent corepressor with numerous interacting proteins. Growth factor–activated Pak1 phosphorylates CtBP selectively on Ser158 within a putative regulatory loop, triggering CtBP cellular redistribution within the nucleus and to the cell cytoplasm and blocking CtBP corepressor functions (30). In the presence of NADH, Pak1 superphosphorylates CtBP and inhibits CtBP dehydrogenase activity, suggesting that preferential phosphorylation of activated CtBP may alter secondary structures and influence enzymatic and corepressor functions (30). Pak1 regulation of CtBP offers a new transcriptional regulatory pathway whereby a signaling kinase can inactivate a corepressor and stimulate target genes by derepression. Such a mechanism of signaling- and NAD-dependent regulation of corepressor function offers a potential route for functional cross-talk with pathways important in cell metabolism, reactive oxygen balance, and cell redox state, to directly influence gene transcription through HER family signaling activity.

In addition, Pak1 could also influence the Notch signaling pathway that plays an important role in determination of cell fate. Recent results suggest that Pak1 interacts and phosphorylates SHARP, a component of the Notch signaling, on Ser3486 and Thr3568. It was found that Pak1-SHARP interaction plays an essential role in SHARP-mediated repression of Notch target genes in human cancer cells (31).

Another example of signaling-triggered phosphorylation-dependent regulation of corepressor function is Pak1 phosphorylation of Snail, a critical regulator of epithelial-to-mesenchymal transition discussed earlier. Repression of E-cadherin transcription seems to be specifically relevant in the late steps of epithelial tumorigenesis due to a causal relationship that exists between the loss of E-cadherin and the invasive properties of tumors. Furthermore, the corepressor function of Snail may be regulated by its intracellular location as mentioned earlier (32). Pak1 phosphorylation of Snail on Ser246 also supports the accumulation of Snail in the nucleus as well as its repressor functions. A Ser246-Ala substitution in Snail or Pak1 knockdown by short interfering RNA blocks Snail phosphorylation on Ser246, leading to increased cytoplasmic accumulation of Snail and attenuation of Snail repressor activity in breast cancer cells. Thus, in principle, Pak1 or its upstream activators such as HER family, which initiate signaling, could control the process of epithelial-to-mesenchymal transition by controlling Snail phosphorylation, and consequently, its functions in breast cancer cells. In addition, because Snail is also regulated by glycogen synthase kinase-3β through phosphorylation, it will be interesting to see whether glycogen synthase kinase-3β– and Pak1-mediated Snail phosphorylation might influence each other (Fig. 1).

**Conclusion.** Because Akt and/or Pak signaling is important in the process of tumorigenesis, any interference with these molecules or with its critical downstream substrates may be a useful therapeutic approach. Because both Akt and Pak1 are prosurvival molecules, interfering with their activity and signaling should induce apoptosis in tumors, leading to tumor regression. Because Akt/Pak1 represents a common convergence

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nodule of receptor tyrosine kinases, phosphatidylinositol 3-kinase, 
RAS, small GTPases and lipid signaling, any potential effect of Akt 
and/or Pak1 signaling on the biology of breast cancer is likely to 
be applicable to other cancer types and should aid in the 
development of targeted anticancer therapies.

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