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Active p21-Activated Kinase 1 Rescues MCF10A Breast Epithelial Cells from Undergoing Anoikis[1]

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Abstract

The protein kinase, PAK1, is overexpressed in human breast cancer and may contribute to malignancy through induction of proliferation and invasiveness. In this study, we examined the role of PAK1 in the survival of detached MCF10A breast epithelial cells to test whether it may also regulate the early stages of neoplasia. MCF10A cells undergo anoikis, as measured by the cleavage of caspase 3 and poly(ADP-ribose) polymerase (PARP), after more than 8 hours of detachment. Endogenous Akt, PAK1, and BAD are phosphorylated in attached MCF10A cells, but these phosphorylation events are all lost during the first 8 hours of detachment. Expression of constitutively active PAK1 or Akt suppresses the cleavage of caspase 3 and PARP in detached MCF10A cells. Co-overexpression of active PAK1 with dominant-negative Akt, or of active Akt with dominant-negative PAK1, still suppresses anoikis. Thus, Akt and PAK1 enhance survival through pathways that are at least partially independent. PAK1-dependent regulation of anoikis is likely to occur early in the apoptotic cascade as expression of dominant-negative PAK1 increased the cleavage of the upstream caspase 9, while constitutively active PAK1 inhibited caspase 9 activation. These results support a role for activated PAK1 in the suppression of anoikis in MCF10A epithelial cells. *Neoplasia* **7,** 638–645

Keywords: Breast carcinoma, apoptosis, cell survival, caspase activation, protein kinases.

Introduction

Epithelial cells undergo apoptosis when they lose contact with the extracellular matrix (ECM), or bind to an inappropriate integrin [1]. This phenomenon has been called anoikis, and it prevents shed epithelial cells from colonizing elsewhere and thus protects against neoplasia [2]. Anoikis is also an important mechanism in the initial cavitation step of embryonic development [3] and in mammary gland involution [4], and has been studied extensively in angiogenesis research [5]. Failure of anoikis possibly contributes to the malignancy of mammary glands and other carcinomas [6–8].

The p21-activated kinase (PAK) family is homologous to the yeast sterile 20 (Ste 20), and regulates a wide variety of cellular responses, including cell morphology, proliferation, and survival. PAKs were first identified in screens for binding targets of Rac and Cdc42 [9]. Multiple pathways lead to activation of PAK1 [10], some of which [e.g., G$\beta\gamma$ subunits that stimulate PAK1 through activation of phosphatidylinositol 3-kinase (PI3-kinase) and Akt] are independent of Rac1/Cdc42 [11]. Activation of PAK1 by diverse signals leads to autophosphorylation at several sites, including threonine-423 (T423) within the activation loop of the kinase. PAK1 phosphorylation at T423 has been linked to its activation, as substitution of the acidic residue glutamic acid (E) at this site yields a constitutively active T423E PAK1 enzyme [12].

PAKs are involved in the regulation of the apoptotic death pathway. In Jurkat cells, for example, expression of dominant-negative PAK1 inhibited fragmentation into apoptotic bodies, and PAK function is also required for the stimulation of JNK by Fas [13]. However, in HeLa and NIH3T3 cells, overexpression of wild-type or constitutively active PAK4 protects these cells from apoptosis in response to serum withdrawal, UV irradiation, and TNFα treatment. Expression of PAK4 inhibits the activation of caspase 3–like enzymes, and specifically promotes the phosphorylation of BAD on serine-112 (Ser-112) [14]. PAK1 is activated by IL-3 (a cytokine) in FL5.12 lymphoid cells, and active PAK1 protects these cells from apoptosis by phosphorylating BAD [15]. Inhibition of PAK1 has also been reported during detachment-induced death of NIH-3T3 fibroblasts [16].

Abbreviations: EGF, epidermal growth factor; ECM, extracellular matrix; GPCR, G protein–coupled receptor; HA, hemagglutinin; LPA, lysophosphatidic acid; PAK, p21-activated kinase; PARP, poly(ADP-ribose) polymerase; PI3-kinase, phosphatidylinositol 3-kinase

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PAK1 has been strongly implicated in breast cancer. It is overexpressed in human breast cancer [17,18], probably, at least in some cases, due to gene amplification [19]. PAK1 has been shown to mediate cellular effects of polypeptide growth factors on the motility and invasiveness of human breast cancer cells, and to promote their anchorage-independent growth [20,21]. In murine models, it was shown that inhibition of PAK1 kinase activity by a dominant-negative fragment or by short-interference RNA drastically reduced transactivation functions of estrogen receptor-α. Mammary glands from mice expressing constitutively active T423E PAK1 (PAK-TE) developed widespread hyperplasia during lactation [22]. Additional work performed by this group revealed that estrogen rapidly activated PAK1 in a PI3-kinase–independent manner. Furthermore, estrogen induced the phosphorylation and perinuclear localization of the cell survival forkhead transcription factor, FKHR, in a PAK1-dependent process. PAK1 directly interacts with FKHR and phosphorylates it [23]. A further connection from PAK1 to mammary hyperplasia is that PAK1 activity stimulates cyclin D1 expression [17].

Detachment-induced apoptosis is suppressed in epithelial cells transformed by *ras* and *src* oncogenes [1]. Active forms of Ras protein are capable of protecting cells from anoikis by stimulating PI3-kinase through direct interaction with the catalytic p110 subunit, leading to the activation of Akt [24]. Ras transformation involves the synergistic effects of the Ras/Raf/MAPK pathway and the PI3-kinase/Akt pathway [25]. In studies of Ras transformation of Rat-1 fibroblasts, it has been shown that PAK is necessary for the cooperative transformation of Rat-1 fibroblasts by Ras, Rac, and Rho [26–29]. Akt may be a key intermediate between Ras and PAK1 in this pathway. In human breast cancer, suppression of anoikis by activated Ras has recently been reported to be independent of both ERK MAP kinases and PI3-kinase/Akt [30].

In this study, we were interested in whether PAK1 plays a role in cell survival in MCF10A human breast epithelial cells. MCF10A cells are derived from MCF10M human epithelial cells, which were obtained from a woman with fibrocystic disease [31]. The first spontaneous immortalization of the MCF10M cells resulted in MCF10A and MCF10F cells, which are routinely used as normal immortalized breast epithelium controls for studies of human breast cancer cells lines [32]. We are able to show that, within 24 hours of growth in suspension, MCF10A cells begin undergoing apoptosis as evidenced by cleavage of caspase 3 and poly(ADP-ribose) polymerase (PARP). Overexpression of active PAK1 or active Akt blocks cleavage of caspase 3 and PARP, revealing a protective role for these kinases in the prevention of anoikis. These results indicate that inappropriate activation of PAK1 could play a role in aberrant cell survival in human breast epithelial cells.

Materials and Methods

Plasmids and Antibodies

Antibodies to the C-terminus of PAK1 (c19) and to caspase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphoPAK1 (Thr-423)/PAK2 (Thr-402), phosphoAkt (Ser-423), total Akt, total BAD, phosphoBAD (Ser-112 and Ser-136), and phospho-p70 S6 kinase (Thr-421 and Ser-424) were purchased from Cell Signalling Technology (Beverly, MA). The caspase 9 antibody was purchased from Stressgen (Victoria, BC, Canada). PARP antibody was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Anti–glyceraldehyde-3-phosphate dehydrogenase (GADPDH) monoclonal antibody was purchased from Calbiochem (San Diego, CA). Lipofectamine 2000 Plus was purchased from Invitrogen (Carlsbad, CA). Plasmids used in this study include pRK7 and pRK7mycPAK1 [10]. Expression vectors pRK7mycPAK-299R and pRK7mycPAK-TE were prepared from pCMV6mycPAK-299R and pCMV6mycPAK-TE, respectively (kind gifts from Jonathan Chernoff), by subcloning *Bam*HI/*Eco*RI fragments into pRK7. Dominant-negative Akt, pCMV6.HA.Akt-K/M, and active Akt, pCMV6.myr-Akt.HA, plasmids were kind gifts from Alex Toker.

Cell Culture and Transfection Assays

Immortalized nonmalignant human breast epithelial MCF10A cells were cultured as described previously [33]. Briefly, MCF10A cells were incubated in 100-mm dishes in DMEM/F-12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. MCF10A cells were transfected with Lipofectamine 2000 Plus reagent with 20 μg of pRK7myc-PAK1 plasmid, 20 μg of pRK7mycPAK-299R, 20 μg of pRK7mycPAK-TE, 10 μg of pCMVHA.Akt-K/M, 10 μg of pCMVHA.Akt, 10 μg of pCMVHA.myr-Akt, or with 20 μg of pRK7 plasmid alone (as a negative control). Control transfections using a pRK7 plasmid with an enhanced green fluorescent protein insert demonstrated that approximately 20% of the cells was strongly fluorescent and a similar additional proportion exhibited much weaker fluorescence (data not shown).

Anoikis Assay

Cells were trypsinized 48 hours posttransfection, transferred to 15-ml conical tubes, washed, collected by centrifugation for 5 minutes at 500*g*, and counted. A total of 1.5×10^6 cells were transferred to either 100-mm culture dishes or 100-mm polyhema-coated Petri dishes, and cultured as previously described [33]. At the indicated times, the suspended cells were washed twice with phosphate-buffered saline (PBS) and then lysed with 200 μl of boiling 2$\times$ Laemmli buffer. The medium from the attached cells was reserved to a 15-ml conical tube. The attached cells were harvested by trypsinization, combined with the appropriate reserved medium, and centrifuged. The cells were washed twice with PBS and then lysed with 200 μl of boiling 2$\times$ Laemlli buffer.

Agonist Treatment and Detection of Active Endogenous PAK1

MCF10A cells were grown to confluency on 100-mm culture dishes and treated as previously described [10,11].