Localization of p21-activated Protein Kinase γ-PAK/Pak2 in the Endoplasmic Reticulum Is Required for Induction of Cytostasis*

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The intracellular localization and physiological functions of the p21-activated protein kinase γ-PAK have been examined in human embryonic kidney 293T and COS-7 cells. At 1–4 days post-transfection, cell division was examined in human embryonic kidney 293T and COS-7 cells. At 1–4 days post-transfection, cell division is inhibited by the expression of wild type (WT) γ-PAK and the mutant S490A, whereas cells expressing S490D and the inactive mutants K278R and T402A grow exponentially, indicating a role for γ-PAK in the induction of cytostasis. WT γ-PAK and S490A are localized in a region surrounding the nucleus identified as the endoplasmic reticulum (ER), as determined by immunofluorescence, whereas K278R, T402A, and S490D lack localization. As shown by sucrose density gradient centrifugation, WT γ-PAK, S490A, and endogenous γ-PAK are distributed among the high density (ER-associated), intermediate density, and low density fractions, whereas the mutants that do not inhibit cell division are present only as soluble enzyme. The amount of endogenous γ-PAK associated with the particulate fractions is increased 4-fold when cell division is inhibited by ionizing radiation. γ-PAK in the ER and intermediate density fractions has high specific activity and is active, whereas the soluble form of γ-PAK has low activity and is inactive. The importance of localization of γ-PAK is supported by data with the C-terminal mutants S490D and Δ488; these mutants have high levels of protein kinase activity but do not induce cytostasis and are not bound to the ER. A model for the induction of cytostasis by γ-PAK through targeting of γ-PAK to the ER is presented in which γ-PAK activity and Ser-490 are implicated in the regulation of cytostasis.

The p21-activated protein kinases include group I PAKs (α-PAK (Pak1), β-PAK (Pak3), and γ-PAK (Pak2)) and group II PAKs (Pak 4, 5, and 6). The members of group I are activated by autophosphorylation through binding of the small G proteins Cdc42 and Rac1, whereas the activation of group II enzymes has not been clearly identified (1–5). Within the group I Pak family, γ-PAK has distinct properties compared with other PAK isoforms. γ-PAK appears to be ubiquitous, whereas α- and β-PAK have greater tissue specificity. γ-PAK is primarily inactive in dividing cells and is transiently activated when cells are subjected to moderate stress conditions such as hyperosmolarity, ionizing radiation, and DNA-damaging drugs (1, 6, 7). Phosphatidylinositol-3-kinase and tyrosine kinase activity have been identified as upstream activators of γ-PAK in response to ionizing radiation and araC (7). A function for γ-PAK was suggested initially by microinjection of active γ-PAK into early frog embryos; active γ-PAK inhibited cell cleavage, whereas inactive γ-PAK had no inhibitory effect (8). γ-PAK is also involved in apoptotic signal transduction and can be cleaved and activated in vitro by caspase 3, a member of the cysteine-aspartic acid protease cascade activated during apoptosis, and in vivo under anti-Fas apoptotic induction (9–11). Because of the cytotoxic nature of γ-PAK, the enzyme activity and protein level are tightly regulated. In addition to transient activation by Cdc42 in response to moderate stress, γ-PAK is degraded through the proteosome pathway after ubiquitination (12). Inactive γ-PAK has been shown to be protected from degradation and to accumulate by association with other proteins, as shown with c-Abl (12).

In contrast, α-PAK responds to growth-related signals, such as platelet-derived growth factor (13), epidermal growth factor (14), inflammatory factors such as interleukin-1 and angiotensin (15) and to sphingosine (16). Subcellular localization has been shown to be important for the physiological functioning of α-PAK. α-PAK localizes to cortical actin structures in growth factor-stimulated cells (13), resulting in cytoskeletal reorganization, including membrane ruffling, filopodia extension, focal complex formation, and a decrease in actin stress fibers (17, 18). Interaction of α-PAK with the adaptor protein Nck leads to translocation of α-PAK to the cellular membrane and stimulation of activity (13, 19, 20).

All members of the group I PAKs contain two functional domains, an N-terminal regulatory domain (residues 1–246 in γ-PAK) and a C-terminal catalytic domain (residues 247–524). The G protein binding site, which is also conserved in the Ste20 PAK isoform in Saccharomyces cerevisiae (21), is located at residues 73–108 in γ-PAK (1). In γ-PAK, Lys-278 coordinates ATP in the active site, and the mutant K278R is kinase inactive (9, 22). In the absence of an activator, γ-PAK is autophosphorylated at five sites but not activated (23, 24). Upon binding of Cdc42(GTP) or cleavage by caspase 3, an additional three sites are autophosphorylated, resulting in activation of the protein kinase. A site shown to be involved in activation of γ-PAK is Thr-402, the conserved threonine in the activation loop; the mutant T402A (mimicking nonphosphorylated threonine) has little protein kinase activity (9, 23). K/R/K/S/T is the preferred recognition sequence phosphorylated by γ-PAK (25). Ser-490 in the sequence KRGS is a potential autophosphorylation site for γ-PAK and for phosphorylation by protein kinase C. Ser-490 is
located adjacent to a region involved in binding to the β-subunit of the trimeric G protein (26). This region is removed in the truncated mutant Δ488 that lacks the C-terminal 36 amino acid residues.

Conditions of hyperosmolarity and the addition of sphingosine to cultured cells result in translocation of the ER protein (27, 28). This region is removed in the particulate fraction and are localized in the endoplasmic reticulum (ER). In contrast, the kinase-inactive γ-PAK mutants K278R and T402A and the C-terminal mutants S490D and Δ488 are present only as soluble enzymes and are present in the nucleus and the cytosol. WT γ-PAK and S490A inhibit cell growth, whereas the other mutants have no inhibitory effect. Taken together, the data show that subcellular localization of active γ-PAK is essential for the induction of cytostasis.

EXPERIMENTAL PROCEDURES

Materials—GTP-γ-S was from Roche Molecular Biochemicals. [γ-32P]ATP was purchased from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Invitrogen. Rabbit anti-calreticulin polyclonal antibody was from Chemicon International. Mouse anti-HA antibody to ensure that identical amounts of proteins were loaded on each lane. To activate γ-PAK by cleavage with caspase 3, the immunoprecipitates were incubated in 30 μl of caspase cleavage buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol, and 0.1% CHAPS) and incubated in the presence or absence of caspase 3 for 30 min at 37 °C. After activation of γ-PAK by cleavage, the immunoprecipitates were incubated in 30 μl of PBS with 1 μg of Cdc42 preloaded with 0.18 mM GTPγS for 5 min at 30 °C. Protein kinase activity was assayed in a volume of 70 μl in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 30 mM 2-mercaptoethanol, 0.2 mM ATP, 1 μg of histone 4, and [γ-32P]ATP (2,000 dpm/μmol) for 30 min at 30 °C; these were kinetically valid conditions. Phosphorylation of histone 4 was analyzed on a Phast gel in 15% polyacrylamide gels followed by autoradiography.

To analyze protein kinase with similar amounts of recombinant WT and mutant γ-PAK protein, the number of cells was adjusted, and the level of protein was determined by Western blotting. Lysates were prepared from 2.4 × 106 cells for WT γ-PAK and S490A, from 9 × 106 cells for Δ488, and from 3 × 106 cells for K278R. The proteins were analyzed by Western blotting as described above. Radiolabeled histone 4 was excised and counted in a scintillation counter. Aliquots (10 μl) of the same immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-HA antibody to ensure that identical amounts of proteins were loaded on each lane. To assay for protein kinase activity with the synthetic heptapeptide S3 (ARRESAA) 1 μM S3 was added to the assay described above in place of histone 4. Phosphorylated S3 was analyzed on P81 phosphocellulose paper, as described previously (25).
for the green fluorescent protein positive signal under an inverted microscope (Eclipse TE300, Nikon). The same sample was fixed with 4% formaldehyde for in situ immunofluorescence staining of γ-PAK with anti-HA antibody as described. The coexpression of γ-PAK and green fluorescent protein was evaluated using a fluorescence microscope (Eclipse E800, Nikon). Total cells (1,000–2,000) and fluorescent cells were counted from multiple images.

Sucrose Density Gradient Fractionation—To correlate the subcellular localization with γ-PAK activity, sucrose density gradient fractionation of the postnuclear supernatant from 293T cells was carried out as described (28, 29) with some modifications. At 48 h post-transfection, ~3 × 10⁶ cells were harvested and washed with PBS and with buffer A as described by Sfeir and Veis (29). The cell pellet was resuspended and kept on ice for 5 min in 250 µl of buffer A and then homogenized with a Dounce homogenizer (pestle B). The homogenization was monitored under a light microscope and generally resulted in >98% cell lysis.

After centrifugation at 1,000 × g for 10 min at 4 °C, the pellet was discarded, and the postnuclear supernatant was fractionated as described below.

Preparation of sucrose density gradients followed the procedure described by Rexach and Schekman (28) with some modifications. The postnuclear supernatant (250 µl) was loaded onto the top of a 2.5-mL gradient and centrifuged for 2 h at 30,000 rpm in a Beckman rotors (SW 60Ti) at 4 °C. Eleven fractions of 250 µl each were collected from the bottom of the tube. The protein in each fraction was precipitated by the addition of an equal volume of 10% trichloroacetic acid and 50% acetone at −20 °C for 1 h. After centrifugation at 16,000 × g for 5 min at 4 °C, the pellet was washed twice with cold acetone, air-dried, resuspended in 30 µl of SDS sample buffer, and analyzed by SDS-PAGE on 7.5% polyacrylamide gels. The proteins were blotted onto a nitrocellulose membrane and probed with antibody to the HA tag. Endogenous γ-PAK was detected with goat anti-γ-PAK polyclonal antibody N19 followed by horseradish peroxidase-goat anti-rabbit IgG.

Calreticulin was detected with rabbit antibody PA3-900 followed by horseradish peroxidase-goat anti-rabbit IgG antibody. Ionizing radiation of 293T cells was performed as described previously (6). Cells were then incubated for 2 h before analysis of localization of γ-PAK activity. To examine γ-PAK activity, Nonidet P-40 was added at a final concentration of 1% to a 30-µl sample from each fraction from the sucrose density gradient. The samples were incubated in the presence and absence of caspase 3 for 30 min at 37 °C and assayed with histone 4 as described above. Phosphorylation of H4 was quantified using a Bio-Rad phosphorimaging IMAGEquant program. Specific activity was determined by assaying for protein kinase activity under kinetically valid conditions (see above), and γ-PAK protein was quantified by immunoblotting. The cleavage of γ-PAK by caspase 3 was confirmed by immunoblotting with anti-HA antibody after SDS-PAGE, as described above, to detect HA-tagged γ-PAK p58 (full-length) and p27 (caspase-cleaved N terminus) in the fractions.

RESULTS

Effects of γ-PAK Expression on Cell Proliferation—To examine the effects of γ-PAK expression on cell proliferation, recombinant WT γ-PAK and three mutants were expressed in COS-7 cells and analyzed over a 4-day period. Expression of γ-PAK was detected by immunofluorescence using anti-HA tag antibody, and antibody to calreticulin was used to determine the total number of cells. Approximately 7% of the cells were transfected, and the number of cells increased ~6-fold over the 4-day period, with a doubling time of 24 h (Fig. 1A). The number of cells expressing WT γ-PAK remained constant over time, whereas the nontransfected cells continued dividing. The approximate number of cells expressing K278R on day 1 was comparable with the WT γ-PAK; however, the cells with K278R continued dividing over the first 3 days and remained at that level on day 4, the time limit for protein expression through transient transfection. S490A had an expression pattern similar to that of WT γ-PAK, whereas expression of S490D was comparable with that of K278R. The results indicate that expression of WT γ-PAK and S490A resulted in induction of cytostasis, whereas K278R and S490D had no effect on cell division.

Experiments with 293T cells showed that the number of cells expressing the WT γ-PAK and S490A remained constant over a 2-day incubation period, whereas the nontransfected cells continued dividing. Thus, the percentage of cells expressing these two forms of γ-PAK was reduced from 21–24% on day 1 to 10–11% on day 2 (Fig. 1B). Cells expressing the kinase-inactive mutants K278R and T402A and the C-terminal mutants S490D and D488 were expressed at around 30% on days 1 and 2, indicating that 293T cells transfected with these mutants continued dividing. Thus, the data supported the conclusions reached with COS-7 cells, that WT γ-PAK and S490A induced a cytostatic response.

Analysis of Expressed Recombinant γ-PAK—The WT and mutant forms of HA-tagged γ-PAK were analyzed after expression in 293T cells. γ-PAK in the supernatant and particulate fractions was analyzed by SDS-PAGE and immunoblotting with antibody to the HA tag. As shown in Fig. 2A, WT γ-PAK was present in both the supernatant and the particulate fractions. The amount of recombinant WT γ-PAK in the particulate fraction was 80% of that in the supernatant. Recombinant γ-PAK comigrated on SDS-PAGE with the major band of endogenous γ-PAK (58 kDa) present in the soluble and particulate fractions (as detected with N19 antibody).

The kinase-inactive mutant K278R was expressed at a level 8.8-fold higher than the recombinant WT enzyme but was
present only in the supernatant (Fig. 2A). A similar expression pattern was observed with T402A, which contained a mutated autophosphorylation site in the activation loop. When two mutants of the potential autophosphorylation site Ser-490 were examined, S490A (mimicking nonphosphorylated serine) was expressed at a level slightly less than WT γ-PAK and was present in both fractions. The level of S490D (mimicking phosphoserine) was 8.1-fold higher than the WT γ-PAK, a level similar to that of the kinase-inactive mutants, and was present only in the supernatant. The kinase-active mutant Δ488, which lacked the C-terminal 36 amino acid residues including Ser-490, was expressed at a 3.3-fold higher level than the WT and was present only in the supernatant. With the double mutants K278R/S490A and K278R/S490D, the amount of expressed protein and the pattern of expression were similar to those of K278R. Wild type γ-PAK and S490A migrated as a 58-kDa protein on SDS-PAGE, whereas K278R, T402A, S490D, and the double mutants migrated at 60 kDa. In contrast, the catalytic domain of γ-PAK was expressed at levels 20–50-fold lower than those of the full-length wild-type protein and was highly unstable in 293T cells as well as in insect cells (data not shown). The level of expression of WT γ-PAK was ~40% of the endogenous γ-PAK protein in 293T cells. The relative levels of expression and mobility on SDS-PAGE of WT γ-PAK and the mutants are the same in COS-7 and 293T cells.

Protein Kinase Activity of Wild Type and Mutant γ-PAK Expressed in 293T Cells—To measure the protein kinase activity of γ-PAK expressed in 293T cells, ~5 × 10^6 cells were collected at 48 h post-transfection. γ-PAK was immunoprecipitated from the cell lysates with anti-HA antibody and assayed with the specific substrate histone 4. Basal γ-PAK activity was observed with WT γ-PAK, S490A, S490D, and Δ488, which was enhanced further upon cleavage with caspase 3 (Fig. 2B). The immunoprecipitate of S490D had higher kinase activity, consistent with the higher protein expression level of this mutant. K278R, and T402A had essentially no protein kinase activity either before or after cleavage, as expected.

To measure the relative specific activity of the different forms of γ-PAK, the number of 293T cells was adjusted to obtain approximately the same amount of γ-PAK; the cells were collected at 48 h post-transfection, and γ-PAK was immunoprecipitated and assayed with histone 4 and peptide S3. As shown by the immunoblot in Fig. 3A (bottom panel), approximately the same amount of γ-PAK protein was present in each sample. In the absence of an activator, similar low levels of basal γ-PAK activity were observed with WT γ-PAK, S490A, and Δ488, with very low activity for S490D. Cleavage with caspase 3 or binding of Cdc42(GTP^S) stimulated phosphorylation of H4 to a similar extent. This was 2–3-fold when assayed with histone 4 or with S3 (Fig. 3, B and C), whereas the activity of S490D was increased 6-fold because of a lower basal activity.

Immunolocalization of Recombinant Wild Type and Mutant γ-PAK—To examine the subcellular localization of γ-PAK, COS-7 cells transfected with WT and mutant forms of γ-PAK were probed with anti-HA tag antibody; fluorescence was visualized by confocal microscopy. WT γ-PAK was localized around the nucleus in a broad band, as shown in Fig. 4. In contrast, K278R was present both in the cytosol and in the nucleus. S490A was localized around nucleus in a pattern similar to that of WT γ-PAK, whereas S490D lacked distinct localization, similar to that observed with K278R. To identify the site of localization of WT γ-PAK and S490A around the nucleus, the cells were probed with antibody against the ER marker calreticulin. As shown by image overlays (yellow), WT γ-PAK and S490A colocalized specifically with calreticulin in the ER. When 293T cells transfected with WT γ-PAK were examined in a similar manner, identical results were obtained. In addition, T402A and Δ488 were present in both the cytosol and the nucleus (data not shown), similar to that observed with K278R and S490D (Fig. 4).

Subcellular Localization of Endogenous and Recombinant γ-PAK by Sucrose Density Gradient Centrifugation—To examine the subcellular localization of γ-PAK further, the post-nuclear supernatant from 293T cells was subjected to sucrose density gradient centrifugation. After centrifugation, endogenous γ-PAK was identified in nontransfected cells by Western blotting with anti-HA antibody, and recombinant γ-PAK was identified by anti-HA antibody. Fractions 1–10 contained a gradient ranging from 55 to 15% sucrose, respectively, whereas fraction 11 was the position of the sample loading. As shown in Fig. 5A, endogenous γ-PAK in actively dividing cells was localized in three places in the gradient, in fractions 1–4 (high density; particulate), in fractions 6 and 7 (intermediate density; particulate), and in fractions 10 and 11 (low density; soluble). The ER marker calreticulin, identified with anti-calreticulin antibody, was colocalized with γ-PAK in the ER (fractions 1–4) and was present in soluble fractions 10 and 11.
counter. Phosphorylation of H4 by WT was quantified by excision of the H4 band and counted in a scintillation counter. The cell lysate was prepared as described in Fig. 2. 

Fractions 6 and 7 (intermediate density), and 9–11. These results correlated well with the immunocytochemistry data showing that native and recombinant WT γ-PAK, as well as S490A, were localized on the ER. It is important to note that in dividing cells, the ER-associated and intermediate density fractions contained 10 and 5% of the total endogenous γ-PAK protein, respectively; the majority of the endogenous γ-PAK was present as the soluble form. In comparison, the majority of the recombinant WT γ-PAK and S490A were located in the particulate fractions. To examine this further, 293T cells were subjected to ionizing radiation to inhibit cell proliferation. Under these conditions, more than half of the endogenous γ-PAK was translocated to the ER (36%) and intermediate density fractions (20%) (Fig. 5B). The mutant K278R was not translocated to the ER after ionizing radiation. 

Because S490D had the same intracellular distribution profile as the inactive mutants of γ-PAK, but also had high protein kinase activity, the role of the C-terminal region in subcellular localization was examined further using the truncated mutant Δ488. Like S490D, Δ488 was detected only as a soluble form (Fig. 5A). These data indicated that the C-terminal region was required for localization of γ-PAK in the ER. A comparison of the data with S490A and S490D suggested that phosphorylation at Ser-490 could have a role in regulating the release of γ-PAK from the ER. The results with the double mutations K278R/S490A and K278R/S490D indicated that localization of S490A in the ER could be overruled by the K278R mutation.

To examine γ-PAK activity in the different subcellular fractions, HA-tagged WT γ-PAK was immunoprecipitated from pooled fractions 1–4, 5–8, and 9–11. The immunoprecipitates were preincubated in the absence or presence of caspase 3 and assayed for protein kinase activity with histone 4. The specific activity was calculated based on the phosphorylation of histone 4 and the amount of γ-PAK protein in the fractions. The ER-associated form of γ-PAK (fractions 1–4) and γ-PAK with the intermediate density fraction (fractions 5–8) were active and were not activated further by cleavage with caspase 3 (Fig. 5C). In contrast, the soluble form of γ-PAK was activated 3-fold by caspase 3.

A comparison of endogenous γ-PAK with that of the recombinant WT was measured in a similar manner. As shown in
Fig. 5. Distribution of the γ-PAK protein and protein kinase activity to the ER as determined by sucrose density gradient centrifugation. The postnuclear supernatant was prepared from transfected and nontransfected 293T cells incubated for 48 h and subjected to sucrose density gradient fractionation. A, endogenous γ-PAK was detected with anti-γ-PAK antibody N19. Recombinant γ-PAK was detected with anti-HA tag antibody. The ER was identified with anti-calreticulin antibody. B, irradiated 293T cells were treated with ionizing radiation, incubated for 2 h, and the lysates were subjected to sucrose density gradient centrifugation as in A. γ-PAK was detected with anti-γ-PAK antibody. 293T cells transfected with the mutant K278R were used as a control. The activity of γ-PAK in different sucrose density fractions was quantified with the NIH Image program. C, the sucrose density gradient fractions (200 μl each fraction) were pooled as 1–4 (ER-associated fractions), 5–8 (intermediate density fractions), and 9–11 (soluble fractions). HA-tagged WT γ-PAK was immunoprecipitated from the pooled fractions, incubated in the presence or absence of caspase 3, and assayed with histone 4. The 32P incorporated into histone 4 was analyzed by SDS-PAGE followed by phosphorimaging quantification.

Fig. 6. Comparison of endogenous and recombinant γ-PAK activity. The sucrose density gradient fractions were carried out as described in Fig. 5. Thirty μl of each fraction was incubated in the presence or absence of caspase 3 and assayed with histone 4; phosphorylation was analyzed by SDS-PAGE and quantified by phosphorimaging. A, activity of γ-PAK in each sucrose density fraction. B, the sucrose density gradient fractions were pooled as 1–4 (ER-associated fractions), 5–8 (intermediate density fractions), and 9–11 (soluble fractions). The specific activity of the recombinant WT was calculated by subtraction of the endogenous γ-PAK activity in nontransfected cells from the total activity in transfected cells. The γ-PAK protein was determined by Western blotting as shown in Fig. 5, and the specific activity was calculated using these values. Cleavage of recombinant γ-PAK is shown after immunoprecipitation and analysis by SDS-PAGE and Western blotting.

Fig. 6A, γ-PAK activity in nontransfected cells was also associated with the ER (fractions 1–4) and in the intermediate density fractions (fractions 5–8). γ-PAK activity associated
with the ER could not be activated further upon cleavage by caspase 3. The soluble form (fractions 9–11) had the lowest activity and could be activated further by cleavage with caspase 3 (Fig. 6A). Upon expression of recombinant WT γ-PAK, total γ-PAK activity was increased ~35% and was distributed in a pattern similar to the endogenous enzyme. γ-PAK in the ER-associated and intermediate fractions was not activated by caspase 3, whereas the soluble form was activated by such cleavage. γ-PAK was cleaved by caspase 3 to an equal extent in all three fractions, as shown in the immunoblots by the appearance of the p27 fragment containing the N-terminal region of γ-PAK (Fig. 6B). This cleavage was dependent on prior dissociation of γ-PAK from the ribosome. When γ-PAK was associated with the ER, the protein was protected from cleavage (data not shown).

The specific activity of γ-PAK was calculated from the activity measurements of endogenous γ-PAK with H4 and the protein content in the sucrose density gradient. The particulate forms of γ-PAK had significantly higher specific activities (20-fold and 27-fold, respectively) than the soluble form (Fig. 6B). Cells transfected with recombinant γ-PAK had ceased dividing, which correlated with significantly higher levels of γ-PAK associated with the particulate fractions; 50% of the γ-PAK protein was associated with the ER, and 15% was present in the intermediate density fractions. The ER-associated and intermediate forms of recombinant WT γ-PAK had 5- and 10-fold higher specific activities compared with the soluble form. The results indicated that endogenous and recombinant γ-PAK in ER and intermediate density fractions were highly active and that the level of γ-PAK associated with particulate fractions was significantly greater when cell growth was inhibited.

DISCUSSION

The effects of γ-PAK on cell proliferation were examined by the expression of WT and mutant γ-PAK in COS-7 and 293T cells. The number of cells expressing WT γ-PAK and S490A remained constant over a 4- to 5-day period, indicating that both WT and S490A inhibit cell growth. With the kinase-inactive and -activable C-terminal mutants S490D and Δ488, the cell number increased exponentially along with the nontransfected cells, indicating that these mutants have no inhibitory effect on cell growth. The inhibitory effect of WT γ-PAK expressed in COS-7 and 293T cells was also observed with NIH 3T3 cells and with cells infected with retroviral expression of γ-PAK (data not shown). The data indicate that expression of the WT γ-PAK or S490A induces cytostasis.

Recombinant WT γ-PAK and S490A are associated with the ER as shown by immunocytochemistry and by sucrose density gradient centrifugation; the protein kinases are also detected in the intermediate density fractions and as soluble enzymes. In contrast, the active mutants S490D and Δ488, and the inactive mutants K278R and T402A, are present in both the nucleus and the cytosol and do not bind tightly to the ER, as determined by sucrose density gradient centrifugation. The ER and intermediate density fractions with WT or S490A contain >50% of the total recombinant γ-PAK protein. These particulate forms of γ-PAK are already active and cannot be activated further, whereas soluble γ-PAK has a low specific activity and can be activated by cleavage with caspase 3. This pattern is the same as that of native γ-PAK in dividing cells, except that only 15% of the endogenous γ-PAK is associated with the ER and intermediate density fractions. Considering that proliferating cells have primarily inactive endogenous γ-PAK, ionizing radiation was used to render 293T cells in the quiescent state. Within 2 h after ionizing radiation, ~50% of endogenous γ-PAK was translocated to the ER. The major difference between cells transfected with WT γ-PAK or S490A, and cells transfected with kinase-inactive mutants K278R and T402A or C-terminal mutants S490D and Δ488, is that the former are unable to undergo cell division, whereas the latter continue dividing. These data indicate that localization on the ER results in activation of γ-PAK, and the increase in association of γ-PAK with the ER correlates directly with inhibition of cell growth.

Recombinant WT and mutant γ-PAKs have different expression patterns and different properties in mammalian cells. WT γ-PAK and S490A migrated as 58-kDa proteins on SDS-PAGE compared with the kinase-inactive mutants K278R and T402A and the active mutant S490D, which migrate as 60-kDa proteins. K278R, T402A, and S490D are expressed at an 8-fold higher level than WT γ-PAK and S490A. Native γ-PAK purified from rabbit reticulocytes also has electrophoretically distinct forms; the 58-kDa form is active, whereas the 60-kDa form has little or no protein kinase activity (8). The differences in electrophoretic mobility of the recombinant forms of γ-PAK during SDS-PAGE correlate with differences in cytostatic activity and could reflect structural differences resulting from mutation or differential phosphorylation of the WT and mutant proteins.

As shown by immunofluorescence, K278R and S490D are expressed at significantly higher levels compared with WT γ-PAK and S490A and do not localize specifically in the ER. This was confirmed in human embryonic kidney cells by immunofluorescence (data not shown) and by sucrose density gradient analysis. Localization of WT γ-PAK and S490A, but not kinase-inactive mutants or C-terminal mutants, suggests that both protein kinase activity and localization of γ-PAK to the ER are required for the induction and maintenance of cytostasis. These conclusions are supported by previous studies showing that γ-PAK is targeted to the particulate fraction by Cdc42 in response to hyperosmolarity and activated at the membrane (6). Cdc42 also results in growth inhibition in mammalian cells (30, 31). This could occur through activation of γ-PAK as well as other pathways. The fact that γ-PAK alone can induce cytostasis indicates that it is a primary effector in inducing and maintaining cell stasis.

A number of substrates have been identified for γ-PAK (1), suggesting that the molecular mechanisms involved in cytostasis are multifold. Translation initiation factors and ribosomal proteins have been identified as substrates for γ-PAK (12, 32, 33), and WT γ-PAK has been shown to inhibit protein synthesis when transfected into 293T cells, whereas K278R has no effect on translation.γ-PAK in the intermediate density fractions appears to be related to γ-PAK in the ER. When samples are treated with 1% Nonidet P-40 before centrifugation, γ-PAK moves from the ER-associated fractions to the intermediate density fraction but not the soluble fraction (data not shown). Thus γ-PAK associated with the ER and the intermediate density fractions appears to have a common source. Unpublished data indicate that γ-PAK in the intermediate density fractions is bound to ribosomes, suggesting that γ-PAK on the ER is also bound to ribosomes. Because translation is a major function of the ER, inhibition of translation by γ-PAK on the ER could lead to inhibition of cell proliferation, although other pathways would also be involved. In this regard, γ-PAK has been shown to phosphorylate a wide diversity of proteins (1), and the presence of γ-PAK could also result in phosphorylation of newly synthesized proteins in response to stress.

Because of the cytostatic effects of targeted γ-PAK, the amount of the protein kinase in the cells is tightly regulated, whereas the levels of nontargeted mutants of γ-PAK are up to 8-fold higher. The differences in expression levels of the WT

2 J. Ling, S. J. Morley, and J. A. Traugh, manuscript in preparation.
3 Z. Huang, K. Orton, L. Xu, and J. A. Traugh, manuscript in preparation.
which are not localized on the ER are of two types, the kinase-

functioning of other PAK family members, including

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bition of cell division. Similarly, cells overexpressing WT

and S490D, and has a role in mediating the cytostatic effects of

ation of Ser-490 or removal of the C-terminal region (indicated by the


cytostasis is shown. The C terminus, including Ser-490, regulates the localiza-

endothelial cells and do not inhibit cell proliferation. The summarized

ER localization of 3-PK/Pak2 and Cytostasis

Table I

Effects of expression of wild type and mutant 3-PK

| 3-PK     | Expression* | Protein kinase activity* | Association with ER | Cytostatic effect* |
|----------|-------------|-------------------------|--------------------|-------------------|
| Wild type | +           | +                       | +                  | +                 |
| K278R    | ++          | -                       | -                  | -                 |
| S490A    | +           | +                       | -                  | -                 |
| S490D    | ++          | +                       | -                  | -                 |
| T402A    | ++          | -                       | -                  | -                 |
| Δ488     | ++          | -                       | -                  | -                 |

* The level of expression of recombinant HA-3-PK was determined by immunoblotting against the HA tag.

† The protein kinase activity was determined with immunoprecipitated 3-PK as assayed with histone 4.

‡ The association of 3-PK with ER was determined by immunofluorescence and sucrose density gradient centrifugation.

§ The cytostatic activity of 3-PK was determined by measuring cell proliferation after transient transfection of 3-PK.

enzyme and S490A, and the kinase-inactive mutants and

S490D, suggest that mammalian cells can tolerate higher levels

of nontargeted 3-PK than WT 3-PK. It is of interest to note that in addition to regulation of 3-PK activity by Cdc42,

sphingosine, and caspase cleavage, the level of WT 3-PK is

ightly regulated by degradation through the proteosomal path-

way (12). Generally, stress-related proteins, which result in

 irreversible damage if overexpressed, are tightly regulated in

mammalian cells. For instance, WT c-Abl, the tyrosine kinase

that has growth-suppressive and apoptotic activities, and the

retinoblastoma tumor suppressor protein RB are expressed at

significantly lower levels than the corresponding inactive mu-

tant proteins (34, 35). We have shown previously that c-Abl and

3-PK are associated in vivo and are cross-phosphorylated (12).

In that complex 3-PK is protected from degradation and

reaches levels approaching those of the kinase-inactive mu-

tants. It is important to note that the cytostatic properties of

3-PK are also observed upon expression of 3-PK in E. coli.

The majority of endogenous 3-PK (85%) is present as soluble

enzyme, whereas around 35% of the recombinant WT

3-PK and S490D is soluble. These differences can be corre-

lated with the growth status of the cells. The cells containing

only endogenous 3-PK are dividing; thus the majority of

3-PK is present as inactive enzyme. In cells treated by ioniz-

ing radiation, >50% of the endogenous 3-PK becomes associ-

ated with the particulate fractions, concomitant with the inhibi-

tion of cell division. Similarly, cells overexpressing WT

3-PK and S490A are not dividing, and active 3-PK accumu-

lates in the ER. Intracellular localization is also involved in the

functioning of other 3-PK family members, including α-3-PK.

Interaction of α-3-PK with adaptor proteins such as Nck is

implicated in translocation and stimulation of α-3-PK activity

by growth factors (13, 19, 20). Activation of Cdc42 localizes

α-3-PK to areas of membrane ruffling and reformation of the

cytoskeleton (13, 36).

Δ488, a truncated mutant simulating a spontaneous frame-

shift mutant, provides further evidence that Ser-490 and the

C-terminal regulatory region have an important role in subcel-

lular localization and induction of cytoptasis. Δ488 has protein

kinase activity similar to that of the WT but is expressed at

higher levels than WT 3-PK and exists only as a soluble form.

The lack of specific localization observed with S490D and Δ488

suggests that the C terminus is involved in regulating the

association of 3-PK with the ER and that Ser-490 has a key

function in this regulation.

The properties of WT and mutant forms of 3-PK are sum-

marized in Table I. 3-PK proteins that are localized on the

ER, including WT and S490A, are expressed at lower levels in

mammalian cells and have cytostatic effects. Mutants of 3-PK

which are not localized on the ER are of two types, the kinase-

inactive mutants K278R and T402A, and the active C-terminal

mutants Δ488 and S490D; these enzymes are expressed at up
to 8-fold higher levels than WT 3-PK and S490A in mamma-

lian cells and do not inhibit cell proliferation. The summarized

results suggest that 1) protein kinase activity alone is not

sufficient for the cytostatic effect of 3-PK; 2) localization to

the ER is important for cytoptasis; 3) the C-terminal region of

3-PK is involved in localization, as shown with Δ488, S490A,

and S490D, and has a role in mediating the cytostatic effects of

3-PK. Ser-490 in the sequence KRS is a potential recogni-

tion and/or phosphorylation site for several protein kinases

including γ-PK, and phosphorylation at this site, as shown

with S490A and S490D, appears to be important in regulating

localization of 3-PK in the ER. Ser-490 has not been identified

as a phosphorylation site in studies examining the autophos-

phorylation or phosphorylation of γ-PK (24); however if Ser-

490 is phosphorylated only when 3-PK is associated with the

ER, it would not have been detected in those studies.

To evaluate the significance of Ser-490 in 3-PK, the tertiary

structure of the catalytic domain of γ-PK was modeled using

the SWISS-MODEL program based on the x-ray crystal struc-

tures of α-PK (37). The two predicted subdomains of γ-PK


![Fig. 7. Regulatory sites and proposed model for γ-PK in cyto-
ptasis. A, the tertiary structure of the catalytic domain (amino acids

228–521) of γ-PK was predicted using the SWISS MODEL program at

www.expasy.ch/swissmod/course/text/submit.htm. The reference pro-

tein used for modeling was the catalytic domain of human α-PK (37).

B, a model for localization of γ-PK on the ER and induction of cyto-
ntasis is shown. The C terminus, including Ser-490, regulates the localiza-
inγ-PK to the ER as described under “Discussion.” Phosphory-
lation of Ser-490 or removal of the C-terminal region (indicated by the

Δ488 mutant) and/or a lack of autophosphorylation of Thr-402 could
prevent γ-PK from associating with the ER or enhance dissociation
from the ER.

| ER Associated Active | Soluble | Not Activated |
|----------------------|---------|---------------|
| Cytostasis           |         |               |
| No Cytostatic Effect  |         |               |

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form a “catalytic cavity,” with Lys-278 located inside (Fig. 7A). Thr-402 in γ-PAK corresponds to the highly conserved threonine in many protein kinases, which is located in the activation loop (38–40). Thr-402 is an autophosphorylation site and is required for activation of γ-PAK (9, 23). Ser-490 is localized on the opposite side of the catalytic domain, on the surface of an α-helix bundle that may serve as an “interface” to interact with other proteins. The β subunit of the heterotrimeric G protein has been shown to bind to Ste20, mouse mPAK3, rat α-PAK, and yeast Cla4 (26) at a sequence corresponding to residues 505–518 on γ-PAK, which lies in an α-helix near Ser-490. Because of the proximity of Ser-490 to the G protein binding region, it is possible that the trimeric G protein β subunit may also participate in the cytosolastic response. Thus, Ser-490 may regulate the interaction of γ-PAK with other components leading to ER localization of γ-PAK.

The absence of phosphate on Ser-490 alone (mimicked by S490A) is not sufficient to lead to ER localization of γ-PAK. As shown in Fig. 5, mutant K278R/S490A is not localized on the ER and has no cytosotastic properties (data not shown). This implies that autophosphorylation and activation of γ-PAK are required for ER localization. A schematic model for regulating the targeting and functioning of γ-PAK in the induction of cytostriction is shown in Fig. 7B. When Ser-490 is not phosphorylated and Thr-402 is phosphorylated, γ-PAK would be active and associated with ER, and cells would undergo cytosostasis. When Ser-490 is phosphorylated, or when Thr-402 is not phosphorylated, γ-PAK is not associated with ER, and cells could proliferate normally. Deletion of the C-terminal region containing Ser-490 also prevents ER association and thus would prevent induction of cytosostasis.

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