

Protein kinase B phosphorylates AHNAK and regulates its subcellular localization

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AHNAK is a ubiquitously expressed giant phosphoprotein that was initially identified as a gene product subject to transcriptional repression in neuroblastoma. AHNAK is predominantly nuclear in cells of nonepithelial origin, but is cytoplasmic or associated with plasma membrane in epithelial cells. In this study we show that the extranuclear localization of AHNAK in epithelial cells depends on the formation of cell–cell contacts. We show that AHNAK is a phosphorylation substrate of protein

kinase B (PKB) *in vitro* and *in vivo*. Nuclear exclusion of AHNAK is mediated through a nuclear export signal (NES) in a manner that depends on the phosphorylation of serine 5535 of AHNAK by PKB, a process that also plays a major role in determining extranuclear localization of AHNAK. AHNAK is a new PKB substrate whose function, though unknown, is likely to be regulated by its localization, which is in turn regulated by PKB.

Introduction

AHNAK was first identified as a gene whose transcription is repressed in cell lines of neuroblastoma and some other tumors (Shtivelman et al., 1992). AHNAK is a protein of an exceptionally large size (700 kD) that is encoded by an intronless gene on chromosome 11q23 (Shtivelman et al., 1992; Kudoh et al., 1995). AHNAK protein has three structural domains: a relatively short NH₂ terminus of 251 amino acids, a large central domain of 4,392 amino acids composed entirely of highly conserved repeated elements, and a unique COOH terminus of 1,000 amino acids (Shtivelman et al., 1992). The central domain contains 39 conserved repeated elements, most of which are 128 amino acids in length but many contain internal “deletions.” In turn, these repeats display a recurrent motif of seven amino acids marked by central proline residue and alternating hydrophilic and hydrophobic residues. Structurally, this sequence resembles a β -strand with a periodicity of 2.33. This model predicts a polyionic rod with hydrophobic residues facing inward and charged residues facing outward, resulting in clear separation of the hydrophobic and hydrophilic residues (Shtivelman et al., 1992).

Expression of AHNAK RNA and protein is regulated in a cell cycle–dependent manner. The highest levels of both RNA and protein are observed in the G1 or G0 phase of the

cell cycle and are decreased in proliferating cells (Shtivelman and Bishop, 1993). At the same time, total phosphorylation of AHNAK in quiescent cells such as serum-starved fibroblasts is dramatically diminished (Shtivelman and Bishop, 1993). Neuroblastoma cells express low levels of highly phosphorylated AHNAK, but upon treatment with retinoic acid that induces gradual growth arrest and terminal differentiation levels of both AHNAK RNA and protein are increased, whereas phosphorylation of AHNAK is significantly reduced (Shtivelman and Bishop, 1993). These results indicate that high levels of unphosphorylated AHNAK might be required in the G1 phase, perhaps contributing to the maintenance of the quiescent state. Alternatively, the phosphorylated form of AHNAK might perform some cell division–related function and might be rendered inactive by dephosphorylation. Phosphoamino acid analysis showed that AHNAK is phosphorylated on serine and threonine residues (Shtivelman and Bishop, 1993).

We first described subcellular localization of AHNAK to be restricted mostly to cell nuclei and Golgi apparatuses of nonepithelial cells (Shtivelman et al., 1992). Later, AHNAK was identified independently as a protein associated with the plasma membrane in epithelial cells (Hashimoto et al., 1993; Masunaga et al., 1995). At low Ca²⁺ concentrations AHNAK assumes cytoplasmic localization, but an increase in extracellular calcium results in translocation of AHNAK to the plasma membrane (Hashimoto et al., 1995). The COOH terminus of AHNAK was shown recently to be responsible for subcellular localization of AHNAK (Nie et al., 2000).

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Recent reports from other laboratories indicate that AHNAK might play a role in signal transduction. AHNAK was identified as a protein that binds and activates phospholipase C- γ in the presence of arachidonic acid (Sekiya et al., 1999). In cardiomyocytes, AHNAK associates with the $\beta 2$ subunit of the cardiac L-type calcium channel (Haase et al., 1999). Recently, AHNAK was shown to bind S100b, a calcium- and zinc-binding protein (Gentil et al., 2001) in a Ca^{2+} -dependent manner. Transcription of AHNAK is subject to strong downregulation by transforming Ras alleles in rodent fibroblasts (Zuber et al., 2000), which might be related to the elevated levels of AHNAK in resting cells as we described previously (Shtivelman and Bishop, 1993).

Protein kinase B (PKB)* is a member of the second messenger-regulated subfamily of protein kinases implicated in signaling downstream of growth factor and insulin receptor tyrosine kinases and of phosphatidylinositol 3-kinase (PI 3-kinase). PKB is probably best known for its critical role in cell survival pathways in response to a variety of apoptotic factors, though the downstream targets of this enzyme are far from being fully explored (Downward, 1998; Datta et al., 1999).

PKB activation occurs after stimulation with growth and survival factors, by extracellular stimuli such as cell–matrix and cell–cell contacts, and in response to hypoxia (for review see Downward, 1998; Vanhaesebroeck and Alessi, 2000). Activation of PKB is initiated by the binding of 3'-phosphorylated phosphoinositide products of PI 3-kinase (PI 3,4,5-triphosphate and PI 3,4-biphosphate) to the pleckstrin homology (PH) domain. This binding results in targeting of PKB to the plasma membrane and activating phosphorylation of threonine 308 and serine 473 by other kinases such as proline-directed kinase PDK-1 (Vanhaesebroeck and Alessi, 2000). Activated PKB was shown to be responsible for survival signals transduced by PI 3-kinase in the context of several model systems for apoptosis (Kandel and Hay, 1999).

PKB activation has numerous consequences, affecting transcription, protein synthesis, glucose metabolism, apoptosis, and the cell cycle (Downward, 1998; Meier and Hemmings, 1999). However, the signal transduction pathways downstream of PKB activation are still being unraveled. Activated PKB directly phosphorylates Bad (del Peso et al., 1997), caspase 9 (Cardone et al., 1998), IKK- α (Ozes et al., 1999; Romashkova and Makarov, 1999), and apoptosis signal-regulating kinase ASK1 (Kim et al., 2001), promoting cell survival. PKB phosphorylates several nuclear targets and is itself capable of nuclear translocation upon growth factor stimulation (Andjelkovic et al., 1997; Meier et al., 1997; Borgatti et al., 2000). The most studied nuclear targets are forkhead transcription factors (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999). Phosphorylation of AFX, FKHR, and FKHL1 leads to their nuclear

*Abbreviations used in this paper: caPKB, constitutively active PKB; EGFP, enhanced GFP; GFP, green fluorescent protein; GST, glutathione S-transferase; LMB, leptomycin B; MAGUK, membrane-associated guanylate kinase; NES, nuclear export signal; NLS, nuclear localization signal; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.

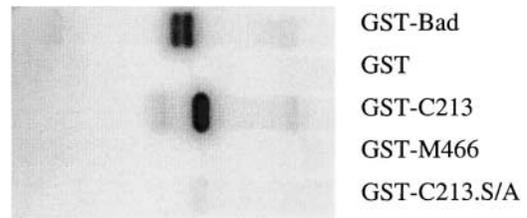


Figure 1. **AHNAK is a phosphorylation substrate of PKB in vitro.** Purified PKB was tested with the GST fusion proteins indicated as described in the Materials and methods. GST–Bad served as a positive control for phosphorylation and GST as a negative control. GST–C213 contains the last 213 amino acids of AHNAK fused to GST. The S/A construct contains a substitution of serine to alanine within the PKB consensus site. GST–M466 contains part of the AHNAK middle domain consisting of four repeats.

exclusion and inhibition of transcriptional activity, resulting in inhibition of apoptosis and/or stimulation of proliferation. Recently, cell cycle inhibitor p21 was shown to be directly phosphorylated by PKB (Zhou et al., 2001), with resulting cytoplasmic sequestration of p21 and inhibition of its antiproliferative activity.

In this study we show that AHNAK is a new direct target of PKB phosphorylation in vitro and in vivo, and that phosphorylation of AHNAK by PKB contributes to nuclear exclusion of AHNAK in epithelial cells.

Results

AHNAK is a phosphorylation substrate of PKB in vitro

Examination of the amino acid sequence of AHNAK revealed the presence of a single consensus phosphorylation site for PKB surrounding serine residue 5535 (RHRSNSF) in the COOH-terminal domain of AHNAK. We have examined the functionality of the putative PKB phosphorylation site at serine 5535 in the in vitro phosphorylation assays. Three glutathione S-transferase (GST)–AHNAK fusion polypeptides were produced and purified from bacteria to test as PKB substrates. GST–C213 encompassed the last 213 amino acids of AHNAK (between positions 5431 and 5643), including the PKB consensus phosphorylation site at 5535. In GST–C213S/A serine 5535, the hypothetical phosphorylation target of PKB was converted to an alanine. The third GST fusion (GST–M466) contained 466 amino acids from the middle domain of AHNAK, encompassing three and a half AHNAK repeats that contain multiple PKC and casein kinase II consensus phosphorylation sites but no PKB consensus sites (unpublished data). These polypeptides and the GST–Bad fusion polypeptide (positive control for PKB phosphorylation) were used in the in vitro kinase assays with active PKB purified from insect cells. Out of the three GST–AHNAK polypeptides only GST–C213 was phosphorylated in vitro; substitution of serine to alanine in GST–S/A completely abolished the appearance of a phosphorylated band (Fig. 1). The unrelated AHNAK polypeptide GST–M466 was not phosphorylated either. These results show that serine 5535 of AHNAK functions as an in vitro substrate of PKB.

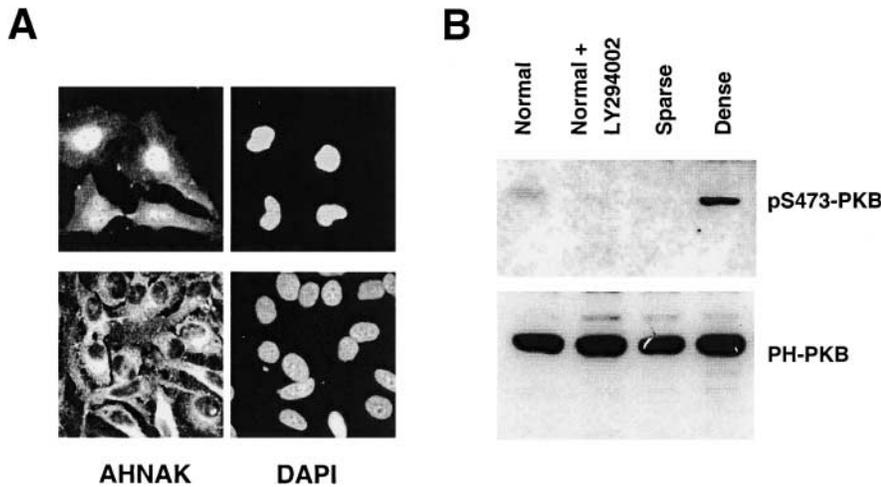


Figure 2. Relocalization of AHNAK to the cytoplasm in dense cultures correlates with an increase in PKB activity. (A) Sparsely growing (top) or dense (bottom) HeLa cells were stained for endogenous AHNAK with a polyclonal anti-AHNAK antiserum against the repeated domain described previously (Shtivelman and Bishop, 1993) and goat anti-rabbit Cy-3-conjugated antibodies. (B) Extracts from HeLa cells grown at normal (subconfluent) density with or without LY294002 treatment (30 μ M for 1 h), at low density (sparse), and confluent culture (dense) were subjected to Western blotting with an antibody specific to phosphoserine 473 of PKB (pS473-PKB) to detect activated PKB or antibody to PH domain (PH-PKB) to detect PKB protein.

PKB activity is increased in densely growing HeLa cells and correlates with the nuclear export of AHNAK

We have shown previously that AHNAK is a predominantly nuclear protein in different cultured cells of nonepithelial origin by subcellular fractionation and immunofluorescent staining (Shtivelman and Bishop, 1993). However, a plasma membrane localization of AHNAK was reported in epithelial cells (Hashimoto et al., 1993, 1995). We have performed immunofluorescent staining of endogenous AHNAK in a HeLa cervical carcinoma cell line. In freshly plated cultures of HeLa cells that have not reached high density, AHNAK was found in the nuclei with a weak diffuse cytoplasmic staining (Fig. 2 A), confirming our previous observations. However, we have noticed that in dense cultures of HeLa cells that have been plated for >2 d, AHNAK assumes mostly cytoplasmic localization (Fig. 2 A).

We have considered the possibility that redistribution of AHNAK to the cytoplasm of dense HeLa cells is regulated through phosphorylation by PKB. In addition to our data on phosphorylation of AHNAK by PKB *in vitro*, several indirect lines of evidence were supportive of this hypothesis: (1) the COOH terminus of AHNAK (where the PKB consensus site is located) is responsible for its subcellular localization (Nie et al., 2000); (2) phosphorylation by PKB induces cytoplasmic sequestration of several known substrates, among them FKHR (Biggs et al., 1999) and p21 (Zhou et al., 2001); and (3) PKB is activated by the formation of cell-cell contacts (Pece et al., 1999; Watton and Downward, 1999). Based on these data we have examined a possible correlation between PKB activity in HeLa cells and AHNAK localization. As shown in Fig. 2 B, subconfluent HeLa cultures have relatively low levels of active PKB, as detected by antibodies specifically recognizing PKB phosphorylated on serine 473. Phosphorylation of PKB is fully inhibitable by treatment with the specific PI 3-kinase inhibitor LY294002. Sparse cultures have barely detectable levels of phosphorylated PKB; however, dense cultures have much more activated PKB, whereas the total levels of PKB protein remain constant under all conditions examined. These data show a correlation between the translocation of AHNAK from nuclei to the cytoplasm and phosphorylation, and therefore activation of PKB.

Inhibition of PI 3-kinase activity leads to nuclear redistribution of AHNAK

Next, we examined the effect of inhibition of PKB activity on localization of AHNAK. Cells were treated for different lengths of time with LY294002 before immunofluorescent staining. These experiments were performed with HeLa and MDCK cells derived from immortalized canine kidney epithelium. AHNAK was nuclear in freshly plated (24 h or less) MDCK cells, but redistributed to plasma membrane in cultures allowed to reach confluence for 48–72 h (Fig. 3).

When dense cultures of HeLa or MDCK cells were treated with LY294002, AHNAK translocated to the nucleus from either the cytoplasm of HeLa cells or from the plasma membrane in MDCK cells (Fig. 3). The process of translocation was rapid, with many cells demonstrating nuclear AHNAK after just 15 min of incubation with LY294002 (unpublished data), and the majority of cells contained nuclear AHNAK after a 1-h incubation. These results indicate that phosphory-

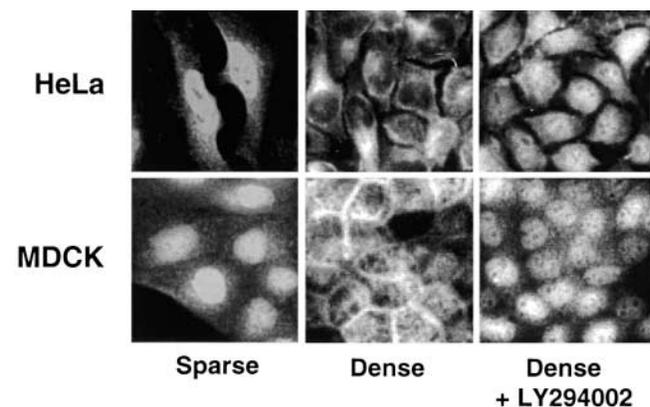


Figure 3. Localization of endogenous AHNAK in cells at different densities and nuclear translocation after inhibition of PI 3-kinase pathway. Cells were processed for indirect immunofluorescence 1 d after plating (sparse) or 3 d after plating (dense). Dense cultures were treated for the last 1 h before fixation with the PI 3-kinase inhibitor LY294002 at 30 mM. Cells were stained as described in the legend to Fig. 2. Treatment of sparse cultures with LY294002 did not affect localization of AHNAK (unpublished data).

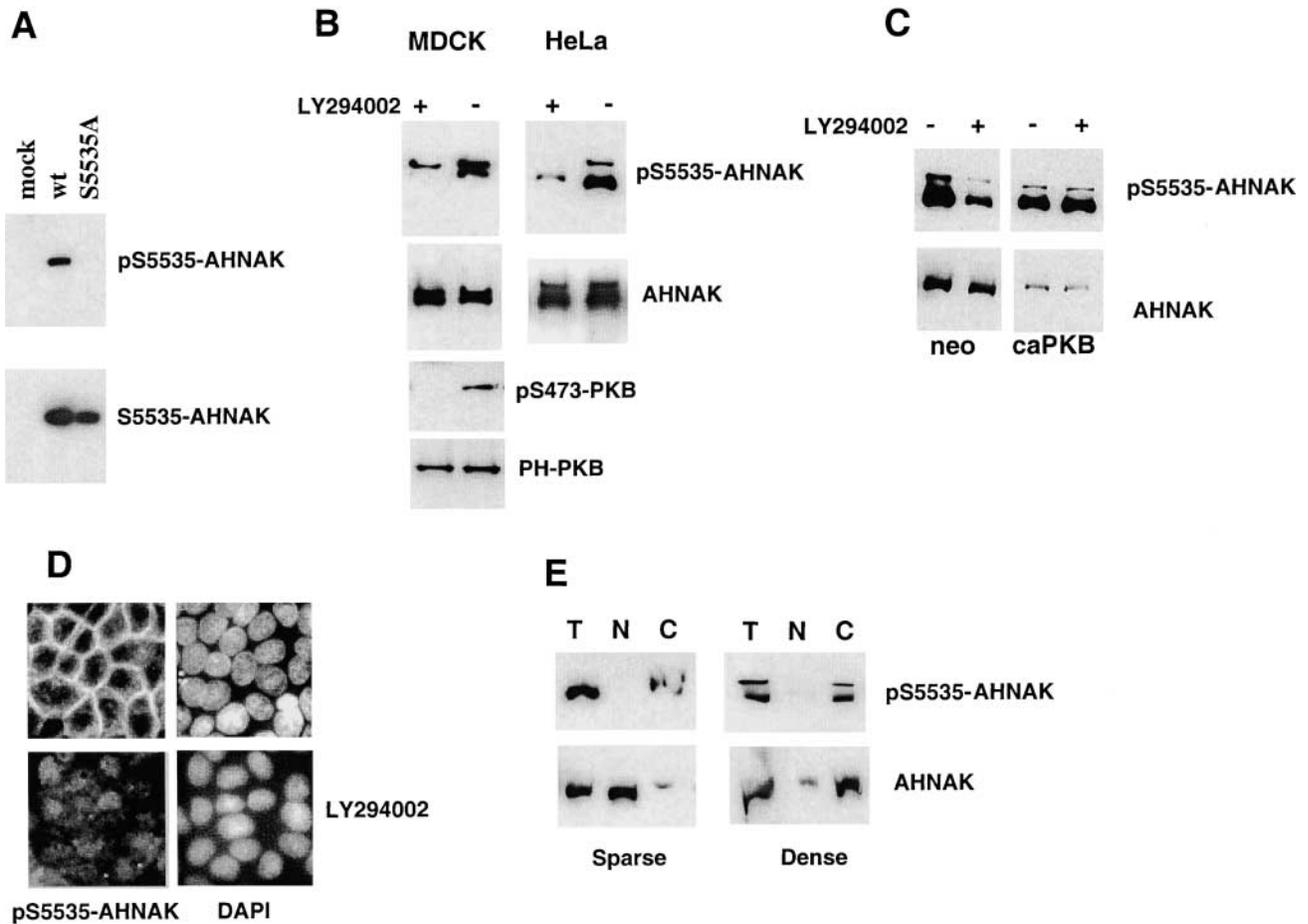


Figure 4. Characterization of phosphospecific antibodies against PKB-phosphorylated AHNAK and immunofluorescent detection of phosphorylated AHNAK. (A) Purified phosphospecific antibodies (pS5535-AHNAK) were used for Western blot analysis of HEK293 cells transiently transfected with two COOH-terminal expression constructs of AHNAK differing only in one amino acid residue; serine 5535 was either intact (wt) or substituted by alanine (S5535A). As a control, an identical Western blot was detected with antibody fraction from the same rabbit before immunoaffinity purification on phosphopeptide column. (B) Reactivity of phosphospecific antibodies with the endogenous AHNAK is greatly reduced in cells treated with LY294002. Western blot analysis of AHNAK was performed with cells extracts prepared before and after treatment with 30 μ M LY294002 for 1 h using purified phosphospecific antibodies. Antiserum against repeat region of AHNAK was used as a control for the total AHNAK protein levels (anti-AHNAK). Activity of PKB in cell extracts was monitored by anti-PKB antibodies as described in the legend to Fig. 2. (C) Western blot analysis of AHNAK phosphorylation on serine 5535 in MDCK neo (control) and caPKB clone expressing caPKB. Cells were either untreated or treated with 30 μ M LY294002 for 4 h. Blots were probed with antibodies indicated as in B. (D) Purified phosphospecific antibodies detect preferentially plasma membrane-associated AHNAK in dense MDCK cells but react only weakly with the nuclear AHNAK in cells treated for 1 h with LY294002. Inclusion of the immunizing phosphopeptide abolished reactivity of antibodies with cells (unpublished data). (E) Western blot analysis of AHNAK contents in subcellular fractions prepared from sparsely and densely growing cells. HeLa cells were fractionated into nuclear (N) and cytoplasmic (C) fractions by hypotonic lysis. Aliquots of fractions and total lysates (T) representing approximately equal cell numbers were subjected to electrophoresis and Western blotting with the indicated anti-AHNAK antibodies.

lation by PKB might prevent nuclear translocation or mediate nuclear exclusion of AHNAK.

AHNAK is phosphorylated by PKB *in vivo* and assumes predominantly extranuclear localization

The unusually large size of AHNAK and the presence of numerous phosphorylation sites for a variety of kinases within its sequence precluded phosphopeptide mapping as a means of identification of the PKB-phosphorylated residue. To determine whether PKB phosphorylates AHNAK *in vivo*, we generated antisera to phosphopeptide containing serine 5535. Antibodies specifically recognizing AHNAK phosphopeptide containing phosphorylated serine 5535 were purified and their specificity was examined. Fig. 4 A shows that

purified phosphospecific antibodies specifically recognize an exogenously expressed COOH-terminal polypeptide of AHNAK, but not a version that has serine 5535 mutated to an alanine residue.

These phosphospecific antibodies were used to examine the phosphorylation status of serine 5535 of the endogenous AHNAK protein in MDCK and HeLa cells subjected to treatment with LY294002. As seen in Fig. 4 B, the reactivity of the purified phosphospecific antibodies with endogenous AHNAK is greatly diminished upon treatment with PI 3-kinase inhibitor. An identical pattern was observed with phosphospecific antibodies purified independently from two additional immunized rabbits (unpublished data). As a control for the effect of LY294002, the activating phosphoryla-

tion of PKB on serine 473 is shown to be inhibited in LY294002-treated cells (Fig. 4 B).

To formally prove that AHNAK is indeed phosphorylated specifically by PKB *in vivo*, we have stably introduced into MDCK cells a constitutively active PKB (caPKB). This form of PKB is not responsive to PI 3,4,5-triphosphate signaling due to removal of the PH domain (Stokoe et al., 1997). In addition, two residues that are phosphorylated in activated PKB, serine 473 and threonine 308, were changed to aspartates in order to mimic a constitutively phosphorylated active form of the enzyme (Stokoe et al., 1997). Examination of the activity of caPKB in control and caPKB-expressing MDCK clones indeed showed that caPKB remains fully active when cells are treated with LY294002 (unpublished data). Phosphorylation of AHNAK was examined in control MDCK clones and in caPKB-expressing clones with or without LY294002 treatment. Fig. 4 C shows that phosphorylation of AHNAK is diminished in MDCKneo but not in MDCK-caPKB cells in the presence of LY294002. Thus, caPKB phosphorylates AHNAK even when the entire PI 3-kinase pathway is inhibited. This proves that PKB and not another kinase is responsible for phosphorylating serine 5535 of AHNAK *in vivo*.

The anti-phosphoserine 5535 antibodies were used for analysis of subcellular localization of PKB-phosphorylated AHNAK. As shown in Fig. 4 C, these antibodies efficiently detect the plasma membrane-localized AHNAK in dense MDCK cells, confirming that AHNAK anchored at the membrane is indeed phosphorylated by PKB. However, the reactivity of the antibodies with the nuclear-localized AHNAK in LY294002-treated cells is greatly reduced (compare Fig. 3 with Fig. 4 D). Similarly, the phosphospecific antibodies efficiently recognize the cytoplasmic AHNAK in dense HeLa cells (unpublished data).

To confirm independently the results on nuclear exclusion of PKB-phosphorylated AHNAK obtained through cell staining, we have performed biochemical fractionation of sparse and dense HeLa cultures. We examined the distribution of the total AHNAK protein versus the PKB-phosphorylated pool of AHNAK in cell fractions. Fig. 4 E shows that phosphoserine 5535-AHNAK is not found in the nuclei irrespective of cell density, not even in sparse cultures where the bulk of AHNAK is nuclear. Thus, the fractionation results are in full agreement with the results of cell staining with AHNAK antibodies.

The exogenously expressed COOH terminus of AHNAK assumes nuclear or cytoplasmic localization depending on the phosphorylation status of serine 5535

To directly demonstrate a role for PKB phosphorylation in localization of AHNAK we examined localization of site-specific AHNAK mutants affecting PKB site. We have subcloned the COOH terminus of AHNAK into vector pEGFP.C1 to create a fusion of the enhanced green fluorescent protein (EGFP) with the last 998 amino acids of AHNAK (EGFP-C998). Site-specific mutations of AHNAK shown in Fig. 5 A were introduced into EGFP-C998.

Serine 5535 within PKB consensus site was changed either to alanine to prevent phosphorylation by PKB (S/A) or

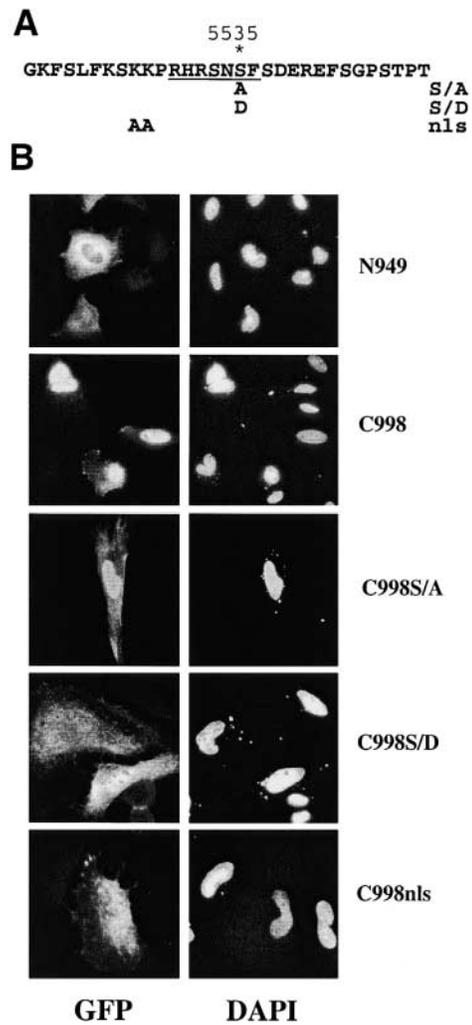


Figure 5. **Localization of the COOH-terminal domain of AHNAK fused to EGFP is regulated by the phosphorylation status of serine 5535.** (A) Amino acid sequence of the region of AHNAK around the PKB phosphorylation site and mutations introduced into it. (B) Localization of the EGFP-COOH-terminal AHNAK fusion proteins in transiently transfected HeLa cells.

to aspartate to mimic constitutive phosphorylation (S/D). In addition, we have substituted two lysine residues within the sequence immediately adjacent to the PKB consensus site by alanines (C998nls, Fig. 5). This was done in order to examine the possibility that the basic cluster immediately adjacent to PKB phosphorylation site might serve as a nuclear localization signal, though it must be noted that short stretches rich in basic residues are encountered several times within the COOH-terminal 998 amino acids of AHNAK (Shtivelman et al., 1992). We were aware of the possibility that changing the charge of one of these basic clusters within the context of a polypeptide of 1,000 amino acids might not necessarily affect its localization. However, the proximity of this particular basic cluster to the PKB phosphorylation site prompted us to examine it in localization studies. We have also prepared a green fluorescent protein (GFP) fusion construct with the NH₂-terminal domain of AHNAK. A total of 949 NH₂-terminal amino acids composed of the unique 251 amino acids and the following five

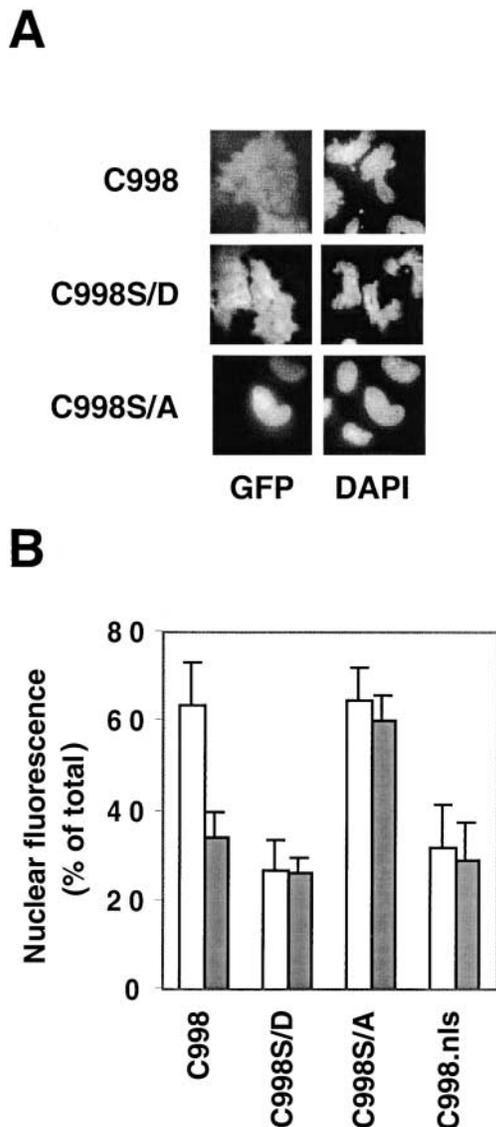


Figure 6. Localization of the COOH-terminal domain of AHNAK fused to EGFP is subject to regulation by cell density only when the PKB phosphorylation site is intact. (A) The indicated EGFP fusion constructs were transfected into dense HeLa cultures, plated for 2 d, and fixed 24 h later. (B) Quantitative analysis of the subcellular distribution of transfected COOH terminus of AHNAK in dense versus sparse HeLa cells. Experiments were performed at least three times with each construct and at least 200 fluorescent cells were counted per each experiment. White bars represent data obtained with cells plated at low density; gray bars represent data obtained with cells plated at high density. Error is expressed as a standard deviation of the mean values.

“AHNAK repeats” were contained within encoded polypeptide (EGFP-N949).

Expression of the proteins of expected size from all constructs was confirmed by Western blotting (unpublished data). Localization of EGFP-AHNAK fusions was examined in transient expression assays with HeLa cells. The NH₂ terminus of AHNAK directed exclusively cytoplasmic localization of EGFP (Fig. 5 B), demonstrating that signals regulating nuclear import of AHNAK are located within its COOH terminus, in agreement with the previously published results

(Nie et al., 2000). The unaltered COOH terminus construct and S/A mutant localized mostly to nuclei, whereas the S/D and nuclear localization signal (NLS) mutants were predominantly cytoplasmic, though some of the fluorescence was retained in the nuclei of transfected cells (Fig. 5). The cytoplasmic localization of the C998nls mutant implies a functional role of the basic cluster adjacent to PKB phosphorylation site (Fig. 5 A) in directing nuclear import of AHNAK. The results obtained with the PKB site mutants provide a confirmation for the functional role of PKB phosphorylation in regulating localization of AHNAK. It should be noted that we had not observed a complete nuclear exclusion of AHNAK with either C998S/D or nls mutants. This implies that the NH₂ terminus or repeated middle domain of AHNAK contributes to the extranuclear localization of AHNAK under conditions of high cellular density. However, the observed cytoplasmic localization of the C998S/D and C998nls mutants confirmed our hypothesis about the key role of PKB in regulating localization of AHNAK protein.

During the course of these experiments we have noticed that expression of EGFP-AHNAK fusion proteins occurs at very low levels and is extremely unstable, resulting in the disappearance of the green fluorescence within 48 h after transfection. This was more pronounced for the COOH-terminal AHNAK fusions with cytoplasmic localization, which were expressed at very low levels compared with nuclear localized polypeptides. Moreover, cells expressing the COOH-terminal domain of AHNAK tended to be of a much larger size on average than untransfected cells and frequently displayed enlarged, multilobular, or multiple nuclei. We have never observed transfected cells undergoing normal mitosis (unpublished data). These observations are very similar to those of Nie et al. (2000), and suggest that expression of the COOH terminus of AHNAK is incompatible with cell division and/or survival.

Localization of the COOH-terminal EGFP-AHNAK fusion proteins is subject to regulation by cell density similar to that of endogenous AHNAK

To examine whether the COOH-terminal domain of AHNAK changes its localization depending on formation of cell-cell contacts, we have performed transient transfection experiments of dense cultures of HeLa cells. As shown in Fig. 6, we found that the EGFP-C998 fusion of COOH terminus of AHNAK undergoes a change in subcellular localization depending on culture density similar to that shown for endogenous AHNAK. Whereas predominantly nuclear in subconfluent cultures, it is found in cytoplasm of densely plated cells (Fig. 6). However, neither of the two constructs with alterations in the PKB phosphorylation site nor the construct with a mutated NLS are capable of assuming a different localization depending on cell density (Fig. 6). The S/A mutant that cannot be phosphorylated by PKB is mostly retained in the nuclei of transfected cells, whereas the S/D mutant mimicking constitutive phosphorylation and the NLS mutant are retained in the cytoplasm independently of cell density of transfected cultures (Fig. 6 B). These results confirm that shuttling of AHNAK as a function of cell density is regulated at least partially through phosphorylation at the PKB site and the adjacent nuclear localization signal.

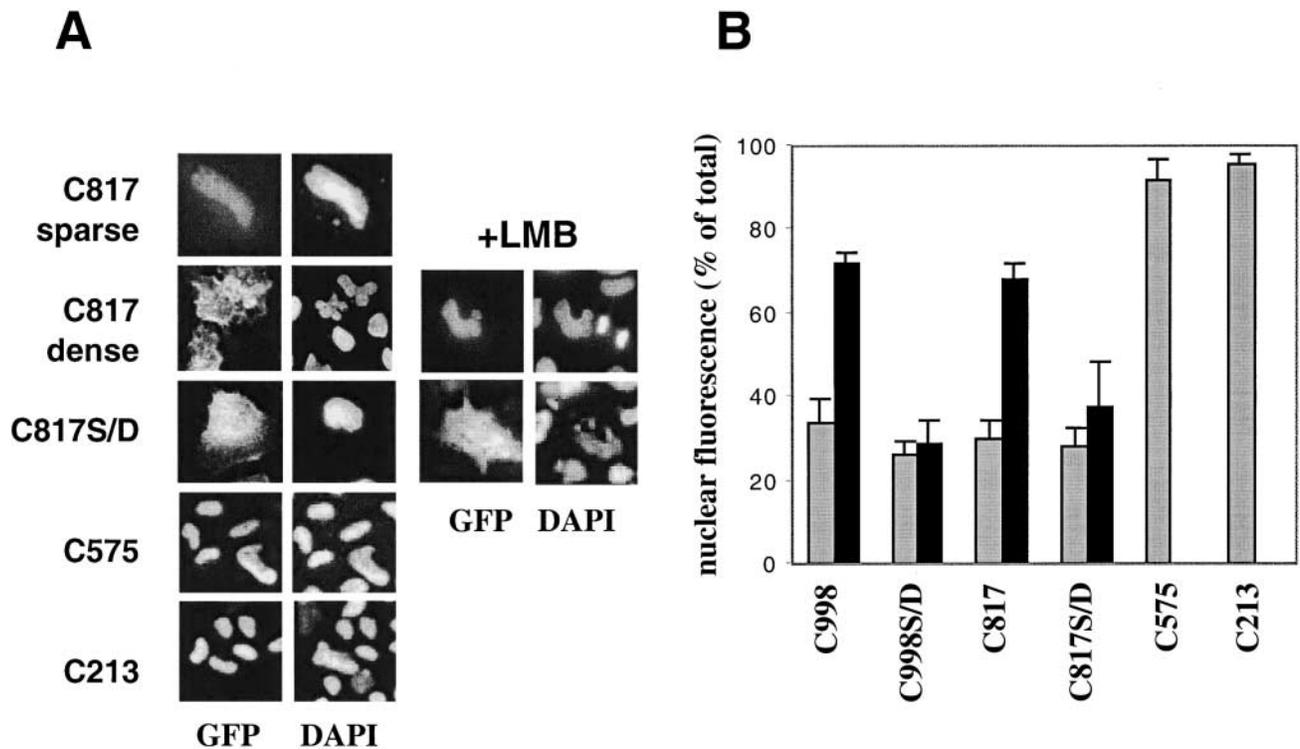


Figure 7. **The region in the COOH terminus of AHNAK between amino acids 4826 and 5068 is necessary for the extranuclear localization of AHNAK and might contain NES.** (A) Localization of the indicated EGFP fusion constructs in transiently transfected HeLa cells 24 h after transfection. Cells shown in the right panels were treated with 20 ng/ml LMB for 1 h. (B) Quantitative analysis of the subcellular distribution of transfected AHNAK polypeptides in dense HeLa cells was performed as described in legend to Fig. 6. Gray bars represent localization in untreated dense cells; black bars represent cultures treated with LMB at 20 ng/ml for 1 h.

Phosphorylation of serine 5535 alone is not sufficient to prevent nuclear accumulation of the COOH terminus of AHNAK

To further analyze the specific role of phosphorylation by PKB in nuclear exclusion of AHNAK, we have conducted experiments aimed at determining the smallest domain of AHNAK whose localization is still regulated by PKB. We have constructed three additional EGFP-AHNAK fusion constructs encoding progressively shorter COOH-terminal fragments of AHNAK (Fig. 7). EGFP-C817 assumed predominantly nuclear localization upon transfection into sparsely plated cells, but in dense cells was found in cytoplasm of most cells (Fig. 7) behaving essentially as EGFP-C998. Introduction of serine 5535 to aspartate substitution into EGFP-C817 had the same effect on its localization as on localization of the longer EGFP-C998 resulting in nuclear exclusion of most of the protein irrespective of cell density (Fig. 7). Two shorter EGFP fusion constructs were prepared containing the last 575 and 213 amino acids of AHNAK. Both of them produced polypeptides that were overwhelmingly nuclear upon transfection into HeLa cells. Density of transfected cells had no effect on the nuclear localization of shorter EGFP fusions (Fig. 7).

We have the nuclear-localized polypeptides produced from EGFP-C575 and -213 to see whether they are phosphorylated by PKB on serine 5535. Extracts from transiently transfected 293 cells were electrophoresed and blotted with phosphospecific antibodies to phosphoserine 5535; both polypeptides were found to be phosphorylated (unpublished

data). Moreover, introduction of serine 5535 to aspartate substitution into EGFP-C575 and EGF-C213 did not prevent nuclear accumulation of these polypeptides (unpublished data). Because the molecular size of even the smaller polypeptide >50 kD, the possibility of free diffusion in and out of the nucleus could be excluded. Apparently the removal of the 236 amino acids between EGFP-C817 and -C575 results in nuclear retention of the remaining COOH-terminal polypeptide, irrespective of the phosphorylation status of serine 5535. This implies that nuclear exclusion of PKB phosphorylated AHNAK relies on additional signals provided by the amino acid sequence between positions 4826 and 5071. We have noticed also that removal of that region resulted in significantly higher expression levels of EGFP-AHNAK fusions in transfected cells as evaluated by microscopy and Western blot analysis (unpublished data).

Identification of nuclear export signal in AHNAK sequence

Close inspection of the predicted amino acid sequence of AHNAK between positions 4826 and 5071 revealed the presence of a leucine-rich sequence at positions 4875–4884 that fits the consensus nuclear export signal (NES) sequence (Fornerod et al., 1997; Henderson and Eleftheriou, 2000). We have examined the two fusion polypeptides (EGFP-C998 and -C817) that contain this region to see whether they are responsive to treatment with leptomycin B (LMB), a specific inhibitor of nuclear export that is mediated in a CRM1-dependent manner (Nishi et al., 1994; Fukuda et al., 1997). As shown in Fig.

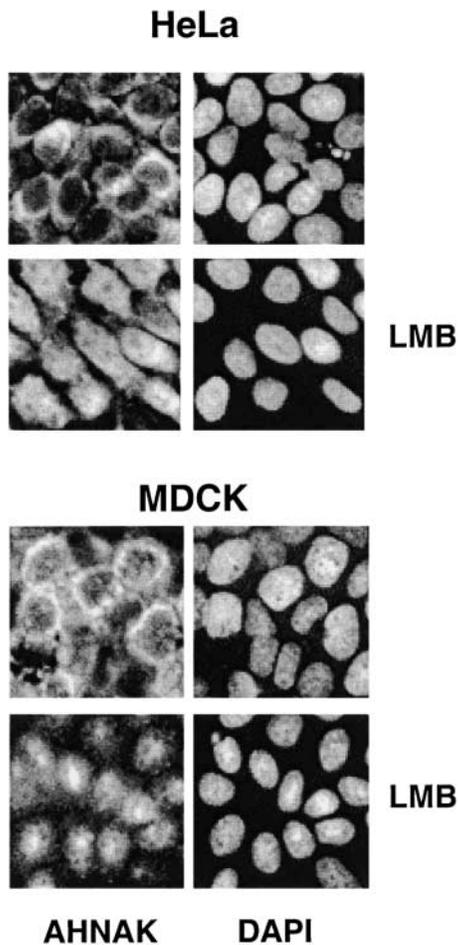


Figure 8. Localization of endogenous AHNAK in HeLa and MDCK cells treated with LMB. Dense cultures either untreated or treated with LMB at 20 ng/ml for 5 h were stained for endogenous AHNAK with a polyclonal anti-AHNAK antiserum against the repeated domain.

7, both polypeptides are retained in the nuclei of dense cultures treated with LMB. However, an aspartate substitution of serine 5355 in both fusion proteins prevented their nuclear accumulation in response to treatment with LMB, indicating that phosphorylation by PKB is involved in regulation of CRM1-mediated nuclear export of AHNAK polypeptide.

To examine whether endogenous AHNAK is also subject to CRM1-mediated nuclear export we have examined the localization of AHNAK in dense HeLa and MDCK cells treated with LMB. Treatment of densely growing HeLa cells with LMB resulted in nuclear redistribution of endogenous AHNAK from a predominantly cytoplasmic localization (Fig. 8). Similarly, treatment of dense MDCK cells with LMB resulted in the disappearance of the membrane-associated AHNAK and its nuclear translocation in majority of cells.

To verify the presence of NES between positions 4875 and 4884 in the AHNAK protein, fusion protein EGFP-C755 was generated spanning the COOH-terminal sequences of AHNAK starting immediately after the putative NES at position 4887. This fusion protein was overwhelmingly nuclear in both sparsely or densely growing HeLa cells (Fig. 9). Because localization of the EGFP-C817 was cytoplasmic in

densely growing cells and responsive to treatment with LMB, whereas EGFP-C755 was nuclear under all conditions, the possibility of a functional NES within the first 62 amino acids of C817 was further strengthened. To examine whether constitutive phosphorylation of AHNAK at serine 5535 could interfere with nuclear localization of EGFP-C755, an aspartate substitution was introduced into this construct. EGFP-C755S/D was found predominantly in the nuclei of transfected cells similar to shorter fusions EGFP-C581 and C213 with serine 5535 to aspartate substitution described above (Fig. 9). This is compatible with the explanation that the putative NES absent from EGFP-C755 is necessary for the nuclear export of PKB-phosphorylated AHNAK.

To confirm the functionality of the leucine-rich sequence at positions 4875–4884 as an NES, we have introduced potentially disabling mutations by changing the key leucine and isoleucine residues within it to alanines in both EGFP-C998 and -C817 fusion proteins. The resulting polypeptides EGFP-C998nes and EGFP-C817nes showed a predominantly nuclear localization when transfected into dense HeLa cells (Fig. 9) confirming that the stretch of amino acids between 4875 and 4884 serves as an NES.

Discussion

In this study we show that localization of AHNAK in epithelial cells is subject to regulation by formation of cell–cell contacts and that this regulation involves phosphorylation of AHNAK by PKB. AHNAK is excluded from the nuclei of dense cells by a mechanism involving CRM1-dependent nuclear export in a phosphorylation-dependent manner.

AHNAK is a protein that shuttles between different cellular compartments of epithelial cells in response to signals received from the extracellular environment. It was shown previously that calcium concentration is one such signal and that in low-calcium AHNAK translocates to the cytoplasm and nucleus from its normal localization at the plasma membrane of keratinocytes (Hashimoto et al., 1995). In this work we have identified the formation of cell–cell contacts as a trigger that provokes plasma membrane translocation of AHNAK from its nuclear localization in isolated MDCK cells. Our study was conducted with two cell lines of epithelial origins: MDCK cells that are immortalized but preserve the differentiation features of polarized epithelium, and HeLa cells derived from a cervical carcinoma. Unlike MDCK, HeLa cells could have lost many of the specialized cellular contact structures of the normal epithelium, and that could be the reason for the lack of AHNAK association with the plasma membrane of HeLa cells. Nevertheless, the mechanisms of the nuclear exclusion of AHNAK in dense cultures seem to be similar in MDCK and HeLa cells in that they are regulated by PKB phosphorylation of AHNAK at serine 5535.

AHNAK phosphorylated by PKB is found predominantly in the cytoplasm of HeLa cells or associated with the plasma membrane of MDCK cells. In turn, inhibition of the PI 3-kinase pathway results in nuclear translocation of AHNAK in both cell lines, similar to plating cells at low density. This is unlikely to be a mere coincidence, as PKB was shown to be activated by the formation of cell–cell contacts (Pecce et al., 1999; Watton and Downward, 1999) and we have indirectly

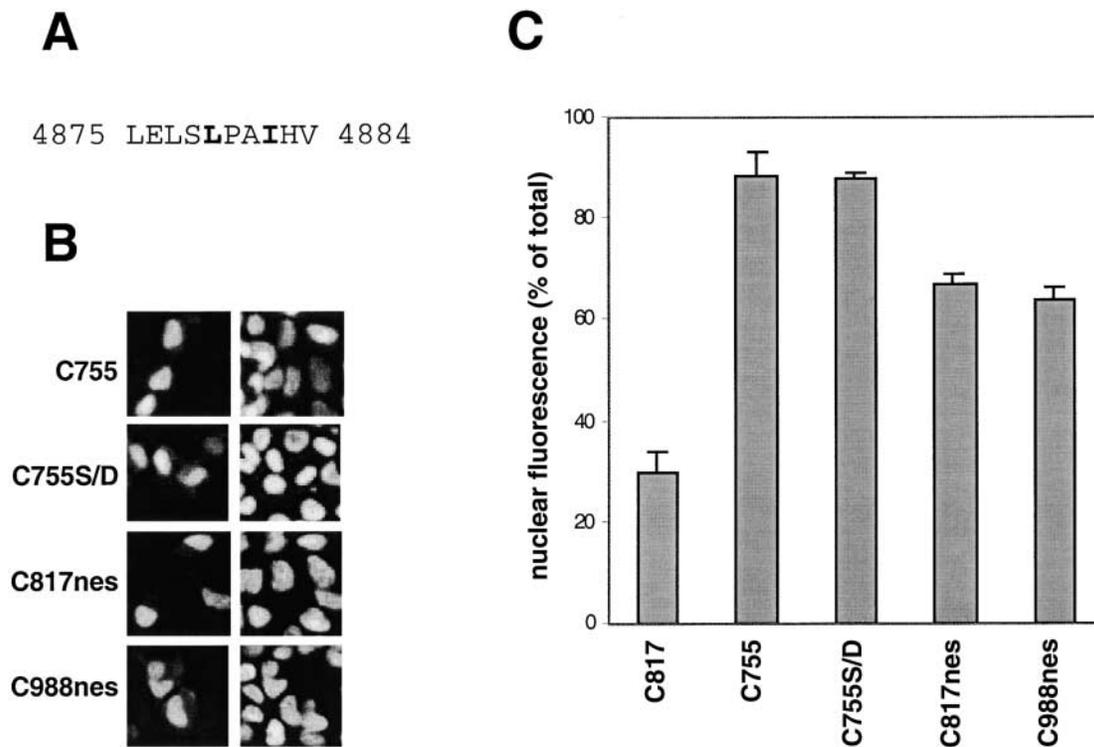


Figure 9. **The region between amino acids 4875 and 4884 serves as an NES.** (A) Sequence of the putative NES in AHNAK. The amino acid residues substituted by alanine residues are in bold. (B) Removal of the putative NES or introduction of mutations into Leu 4879 and Ile 4882 result in nuclear retention of AHNAK. Constructs were transfected into dense HeLa cells. (C) Quantitative analysis of the subcellular distribution of transfected AHNAK polypeptides in dense HeLa cells was performed as described in legend to Fig. 6.

confirmed these observations here by detecting a higher PKB activity in confluent cultures of HeLa cells. Significantly, PKB activation occurs rapidly in MDCK cells that are allowed to form E-cadherin-mediated junctions by restoration of calcium concentration in cultures treated with a calcium chelator (Pece et al., 1999). PKB was reported to be activated by an increase in calcium concentration via Ca^{2+} /calmodulin-dependent protein kinase kinase in a PI 3-kinase-independent manner (Yano et al., 1998). Calcium signaling was previously implicated in the regulation of AHNAK localization (Hashimoto et al., 1995) and this work shows a role for PKB phosphorylation in the shuttling of AHNAK. Based on these results, we suggest that AHNAK could be part of a signal transduction pathway initiated at the calcium-dependent E-cadherin-mediated homophilic adhesion sites resulting in activation of PKB, translocation of AHNAK, and a consequent impact on cell survival and/or proliferation.

The region of AHNAK that regulates its localization was shown to be contained within the COOH terminus of the protein (Nie et al., 2000), but no known motives potentially involved in this regulation have been identified before this study. We show that in addition to the PKB phosphorylation site there is an NES that causes nuclear exclusion of the phosphorylated COOH terminus of AHNAK. The phosphorylation by PKB is apparently a necessary prerequisite for the nuclear export of AHNAK, because constitutively unphosphorylated mutants of the serine 5535 encompassing NES fail to be excluded from the nuclei of densely growing cells. At the same time, removing or mutating NES results in

nuclear retention of the AHNAK COOH terminus irrespective of the phosphorylation status of serine 5535.

Two potential explanations are compatible with the results described here. First, phosphorylation by PKB retains AHNAK in the cytoplasm or tethers it to plasma membrane of epithelial cells, thus preventing nuclear import. The second possibility is that AHNAK is continuously and maybe constitutively imported into the nucleus, but phosphorylation of AHNAK by nuclear PKB facilitates its nuclear export in a CRM1-dependent manner. Our results point to the second explanation as a more plausible one because COOH-terminal constructs of AHNAK constitutively phosphorylated on serine 5535 but lacking NES accumulate within the nuclei of transfected cells, indicating that phosphorylated AHNAK is capable of nuclear import. The most plausible scenario could be that AHNAK is nuclear in sparse cells but formation of cell-cell contacts leads to activation of PKB that translocates to the nucleus, phosphorylates AHNAK, and leads to its nuclear exclusion via the LMB-inhibitable pathway. To support this hypothesis, it has been shown that activated endogenous PKB translocates to the nucleus (Andjelkovic et al., 1997; Meier et al., 1997). In addition, phosphorylation of the transcription factor AFX and the oncogene TCL1 by PKB was shown to occur in the cell nucleus (Pekarsky et al., 2000; Brownawell et al., 2001). Mechanisms of cytoplasmic retention of PKB-phosphorylated AHNAK remain unclear but are unlikely to involve 14-3-3 proteins because we could not detect binding of AHNAK to 14-3-3 (unpublished data).

Epithelial cell proliferation is subject to strict regulation by extracellular signals provided by cell–cell contacts and substrate adherence. Peripheral proteins that are positioned strategically at sites of cell–cell or substrate adherence are known to be involved in the integration of the extracellular stimuli with the signal transduction mechanisms. Sometimes these proteins themselves are capable of traveling from the plasma membrane to the nucleus, apparently fulfilling a messenger function. There is now a growing list of proteins that are associated with specialized cell–cell contacts such as tight junctions, adherens junctions, and desmosomes, which are capable of translocation to cell nuclei under certain defined conditions or developmental stages. One of them is ZO-1, a protein found in both tight and adherens junctions that translocates to the nucleus in cells that have not yet formed cell–cell contacts (Gottardi et al., 1996). ZO-1 is a mammalian homologue of a *Drosophila* protein dlg, a tumor suppressor (Woods and Bryant, 1991) and a “founding member” of the membrane-associated guanylate kinase (MAGUK) family of proteins. The MAGUK family members share a common modular structure that invariably includes one or more PDZ domains (for review see Anderson, 1996). Another MAGUK, MAGI-1c, is also capable of nuclear relocalization from cell cortex (Dobrosotskaya et al., 1997). A protein named periaxin (Gillespie et al., 1994), though not a member of MAGUK family, contains two PDZ domains and is capable of nucleocytoplasmic shuttling (Sherman and Brophy, 2000). Periaxin is found in the nuclei of embryonic Schwann cells but translocates to plasma membrane processes of the myelinating Schwann cells (Gillespie et al., 1994). Interestingly, periaxin is the only protein in the database that shares a significant degree of homology with the repeated domain of AHNAK (unpublished data and Kingsley et al., 2001). Recent report indicates that expression and subcellular localization of AHNAK in embryonic tissues is subject to a dynamic developmental regulation (Kingsley et al., 2001).

Another well known example of protein shuttling in epithelial cells involves β -catenin (for review see Polakis, 2000). β -Catenin binds to E-cadherin in the adherens junctions complex (Orsulic et al., 1999) but translocates to the nucleus upon activation of the Wnt signaling pathway. Nuclear β -catenin binds to and activates the LEF-1/TCF transcription factors to induce expression of genes involved in tumorigenicity and invasion (Polakis, 2000). The mechanisms governing shuttling of β -catenin are quite complex and involve a large protein encoded by the tumor suppressor gene adenomatous polyposis coli implicated in transport of β -catenin out of the nucleus to cytoplasm for further degradation (Henderson, 2000; Neufeld et al., 2000b; Rosin-Arbesfeld et al., 2000). Adenomatous polyposis coli itself is a shuttling protein and is exported out of the nucleus in a CRM1-dependent manner (Henderson, 2000; Neufeld et al., 2000a; Rosin-Arbesfeld et al., 2000). Shuttling of proteins between different cellular compartments plays a major role in the Wnt pathway. The regulated shuttling of AHNAK in epithelial cells might play a role in a yet to be identified signal transduction pathway. Due to its ability to change subcellular localization quite rapidly, AHNAK might be transmitting signals from the cell periphery to the

nucleus or even physically transferring proteins to the required locales within the cells.

An obvious question that arises from analysis of AHNAK distribution in HeLa and MDCK cells is that extranuclear localization of AHNAK might be related to an altered proliferation rate of densely growing cultures. We have found that not to be the case for dense HeLa cultures, but cessation of proliferation occurs in MDCK cells with mature cell–cell contacts (usually >72 h after plating). This indicates that a rather direct correlation exists between nuclear exclusion of AHNAK in epithelial cells and formation of cell–cell contacts, whereas potential links to proliferation rates remain to be explored. However, it is possible that the plasma membrane–anchored AHNAK is somehow involved in the eventual growth arrest of normal epithelial cells. The relationship between localization of AHNAK and cell proliferation promises to be complicated and cell type dependent. For example, our unpublished data show that AHNAK is nuclear in glioblastoma cell lines but predominantly cytoplasmic in proliferating neuroblastoma cells. However, in terminally differentiated neuroblastoma cells AHNAK translocates to the nuclei and to the neuronal processes (unpublished data). It is yet unclear whether relocalization of AHNAK in growth-arrested neuroblastoma cells is a direct consequence of proliferative quiescence induced by retinoic acid, or rather a consequence of a dramatic change in cellular morphology from an undifferentiated to a neuronal phenotype.

In conclusion, AHNAK is a new PKB phosphorylation substrate whose subcellular localization, like that of several other PKB targets, is subject to regulation by PKB phosphorylation. All identified PKB substrates share one common feature: they play critical roles in most important pathways governing cell survival and proliferation and belong to different classes of proteins ranging from transcription factors and proapoptotic proteins to CDK inhibitors and metabolic regulators. Though the function of AHNAK remains a mystery, its unusual structural features and unique flexible and highly regulated localization patterns might indicate an important role of this protein in key signal transduction processes.

Materials and methods

Cell culture

HeLa cells were maintained in high-glucose DME with 10% FCS. MDCK cells were maintained in DME with 5% FCS. For transfections and immunofluorescence, cells were plated on eight-well chamber slides at desired densities, fixed 24 h after transfection, and processed. Transfections were carried out using Eugene (Roche) according to the manufacturer's protocol. Usually 200 ng of plasmids was transfected per well. Treatment before fixing cells included LY290042 (Calbiochem) at 30 or 50 μ M or LMB (Sigma-Aldrich) at 20 ng/ml.

Plasmid constructs

EGFP and GST fusion constructs were generated using PCR with oligonucleotides designed to amplify desired regions of AHNAK cDNA and introduce restriction sites for subcloning into pEGFP.C1 (all COOH-terminal constructs), pEGFP.N1 (the NH₂ terminus of AHNAK), or pGEX.KG. Mutations were introduced through whole-plasmid PCR with the Pfu Turbo polymerase (Stratagene) using oligonucleotides with desired changes. The starting plasmid was digested with Dpn1 before transformation of *Escherichia coli*. All introduced amino acid substitutions were confirmed by sequence analysis. Expression of proteins of expected size from all constructs was confirmed by transient transfection into HEK293 cells and Western blot analysis of lysates with appropriate antibodies.

Cell staining

Cells in chamber slides were fixed in 4% buffered paraformaldehyde for 15 min, permeabilized in 0.1% Triton X-100 for 7 min, blocked in 2% BSA for 30 min, and incubated with anti-AHNAK antibodies FEN (Shtivelman and Bishop, 1993) or phosphospecific antibodies to AHNAK in 1% BSA for 1 h at room temperature. After PBS washes, cells were incubated with Cy3-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratory) and nuclei were counterstained with DAPI.

Purification of phosphospecific antibodies

Rabbits were immunized with phosphopeptide FKSKKPRHRSNpSFSDER corresponding to positions 5524–5540 in the AHNAK amino acid sequence and synthesized at the Biomolecular Resource Facility of The University of California at San Francisco (San Francisco, CA). The phosphopeptide was conjugated to maleimide-coupled BSA (Pierce Chemical Co.). Antisera were passed three times through an affinity column of the nonphosphorylated form of the immunizing peptide (Sulfolink; Pierce Chemical Co.). The bound antibodies were eluted each time to produce a fraction recognizing both nonphosphorylated and phosphorylated forms of AHNAK. The final flowthrough was then passed through a column of the immunizing phosphopeptide. The retained antibodies were eluted, concentrated, and used for analyses in the presence of the nonphosphorylated peptide to ensure specific detection of phosphoserine 5535.

Western blot analysis

Western blots for AHNAK were performed with protein extracts separated on 4% polyacrylamide gels; blotted using Novagen/InVitrogen module, incubated with appropriate antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Dako), and developed using ECL (Amersham Pharmacia Biotech).

Phosphorylation of AHNAK by PKB in vitro

0.2 μ g of partially active glu-glu-tagged PKB purified from Sf9 cells (Stokoe et al., 1997) was added to 0.5 μ g of GST or GST fusion proteins in a kinase buffer consisting of 20 mM Tris HCl, pH 7.5, 1 mM EDTA, 75 mM NaCl, and 10 mM MgCl₂. 20 μ M ATP containing 5 μ Ci [γ -³²P]ATP was added to initiate phosphorylation in a total reaction volume of 20 μ l. After 20 min, the reaction was terminated by the addition of 2 \times SDS sample buffer, the samples separated by SDS-PAGE, and phosphorylation detected by PhosphorImager analysis. GST-BAD (0.5 μ g) was used as a positive control for phosphorylation by PKB.

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