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Akt1 contains a functional leucine-rich nuclear export sequence

Motoyasu Saji [a,1], Vasily Vasko [a,b,c,1], Faiza Kada [b], Elena Hardy Allbritton [b], Kenneth D. Burman [b], Matthew D. Ringel [a,*]

[a] The Ohio State University and Arthur G. James Comprehensive Cancer Center, Columbus, OH, USA
[b] The Washington Hospital Center/MedStar Research Institute, Washington, DC, USA
[c] Department of Pediatrics, Uniformed Service, University of Health Science, Bethesda, MD, USA

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Abstract

Nuclear Akt1 expression and Akt activation are common in cancer invasion. However, the mechanisms for this association and its causal role in invasion are uncertain. In an effort to identify potential mechanisms for regulating Akt subcellular localization, we analyzed the Akt gene sequences and identified a highly conserved leucine-rich potential nuclear export sequence (NES). Initial experiments demonstrated that leptomycin B induced nuclear Akt1 localization. Transient expression experiments demonstrated that, in comparison to wild-type Akt1, NES-mutated (AKT/NES) Akt1 has reduced interactions with CRM-1 and persistent nuclear localization. Subsequent stable transfection experiments in Akt1$^{-/-}$ fibroblasts confirmed that expression of AKT/NES resulted in persistent nuclear localization and activation1. Finally, stable expression of AKT/NES in Akt1$^{-/-}$ fibroblasts was sufficient to enhance cell migration in vitro. Thus, Akt1 contains a functional NES and mutation of the NES results in nuclear-predominant Akt1 activation that is sufficient to induce migration.

Keywords: Thyroid cancer; Cell motility; Metastasis; Invasion; CRM-1

Akt (PKB), a central signaling molecule in the phosphoinositol-3-OH kinase (PI3 kinase) pathway, is activated in multistep manner that initially involves ATP-dependent phosphorylation and membrane localization and insertion via its pleckstrin homology domain [1]. After localizing to the membrane, Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK-1) at threonine 308 (for Akt1) [2] and by integrin-linked kinase, DNA-dependent protein kinase, and/or other factors at serine 473 [3,4]. Akt interacts with several chaperone proteins, resulting in phosphorylation of substrates in the cytoplasm. Akt also enters the nucleus, where it phosphorylates nuclear targets. The process of intracellular movement of Akt appears to be facilitated by its phosphorylation, although the requirement of phosphorylation for nuclear Akt localization is uncertain, and potential differences between the three Akt isoforms have not been fully explored (reviewed by Brazil et al. [5]). The role of subcellular localization as a determinant of Akt activation is uncertain. Because Akt localization appears to differ in invasive cancer cells compared with non-invasive cancer cells (see below), determining the mechanisms for Akt nuclear import and export may represent an important step in clarifying the signals involved in cancer progression.

Akt is known to play a key role in cell motility and its activity has been associated with cancer cell invasion for numerous malignancies. We and others have previously demonstrated increased Akt activity in thyroid cancer tissue compared to normal adjacent thyroid tissue [6,7]. More recently, we demonstrated an association between invasion and metastasis, and nuclear

* Corresponding author. Fax: +1 614 292 1550.
E-mail address: ringel.11@osu.edu (M.D. Ringel).
[1] Both authors contributed equally to this work.

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co-localization of activated Akt and the Akt1 isoform (but not Akt2 or 3) in human thyroid cancer tissue, and in thyroid cancer cells that have migrated through membranes in Boyden chambers in vitro [8]. Interestingly, there was no evidence for differential activation of ERK in invasive thyroid cancer cells and thyroid cancer cell migration in two cell lines was specifically dependent on PI3 kinase activation, suggesting a unique role for this pathway in thyroid cancer cell motility. The precise downstream pathways modulated by Akt that can induce motility have been shown to include p70S6 kinase [9], Rho [10,11], p21 activated kinases (PAK) [12,13], and p27 [14–17]. However, whether nuclear localization of Akt1 and nuclear Akt activity are primary events in thyroid cancer cell motility or invasion, or are secondary to this process is uncertain, and the role of subcellular localization of Akt1 as a determinant of the pathways activated by Akt resulting in cell motility is undefined.

The demonstration that invasive cancer cells are frequently characterized by nuclear Akt activation that is distinct from cancer cells in the central regions of tumors suggests that Akt subcellular cycling may be regulated and could play a role in tumor invasion. The goals of the present study were to define potential regulatory mechanisms for Akt1 nuclear localization and to determine if nuclear Akt1 localization is sufficient to induce cell motility.

Materials and methods

Materials. cDNA encoding wild-type and dominant negative Akt tagged with hemaglutinin (HA) were the generous gifts of Dr. M. Aoki (Scripps Institute, La Jolla, CA). Mammalian expression vectors, pcDNA3.1 and pEGFP-C1, were obtained from Invitrogen (Carlsbad, CA) and BD Biosciences (Palo Alto, CA), respectively. PCR related material, including reverse transcriptase (RT), buffer for RT, dNTPs, and RNase Inhibitor, was purchased from Applied Biosystems (Foster City, CA). Random hexamers, primers, and sequence-specific probes for PCR were from Qiagen (Valencia, CA). Buffers for PCR, TRIzol, Lipofectamine, Lipofectamine 2000, culture medium, and serum were obtained from Invitrogen (Carlsbad, CA). Primary antibodies recognizing phosphorylated and total Akt, and phosphorylated and total PAK were from Cell Signaling Technology (Beverly, MA). Antibodies against Akt1 (#610860) and p27 (#554069) were from BD Biosciences. Antibody against Akt2 (#06-606) was from Upstate Biotechnology (Lake Placid, NY). Antibodies against Akt3 (C-14), hemagglutinin (Y-11), α-tubulin (B-7), lamin A/C (346), and CRM-1 (H-300) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Isoform-specific Akt siRNA was from Cell Signaling Technology. GFP-labeled siRNA was from Upstate Biotechnology. QuikChange Mutagenesis kit was from Stratagene (La Jolla, CA). Leupeptin, pepstatin, 4-amidino-phenylmethane-sulfonyl fluoride (APMSF), and aprotinin were from EMD Biosciences (San Diego, CA). All other materials were obtained from Sigma–Aldrich (St. Louis, MO), unless otherwise described.

cDNA constructs. HA-Akt1 cDNA was excised from pBluescript SK+ using *Hin*dIII and inserted into pcDNA3.1(+) within multiple cloning site [18]. Similarly cDNAs of Akt2 and 3 were inserted into pcDNA3.1 using *Eco*RI and *Apa*I plus *Not*I, respectively. AKT/NES cDNA was created using an in vitro mutagenesis kit (Strategene) using HA-tagged wild-type Akt1 as the base construct. The sequences of the wild-type, L277A/L280A/L282A (NES) mutant, and T308D/S473D (CA) mutant were confirmed by direct sequencing. Once confirmed, the cDNA constructs were excised from pcDNA3.1(+) vector using *Xba*I, which eliminates the HA tag but includes the entire Akt1 cDNA, and placed in pEGFP-C1 vectors.

Akt1 cDNA of S308A/T473A mutant was also from Aoki [19]. Similar to wild-type Akt1, this mutant was inserted into pEGFP-C1 vector using *Xba*I. Akt1 cDNA of K179M was obtained from Dr. M. Shong (Seoul National University, Seoul, Korea).

Cell lines and transfection. Human papillary thyroid carcinoma NPA cells were obtained from Dr. Guy J.F. Juillard (UCLA, Los Angeles, CA) and were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). HEK293 cells were used for initial transient expression experiments and maintained in DMEM medium containing 5% FCS. For transient expression transfection was performed using Lipofectamine reagent in OptiMEM and incubated with plasmid for 16 h. Transfection was confirmed using anti-HA antibodies or by GFP fluorescence detected by fluorescence microscopy (Zeiss AXIOVERT 40 CFL, Thornwood, NY). Murine embryonic fibroblast (MEF) cell lines from Akt1$^{-/-}$ mice and controls were obtained (Dr. M. Birnbaum, University of Pennsylvania, Philadelphia, PA) and cultured as previously described [20]. MEFs were stably transfected with the various Akt constructs in pcDNA3.1(+). After selection of transfected clones by G418 resistance, clones were screened for expression of Akt1 by RT-PCR and for protein using anti-HA and anti-Akt1 antibodies.

siRNA transfection was performed using Lipofectamine 2000 for NPA thyroid cancer cell experiments. For siRNA transfection of MEFs, cells were seeded in six-well dishes. The siRNAs were incubated with RNAiFect (QIAGEN) in DMEM with 5% FCS for 10 min; this transfection mixture was then added to cells in culture medium. Transfected cells were then used for migration experiments and for protein isolation as described in detail below.

Immunohistochemistry. Cells were cultured in LabTek Chamber slides (Nalge Nunc International, Naperville, IL). At low confluence levels (~10–15%), the growth medium was aspirated and cells were fixed using 3.7% formalin solution. After washing in PBS, the cells were incubated with 0.2% Triton solution for 10 min. Incubation with primary antibody was performed overnight at 4 °C. Secondary antibody conjugated with peroxidase or fluorescence was applied for 1 h in the dark at room temperature. Cover slides were then mounted with Prolong antifade kit (Molecular probes, Eugene, OR) and subjected to microscopic examination.

Protein isolation and Western blotting. Cells were washed with ice-cold PBS twice and lysed with ice-cold lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 20 μM (APMSF), 1% Triton X-100, and 0.3 μM okadaic acid]. The cells were scraped, collected into microcentrifugation tubes, and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were transferred into fresh tubes and stored at −80 °C. The protein concentration of cell lysates was determined using a micro-BCA protein assay reagent kit (Pierce, Rockford, IL).

When cytosolic and nuclear proteins were isolated, NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, IL) was used. To confirm separation, Western blot analyses were performed, and tubulin and lamin A/C were used as a marker for cytosolic and nuclear proteins, respectively.

Twenty micrograms of total protein lysate was suspended in reduced SDS sample buffer (Invitrogen) and boiled for 5 min. Protein lysates were subjected to 8% or 4–10% SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes (0.45 μm pore size, Invitrogen) by electrophoretic blotting (Invitrogen). Non-specific binding was prevented by blocking the membrane with TBS-T (0.1%

Tween 20 in 20 mM Tris–HCl, pH 7.6, and 137 mM NaCl) containing 5% non-fat dried milk overnight at 4 °C. Immunoblotting was performed as previously described [6,8].

Immunoprecipitation. Two to 400 μg of total protein was incubated agarose-conjugated antibody against HA for 2 h at 4 °C. Immunoprecipitation and co-precipitation experiments were performed using ProFound Mammalian HA-Tag IP/Co-IP kit (Pierce) as per the manufacturer's recommended protocol.

Cell proliferation. Cells were plated in 12-well plates in growth medium containing serum and medium was changed 24 h after seeding (Day 0). Reaction was terminated by washing ice-cold PBS and 10% perchloric acid at Day 1 and 2, and stored in refrigerator. DNA content was measured simultaneously as previously described [21].

Migration experiments. Three hundred microliters of cell suspension (final concentration of $1–3 \times 10^5$ per well) was placed on the top of the membrane of a Boyden chamber (8 μm pore size, Millipore, Bedford, MA) in DMEM containing 0.2% FCS. The bottom part of the chamber was filled with 400 μl DMEM containing 5% FCS. Cells were then incubated for 8–16 h. At the end of the experiments, cells were fixed and stained using a Diff-Quick staining kit (Dade Behring, Newark, DE). Cells were observed using both low- (10×) and high-power (40×) microscope objectives. Photographs of three representative low-power fields of the total membrane-bound cells were taken. Non-migrated cells remaining on the top of the membranes were removed using a cotton swab. Cells that were not removed are those that migrated to the lower membrane. Three photographs were taken to measure the migrated cells only. All digital photographs were then transformed into gray-scale and then to a bit map using 50% threshold imaging (Adobe Photoshop 7, Adobe Systems, San Jose, CA). The percentage of cells migrated was then calculated using the mean and standard deviations of the four photographs for the total and migrated cells on each membrane. All migration experiments were performed on at least three occasions in duplicate.

Statistical analysis. DNA synthesis and migration results were examined by Student's *T* test or by non-parametric tests depending on the data distribution using StatView (Abacus Concepts, Berkeley, CA). Experiments were repeated at least three times in duplicate. For all analyses, $p < 0.05$ was considered significant.

Results

Identification of a potential nuclear export signal in Akt

While Akt does not contain a nuclear localization signal, evaluation of the Akt1, 2, and 3 sequences reveals a conserved leucine-rich potential CRM-1-binding nuclear export signal (NES, Fig. 1A). Leptomycin B (LMB) interferes with CRM-1–NES interactions. Initial experiments confirmed that NPA thyroid cancer cells express CRM-1 protein (Fig. 1B) and subsequent treatment of NPA cells with LMB results in nuclear accumulation of Akt1 and phosphorylated Akt (pAkt) in the nucleus (Fig. 1C). The effect of LMB on intracellular movement of Akt1 was more pronounced than Akt2 or Akt3 which localized to the nucleus prior to LMB treatment in these cells. In addition, treatment of NPA cells with LMB resulted in an increased tendency for migration in Boyden Chamber assays (data not shown). Specific inhibition of Akt1, but not Akt2 or Akt3, using siRNA reduces NPA cell migration in vitro (Fig. 2), suggesting a specific role for Akt1 in motility of this particular cell line.

Akt1 contains a functional CRM-1-binding nuclear export sequence

Because LMB is not specific for the AKT/NES, we created an Akt1 construct with mutations in the NES domain (L277A/L280A/L282A) to determine if Akt1 binding to CRM-1 was dependent on this sequence (AKT/NES construct). Using immunoprecipitation, AKT/NES displayed reduced interactions with CRM-1 in comparison to wild-type Akt1 (Fig. 3A). Transient

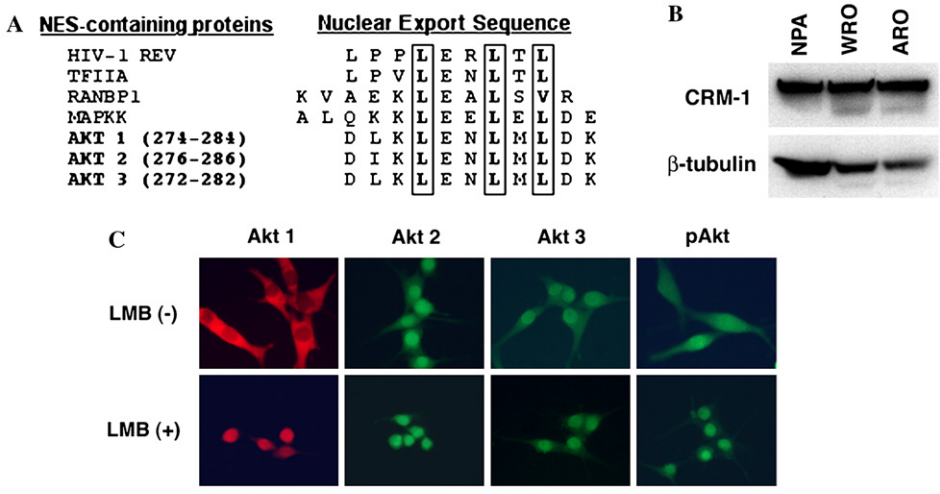

Fig. 1. (A) Akt1, 2, and 3 contain a conserved leucine-rich nuclear export domain. (B) CRM-1 protein levels in human thyroid cancer cell lines NPA, WRO, and ARO are demonstrated by Western blot. (C) Immunofluorescence of Akt1, 2, and 3 using specific antibodies and ser^{473} total phosphorylated Akt (pAkt) [8] before and after treatment with 1 μM leptomycin B (LMB) for 24 h in NPA thyroid cancer cells. Akt1 is redistributed to the nucleus following LMB treatment. Akt2 and 3 tend to be nuclear localized in both conditions, and pAkt becomes mostly nuclear following LMB treatment.

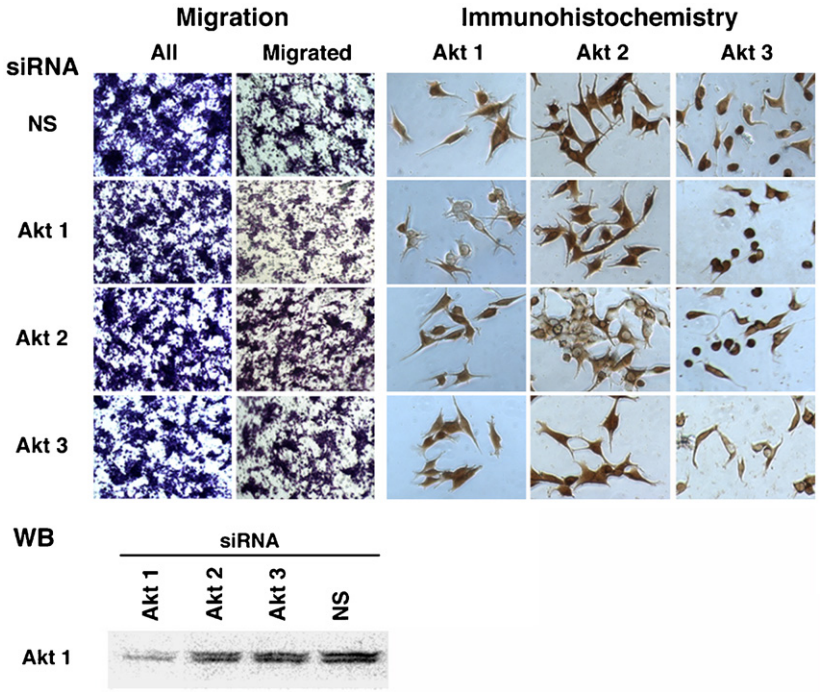

Fig. 2. NPA thyroid cancer cell migration is Akt1-dependent. NPA cells were transfected with Akt isoform specific siRNA or non-specific siRNA. Twenty four hours after transfection, cells were seeded on Boyden chambers in RPMI containing 0.2% FCS in the upper chamber, and 5% FCS in the lower chamber. Cell migration was analyzed 16 h later. Simultaneously, cells were seeded on chamber slides and were analyzed for expression of Akt1, 2, and 3 using isoform-specific antibodies. Suppression of Akt1 expression inhibited migration, while suppression of Akt2 and 3 had minimal effects. Lower panel displays a Western blot confirming the specificity of the Akt1 siRNA.

transfection of a GFP-AKT/NES cDNA construct into HEK293 cells demonstrated preferential nuclear localization in comparison to both wild-type Akt1 and a mobile constitutively active Akt1 mutant cDNA (T308D/S473D, Akt/CA). Inactive forms of Akt1 (K179M and T308A/S473A) localized to similar subcellular compartments as compared to wild-type Akt1 (Fig. 3B), suggesting that phosphorylation of Akt is not required for nuclear localization. Cells were then stimulated with serum or insulin and localization of the constructs was assessed using their GFP-tagged fluorescence (Fig. 3C). Only the AKT/NES construct demonstrated predominantly nuclear localization in both basal and stimulated conditions. Nuclear movement of wild-type Akt1 was induced by insulin in a PI3 kinase-dependent manner, suggesting that activation facilitates Akt nuclear localization.

Akt1 expression is required for cell migration and nuclear Akt1 is sufficient to induce migration

To evaluate the functional consequences of nuclear Akt1 activation, Akt1 null murine embryonic fibroblasts (MEFs) that express Akt2 and 3, but not Akt1 [20] (generous gift from Dr. Morris Birnbaum, University of Pennsylvania), were stably transfected with control vector, wild-type Akt1, or AKT/NES all epitope tagged with HA. To confirm that mutation of the NES region of Akt1 does not abrogate its kinase activity, nuclear and cytosolic extracts were isolated from these cells and immunoprecipitation experiments were performed using a conjugated anti-HA antibody. A greater proportion of AKT/NES localized to the nucleus compared to the other constructs, and kinase assays demonstrated localization of Akt kinase activity in the AKT/NES-expressing cells that corresponds to the localization of the protein (Fig. 4A). Thus, in comparison to mobile forms of Akt1, Akt1/NES is more likely to be nuclear localized and maintain Akt kinase activity. Expression and localization of Akt1 protein was also confirmed in several clones of stable transfectants for each construct by immunofluorescence (Fig. 4B). Cell growth assays did not demonstrate an increase in cell growth induced by overexpression of any Akt1 construct in the Akt1 null MEFs (Fig. 4C).

Because nuclear activation of Akt is associated with cancer invasion and metastasis in human tumors [22–26], and with migration of human thyroid cancer cell lines in vitro [8], we performed Boyden Chamber migration studies using several of the Akt1 null MEF cell lines expressing vector alone, wild-type Akt1, Akt/CA, and AKT/NES; as well as MEFs created from wild-type controls. The wild-type MEFs migrated in Boyden Chambers; a characteristic that was lost in the $Akt1^{-/-}$ MEFs. Re-expression of Akt1 in the $Akt1^{-/-}$ MEF cell line using each of the Akt constructs resulted in

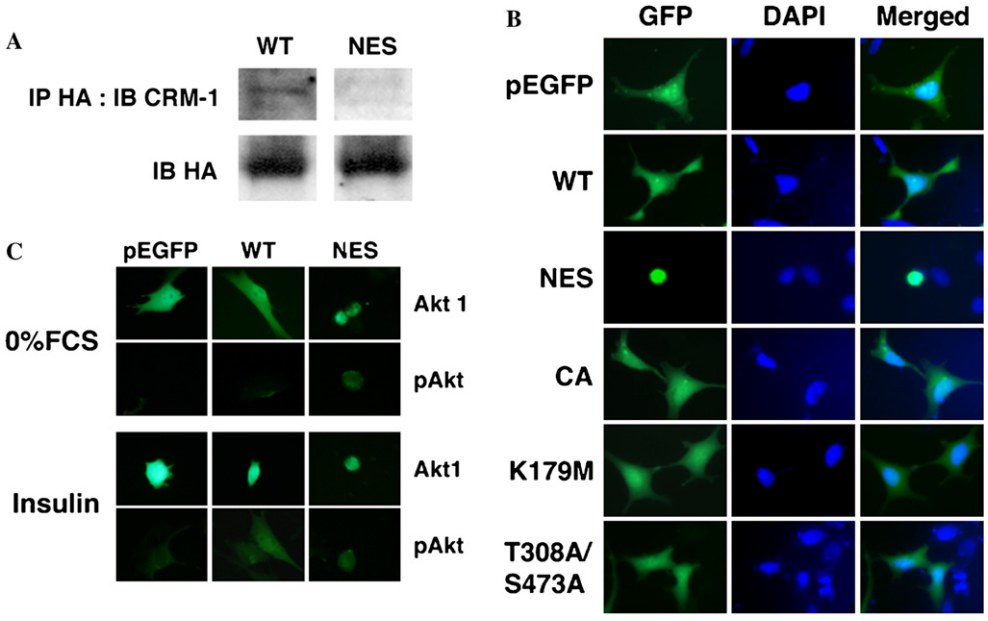

Fig. 3. (A) Mutation of the NES sequence reduces Akt interactions with CRM-1. HEK293 cells were transiently transfected with wild-type HA-tagged Akt1 (WT) or AKT/NES (NES) cDNA and after 48 h, immunoprecipitation was performed using an anti-HA antibody and the precipitated proteins were electrophoresed and blotted using an anti-CRM-1 antibody. For equivalent amounts of precipitate protein (shown with anti-HA immunoblot), AKT/NES displays reduced interactions with CRM-1. (B) Mutation of the NES sequence leads to preferential nuclear localized Akt1. HEK293 cells were transiently transfected with wild-type GFP-tagged Akt1 (WT), AKT/NES (NES), phosphorylation mimicking constitutively active Akt1 (T308D/S473D, CA), kinase dead (K179M), and dominant negative (T308A/S473A). After transfection in the presence of 5% serum, cells were incubated with medium containing 0.2% serum for 24 h. Only the NES protein localizes primarily to the nucleus, while the other constructs displayed both cytoplasmic and nuclear localization. (C) AKT/NES localizes to the nucleus in the absence and presence of insulin. HEK293 cells were transiently transfected GFP-tagged cDNAs encoding wild-type Akt (WT), AKT/NES (NES) or vector control (pEGFP). Twenty-four hours after transfection, cells were shifted to DMEM with no FCS for 24 h, after which 100 ng/ml insulin was added to medium for 1 h. Localization of exogenous Akt1 was examined by GFP fluorescence; total cellular pAkt was assessed by immunofluorescence following formalin fixation using an anti-pAkt antibody. Wild-type Akt was localized to both the cytoplasm and nucleus in the absence of serum or insulin and relocalized to the nucleus following insulin stimulation. In contrast, AKT/NES localized to the nucleus in both conditions and resulted in primarily nuclear activation of Akt.

restoration of migration, confirming that Akt1 expression is required for MEF migration in vitro (Fig. 4D). The cell lines were also tested for anchorage-independent growth. None of the clones exhibited anchorage-independent growth (data not shown).

Discussion

In the present study, we establish the novel findings that Akt isoforms contain a conserved and functional leucine-rich nuclear export sequence, and that nuclear export deficient Akt1 is functionally active and is sufficient to induce cell migration. The potential relevance of these findings to cancer is supported by demonstration that nuclear Akt1 and pAkt are associated with cancer invasion and metastasis in vivo and in vitro [8], and that specific reduction of Akt1 expression using siRNA reduces thyroid cancer cell migration in vitro. Thus, it appears that the observed nuclear Akt1 expression and Akt activation have the potential to be functionally involved in cancer invasion and that an understanding of the regulation of Akt1 subcellular localization may be mechanistically important.

We have focused our studies on Akt nuclear export due to the presence of a conserved NES in the Akt sequences. Indeed, the identification of a nuclear export signal suggests that modification of Akt in this region, either through pre or post-translational mechanisms, could alter cell biology in vivo in a manner that induces cell migration. This possibility is further supported by evidence that mutation of the NES does not alter Akt kinase activity, despite the location of the NES (AA 272–284) in the kinase domain.

It is likely, however, that Akt1 nuclear import can also be regulated in cancer cells. In support of this, we demonstrate that wild-type Akt1 nuclear import is enhanced by its activation. We also confirmed recently published data [27] that Akt nuclear localization does not require its activation since the kinase dead and phosphorylation resistant forms also enter the nucleus. Thus, it appears that phosphorylation of Akt facilitates, but is not required for, its nuclear import in this in vitro model system. While the mechanisms of Akt nuclear import remain poorly defined, enhanced activation, as is noted

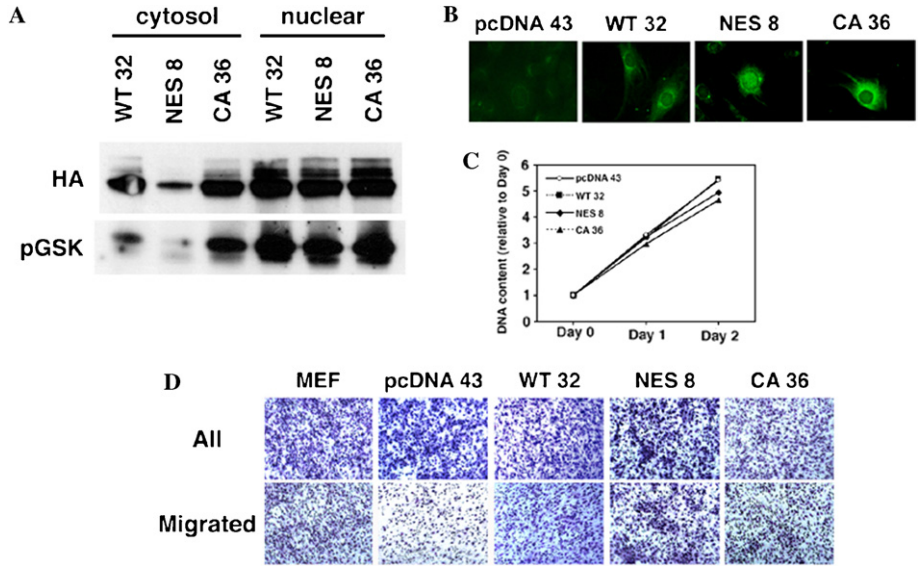

Fig. 4. Akt1$^{-/-}$ MEF cells were stably transfected with HA-tagged wild-type Akt1 (WT), AKT/NES (NES) or phosphorylation mimicking constitutively active Akt1 (T308D/S473D, CA). Multiple clones were obtained, representative clone results are demonstrated. (A) NES demonstrats preferential nuclear localization and maintains kinase activity. Nuclear and cytosolic protein extracts were isolated from the stable transfectants and immunoprecipitation was performed using an anti-HA antibody. After electrophoresis, the NES was preferentially localized to the nucleus and, in comparison to WT, displayed greater levels of nuclear relative to cytosolic Akt activity as detected by kinase assay using recombinant GSK as substrate. Separation of cytosolic and nuclear proteins was confirmed by Western blotting using antibody against α-tubulin and lamin A/C, respectively. (B) Confocal immunofluorescence microscopy using anti-HA antibody demonstrates preferential nuclear and nuclear-membrane localization of the NES construct in comparison to WT or CA. (C) Assessment of cell growth by measurement of DNA content over 48 h in low serum (0.2%) conditions does not demonstrate increased growth induced by overexpression of any of the Akt construct. (D) Boyden chamber assays demonstrate that control murine embryonic fibroblast cells (MEF) migrate efficiently, but that this property is lost in Akt$^{-/-}$ cells and subsequently regained with re-expression of all of the Akt1 constructs.

in invasive cancers, may be involved in increasing nuclear Akt1 import.

Our data also suggest that nuclear Akt1 initiates distinct downstream events that are sufficient to induce cell migration independent of cytoplasmic Akt signaling. Specifically, expression of the AKT/NES construct that is devoid of cytoplasmic activity was sufficient to induce cell migration. As the nucleus is the primary steady-state localization profile of activated Akt1 in the majority of invasive human thyroid cancers, an important role for subcellular localization as a determinant of downstream signaling and subsequent cellular effects is suggested. The mechanisms for nuclear Akt1-induced cell migration are under investigation. Akt1 is known to directly interact with a number of targets in the nucleus as well as in the cytosol. It is likely that these direct interactions are responsible for some of the ability to induce cell migration. Alternatively, the mechanism could be indirect through cross-talk pathways.

In addition to subcellular localization as a determinant of downstream effects on cell motility, there may be importance differences in the abilities of the Akt isoforms to regulate this process. The roles of nuclear Akt2 and 3 nuclear activity were not tested in the present study due to the specific association between nuclear localization of the Akt1 isoform and enhanced nuclear Akt activation in thyroid cancer progression and motility [8] and the effects of Akt1 siRNA on thyroid cancer cell motility. However, as all Akt isoforms contain the conserved NES sequence, it is probable that subcellular localization of Akt2 and 3 is similarly regulated and may also be a determinant of functional consequences and these effects may differ for different cancers.

In summary, all Akt isoforms contain a leucine-rich nuclear export sequence in their kinase domain. In Akt1, this sequence is functional, as its mutation reduces interactions with CRM-1 and results in preferential nuclear localization. Mutation of the NES region of Akt1 does not alter its kinase activity and its expression in Akt1$^{-/-}$ MEFs induces cell migration. These data, in combination with the requirement for Akt1 expression in thyroid cancer cell migration and the associations between nuclear Akt1, Akt activity, and thyroid cancer invasion in vivo, suggest a potential role for subcellular localization of Akt1 as an important and regulatable determinant of cell biology in human disease.

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