[CANCER RESEARCH 63, 4724–4730, August 1, 2003]

Alternative Splicing Disrupts a Nuclear Localization Signal in Spleen Tyrosine Kinase That Is Required for Invasion Suppression in Breast Cancer[1]

Lei Wang, Lindsay Duke, Peter S. Zhang, Ralph B. Arlinghaus, W. Fraser Symmans, Aysegul Sahin, Richard Mendez, and Jia Le Dai[2]

Departments of Molecular Pathology [L. W., L. D., R. B. A., R. M., J. L. D.] and Pathology [P. S. Z., W. F. S., A. S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Spleen tyrosine kinase (Syk) is a candidate tumor (metastasis) suppressor that is highly expressed in mammary epithelial cells. Loss of Syk expression through promoter hypermethylation is associated with increased invasiveness in a subset of breast cancer. Here, we show that in addition to full-length Syk [Syk(L)], an alternatively spliced variant, Syk(S), is frequently expressed in breast cancer cells. Syk(S) is identical to Syk(L), except that it lacks 23 amino acid residues (deletion) within the interdomain B (IDB) of Syk. We also show that the aberrant expression of Syk(S) occurs frequently in primary breast tumors but never in matched normal mammary tissues, suggesting a contribution of Syk(S) to mammary tumor progression. Expression of Syk(L) suppressed breast cancer cell invasiveness. In contrast, Syk(S) expression did not affect the cell invasion potential. This differential phenotypic response is accompanied by their different subcellular localization. Immunocytochemical studies and nuclear and cytoplasmic fractionation experiments indicated that Syk(L) could enter the nucleus, whereas Syk(S) was located exclusively in the cytoplasm. Five basic residues in deletion were found to be critical in determining Syk(L) nuclear transport and invasion suppression activity; mutations completely excluded Syk(L) from the nucleus and blocked Syk(L)-inducible invasion suppression. Moreover, IDB acted as an autonomous nuclear localization signal to facilitate nuclear transport of a heterologous protein. Thus, the IDB of Syk(L) contains a nuclear localization signal that is responsible for Syk(L) nuclear translocation. The correlation of the nuclear localization and invasion suppression function of Syk(L) indicated that nuclear Syk possesses biological activities associated with tumor suppression in mammary epithelial cells.

INTRODUCTION

Syk[3] and its homologues, Zap70, encompass a SYK family of cytoplasmic tyrosine kinase that participates in signal transduction cascades critical for activation and proliferation of a variety of hematopoietic cells (1–5). Syk contains two tandem SH2 domains at the NH_2 terminus, which are separated by an interdomain (IDA), and one catalytic kinase domain at the COOH terminus. The second SH2 domain and the kinase domain are separated by an intervening domain, IDB (Fig. 1). The reported human *SYK* cDNA encodes a protein of 635 amino acids in length (6–8). In addition to this full-length Syk, an alternatively spliced *SYK* transcript (short form) that lacks a 69-bp sequence has been reported (7, 9, 10). The alternatively spliced in-frame transcript creates an Syk protein isoform [Syk(S)] that lacks a 23-residue sequence (DEL) within the IDB (Fig. 1). The expression of Syk(S) in normal tissues is uncommon. The abundance of Syk(S) was reportedly lower than 1% Syk(L) in lymphocyte lines examined; mouse bone marrow was the only tissue type with reportedly high Syk(S) expression (as much as 50%), although the significance of its expression remains unclear (9). In part because of the relatively scarce expression of Syk(S), its physiological and pathological roles and its distinction from Syk(L) have not been studied extensively.

The SYK signal transduction pathway in hematopoietic cells has been worked out in great detail. In these cells, engagement of immune recognition receptor triggers the recruitment of Syk or Zap70, through its tandem SH2 domains, to the doubly phosphorylated immunoreceptor tyrosine-based activation motif of the activated and phosphorylated immunoreceptors (11). There Syk or Zap70 is additionally activated by a combination of autophosphorylation and phosphorylation by Src family protein tyrosine kinases (12). The activated Syk then is available to phosphorylate its protein substrates (13–16), events that are thought to activate pathways responsible for activation of hematopoietic cells. In addition, phosphorylation of certain proteins such as Cbl is believed to modulate the activity of SYK signaling (17, 18), probably through Cbl-mediated Syk ubiquitilation (19). In addition to mediating signals from immunoreceptor activation, Syk relays signals from other receptors, including integrins (20–22), cytokine receptors (23), and G-protein-coupled receptors (24), through mechanisms that differ from SH2-phosphorylated immunoreceptor tyrosine-based activation motif interaction. These observations reflect the complex SYK signaling pathways that are likely to involve multiple partners and a variety of pathways integrated in a tissue-specific manner.

Syk is expressed in mammary gland as well. The first hint of SYK in breast pathobiology came from the observation that Syk expression was lost in some highly invasive breast cancer cell lines (25). The association of the loss of Syk expression with breast cancer invasion is not limited to cultured cells. *In situ* hybridization experiments indicated high levels of *SYK* mRNA in normal mammary epithelial cells, lower levels in ductal carcinoma *in situ*, and none in invasive breast carcinoma (25). The regulation of Syk expression occurs at the transcriptional level, the down-regulation of SYK predicting poorer prognosis for breast cancer (26), and probably other tumor types (27). Alteration of the *SYK* gene has been occasionally observed in hematopoietic malignancy (28), genetic alteration of *SYK*, however, has not been identified in breast tumors. We reported that methylation-mediated gene silencing is causally responsible for SYK loss of expression. Importantly, this hypermethylation at the *SYK* promoter region occurs *de novo* in >30% of primary breast cancers but not in normal mammary gland (29). Aberrant hypermethylation represents an epigenetic mechanism to inactivate a variety of tumor suppressor genes (30). Aberrant hypermethylation of the *SYK* gene suggests, therefore, that Syk plays an important role in mammary malignancy and that Syk may have suppression functions. The invasion-suppressive function of the *SYK* gene was supported by studies showing that cells overexpressing transfected wild-type *SYK* cDNA [*SYK(L)*] exhibited decreased tumorigenicity and metastatic potency (25).

Here, we report frequent expression of Syk(S) in neoplastic mammary tissues, and we present evidence for the differing biological

Received 1/6/03; accepted 5/22/03.

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[1] Supported by funds from Physicians Referral Service of MDACC and United States Department of Defense Grant DAMD17-02-1-0450 (to J. L. D.).

[2] To whom requests for reprints should be addressed, at Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Unit 89, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-8995; Fax: (713) 792-4324; E-mail: jldai@mdanderson.org.

[3] The abbreviations used are: Syk, spleen tyrosine kinase; NLS, nuclear localization signal; IDB, interdomain B; DEL, deletion; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenyindole.

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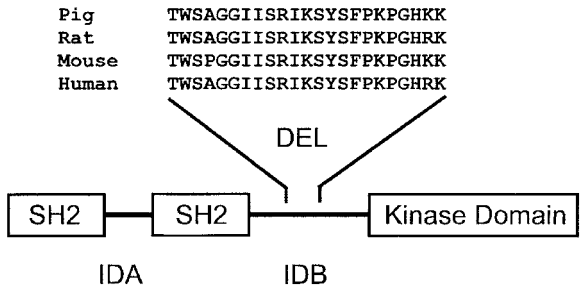

Fig. 1. Domain structure of Syk protein and its alternative splicing variant Syk(S). Syk(S) has 23 amino acid residues (DEL) missing in the IDB region. The sequence homology of the 23-residue DEL across species is shown.

activity of Syk(L) and Syk(S). The structural difference of the two isoforms is shown to contribute to the differing subcellular localizations of the Syk proteins, helping to explain their different biological functions in mammary epithelial cells.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis. The 2.2-kb and the 2.3-kb *Kpn*I/*Xba*I fragments were released from Ku41 and Ku31 (gift from Dr. Shintaro Yagi) as *SYK(L)* and *SYK(S)* cDNAs (7), respectively. The two cDNAs were then subcloned into pcDNA3.1(+) (Invitrogen) to generate pcDNA3.1-SYK(L) and pcDNA3.1-SYK(S), respectively. To generate *SYK(L)* cDNA constructs in which basic residues in DEL were replaced with alanines, a two-step PCR protocol was adopted to introduce mutation of the specified codons using pcDNA3.1-SYK(L) as a template. pcDNA3.1-SYK(L)-M1 or pcDNA3.1-SYK(L)-M2 were generated in which residues R292 and K294 or R304 and K305 were replaced with alanines, respectively. pcDNA3.1-SYK(L)-M3 was also constructed in which all four basic residues were replaced with alanines.

We used PCR to subclone IDB(L) or DEL of Syk in frame into the pEGFP-C3 vector (Clontech) to generate GFP fusion [pEGFP-IDB(L) and pEGFP-DEL]. Two-step PCR was used to generate pEGFP-IDB(L)-M in which three basic residues (K294, K300, and K305) were replaced with asparagines. The entire reading frames of all constructs generated by PCR were confirmed by automated sequencing at the DNA Analysis Core Facility of M. D. Anderson.

Cell Culture and Transfection. All cell lines were purchased from American Type Culture Collection and cultured in recommended media. FuGENE 6 reagent (Roche) was used for cell transfection. To generate stable lines that expressed Syk(L), Syk(S), or Syk(L) mutants, MDA-MB-435S cells were transfected with pcDNA3.1-SYK(L), pcDNA3.1-SYK(S), pcDNA3.1-SYK(L)-M1, pcDNA3.1-SYK(L)-M2, or pcDNA3.1-SYK(L)-M3, or parental vector pcDNA3.1(+) and selected with 800 μg/ml G418 (active concentration; Invitrogen). To prepare individual stable clones, cells were plated with limited dilution, and G418-resistant clones were screened for protein expression by immunoblot analyses. To generate pooled stable cell lines, G418-selected cells were pooled.

Cytosolic and Nuclear Fractionation. Approximately 1×10^7 cells (parental or stable lines) were collected by trypsinization and centrifugation. Cell pellets were then suspended and incubated for 10 min in 800-μl ice-cold buffer A [10 mM HEPES (pH 7.4), 1 mM EDTA, and 1 mM DTT]. Cells were then subjected to Dounce homogenization (pestle B), and the lysis completion was monitored by trypan-blue staining. The supernatant was collected as cytosolic fraction. The nuclear pellets were then washed twice with 400 μl of buffer B [50 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA, 0.5 mM spermide, and 0.15 mM spermine]. The washed nuclear pellets were extracted with 150 μl of buffer C [350 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA, 0.5 mM spermide, and 0.15 mM spermine]. Then the suspension was centrifuged at $20,000 \times g$ for 30 min at 4°C and the supernatant collected as nuclear fraction. Protease inhibitors (Complete, from Roche) were included in all buffers.

Immunoblotting. Total protein lysates were prepared by dissolving tissues or trypsinized cells in 1× SDS sample buffer. Normal and neoplastic tissues were obtained from breast carcinomas resected at the M. D. Anderson Cancer Center and archived in the breast tumor bank. All normal and tumor samples were histologically confirmed.

BCA reagents (Pierce) were used to determine protein concentration. Equal amount of protein preparations (50 μg of total cell lysates, cytosolic, or nuclear fractions) were resolved on SDS-PAGE. For nuclear and cytosolic fractionation experiments, membranes were cut into three parts after blotting, according to the different molecular weight of Oct-1 (nuclear protein control, M_r ~110,000), Syk (M_r ~70,000), and actin (cytoplasmic protein control, M_r ~42,000). Polyclonal antibodies against Syk (N-19), Oct-1 (C-21), and actin (I-19; all from Santa Cruz Biotechnology) were used for immunodetections. For analyses of Syk expression in primary breast cancers, membranes were first blotted with Syk antibody followed by stripping and blotting with actin antibody. Detection was afforded by horseradish peroxidase-conjugated secondary antibodies (Pierce) and Supersignal reagents (Pierce).

Immunohistochemical Assay. Cells cultured on 8-well chamber slides (Nunc) were fixed and permeabilized in 100% methanol (−20°C). Slides were then washed with ice-cold 1× PBS. Cells were incubated at 4°C overnight with Syk primary antibody (N-19, 1:300). After extensive washing with 1× PBS, cells were incubated at 37°C for 1 h with biotin-labeled antirabbit secondary antibody (1:200). Detection of Syk immunoreactive signal was afforded by Vectastain ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine (Sigma). Cells were then counterstained with hematoxylin and examined by light microscopy.

Fluorescence Microscopy. To detect subcellular localization of fusion proteins of GFP and Syk sub-domains (IDB or DEL), COS7 cells cultured on coverslips were transfected with pEGFP-IDB(L), pEGFP-DEL, pEGFP-IDB(L)-M, or parental pEGFP-C3. Twenty-four h after transfection, cells were fixed by 4% paraformaldehyde. To distinguish nuclear and cytoplasmic localization, the fixed cells were incubated with 1 μg/ml DAPI. Fluorescent signals by either GFP or DAPI were examined under the fluorescence microscope (Nikon Labophot-2). GFP fusion protein localization was scored in 100 cells of each experimental group.

Chemoinvasion and Chemotaxis Assays. Chemoinvasion assay was performed using Boyden chambers that had been coated with Matrigel (BD Bioscience). The lower chamber contained DMEM supplemented with 10% fetal bovine serum as chemoattractants. Pooled MDA-MB-435S stable cells ($1–5 \times 10^5$ cells resuspended in 200 μl of serum-free DMEM containing 1% BSA) were plated in the upper compartment of the transwells and incubated for 1–3 days at 37°C. After incubation, cells were fixed and stained with HEMA3 staining kit (Fisher Scientific). Cells remaining in the Matrigel side were removed by cotton swabs. Cells that had invaded through the Matrigel to the bottom side of the transwells were counted in 10 random fields. For chemotaxis assays, a similar protocol was followed, except for using transwells not coated with Matrigel. Four h after plating, cells that had migrated to the bottom side of the transwell were stained and counted.

Reverse Transcriptase-PCR. *SYK* mRNA expression in cultured breast cancer cell lines was examined by reverse transcriptase-PCR using conditions described previously (29). A primer set (forward, 5′-TTTTGGAGGCCGTCCACAAC-3′; reverse, 5′-ATGGGTAGGGCTTCTCTCTG-3′) that spans the 69-bp DEL region was used to amplify *SYK* cDNA. Another primer set (forward, 5′-AATCGGCACACAGGGAAATG-3′; reverse, 5′-AGCTTTCGGTCCAGGTAAAC-3′) was also used to confirm the presence of two transcripts.

RESULTS

Expression of Syk(S) in Breast Cancer Cells. Immunoblotting was used to evaluate the Syk protein expression in breast cancer cell lines. In agreement with our previous observation (29), cells with *SYK* gene promoter hypermethylation (Hs854T, MDA-MB-231, MDA-MB-435S, and MDA-MB-453) did not express Syk protein. In cell lines with unmethylated *SYK* (29), immunoblotting revealed two proteins (Fig. 2*A*). The slower migrating form (apparent molecular weight of M_r ~72,000) corresponded to full-length Syk(L) because it matched the Syk protein expressed in a leukemia line, K562, and a lymphoma line, Raji (Fig. 2*A*). The nature of the faster migrating form (M_r ~68,000) was unknown.

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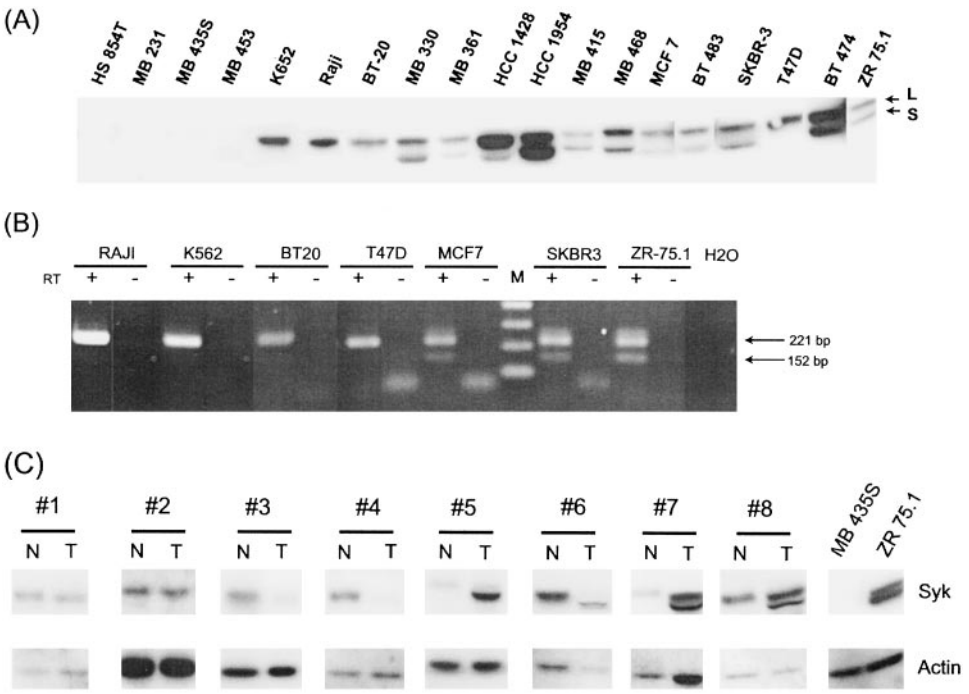

Fig. 2. Expression of Syk(L) and Syk(S) in breast cancer cell lines and primary breast cancers. *A*, immunodetection of Syk protein in a panel of breast cancer cell lines. Total cell lysates were subjected to SDS-PAGE followed by immunoblotting with antisera against Syk (N-19). A leukemia cell line, K562, and a lymphoma line, Raji, were included as positive controls. The long (L) and short (S) forms of Syk are indicated. *B*, reverse transcriptase-PCR was used to determine *SYK(L)* and *SYK(S)* mRNA expression. A primer set adjacent to the deleted 69-bp region was used. The 221- and 152-bp bands represent the *SYK(L)* and *SYK(S)* transcripts, respectively. A reverse transcriptase-negative (−) control was added to rule out false positives resulted from contaminated genomic DNA. These results were confirmed by use of a second primer set. *C*, immunodetection of the Syk(L) and Syk(S) in primary breast cancer and matched neighboring normal tissues. Tissues were homogenized in 1× SDS sample buffer, followed by SDS-PAGE and immunoblotting. After probing with Syk antibody, the membrane was stripped and reblotted with actin antibody to normalize protein loading. Syk-positive (ZR75.1) and -negative (MDA-MB-435S) controls were run in parallel.

A shortened form of *SYK* transcript resulting from alternative splicing that lacked a 69-bp sequence has been reported earlier (7, 9). This spliced reading frame results in the loss of 23 amino acid sequence (DEL) within the IDB. Surmising that the lower band might be the product of this transcription variant, we tested the possibility by using a primer set that spanned the sequences neighboring the DEL to amplify cDNAs from breast cancer cell lines. In cells expressing only the Syk(L), namely BT20, T47D, Raji, and K562 (Fig. 2*A*), only the 221-bp product corresponding to the Syk(L) transcript was amplified (Fig. 2*B*). By contrast, in SKBR3, MCF7, and ZR75.1 cells that expressed both Syk isoforms, we found that a shorter PCR product (152 bp) was present along with the 221-bp band (Fig. 2*B*). The 152-bp products matched the expected size of PCR amplification from *SYK(S)* transcript. These results were confirmed by using another primer set (data not shown). We also sequenced the long and short PCR products. The sequence of the longer PCR product (221 bp) was identical to that of *SYK(L)* cDNA, and the shorter product (152 bp) matched the reported *SYK(S)* cDNA with a 69-bp DEL. Thus, we concluded that the M_r 68,000 protein represented Syk(S) lacking the 23-residue sequence, and the two Syk isoforms in breast cancer cells were detectable by immunoblotting.

Syk(S) Is Expressed in Mammary Tumors. To assess whether Syk(S) is also expressed in primary breast cancers, we performed Syk immunoblotting using 16 nonselected human primary breast tumor cases and their matched pathologically normal breast tissues. Eight representative cases are shown in Fig. 2*C*. Among all samples analyzed, only Syk(L) was detectable in the normal mammary tissues. In tumors, however, three categories of Syk expression patterns were observed. In ~30% (5 of 16) of the cases, Syk(L) was expressed in tumors at a level comparable with that of normal tissues (cases 1 and 2). In ~20% (3 of 16) of the cases, Syk(L) was expressed at a decreased level in breast tumors (down-regulation, cases 3 and 4), which reflected the loss of Syk expression in a subset of primary breast cancers probably because of aberrant promoter hypermethylation. The frequency of Syk(L) down-regulation was lower than that of *SYK* promoter hypermethylation (33% of the cases). In contrast to *SYK* methylation, the Syk expression was not completely shut down. These could have resulted from the heterogeneity of breast cancer and/or contamination of normal tissues in tumors; or it may reflect the high sensitivity of methylation specific PCR, which can detect 0.1% methylated alleles (31). A subpopulation of *SYK*-methylated cells may be reflected by the positive methylation signal. Thus, an overall down-regulation of *SYK* at mRNA and protein levels instead of a complete loss of Syk expression would be expected. In the above two categories, Syk(L) was the only isoform expressed. In the third category, the Syk(S) band was evident in the remaining half (8 of 16) of the cases (cases 5–8, Fig. 2*C*). Syk(S) was found expressed along with Syk(L) in most of the breast cancer cell lines examined (Fig. 2*A*) but was missing in the neighboring normal tissues. The tumor-specific expression of Syk(S) in primary breast cancers suggests its contributory roles for mammary tumor initiation or progression.

Syk(L) but not Syk(S) Suppresses Breast Cancer Invasiveness. Overexpression of Syk(L) has been found to inhibit metastasis and invasion in mouse experimental models (25). To examine whether Syk(L) and Syk(S) exhibit similar biological activity in breast cancer cells, we prepared stable cell lines that expressed the *SYK(L)* and *SYK(S)* cDNA and determined whether the expression of two isoforms affected cell invasiveness. A Syk-negative cell line, MDA-MB-435S, was transfected with pcDNA3.1-SYK(L), pcDNA3.1-SYK(S), or pcDNA3.1 (Neo), and G418-resistant stable clones were pooled. The expression of Syk(L) or Syk(S) was verified by immunoblotting (Fig. 3*A*), and invasion capacity was measured by *in vitro* chemoinvasion assay. Compared with the Neo control, cells expressing *SYK(L)* cDNA exhibited decreased Matrigel invasion (by ~60%). In contrast, invasion activity was not affected by the expression of Syk(S) (Fig. 3*B*). The decreased cell invasiveness was not attributable to the effect of Syk(L) on cell migration because chemotaxis experiments indicated that overexpression of Syk(L) did not affect cell migration (Fig. 3*C*). The 23 residues constituted the only difference between Syk(L) and Syk(S), yet DEL of this short sequence abrogated the invasion suppression activity of Syk(L).

Nuclear Localization of Syk(L) but not Syk(S). Differential biochemical properties may account for different phenotypic functions. As our first attempt to dissect the differences between Syk(L) and

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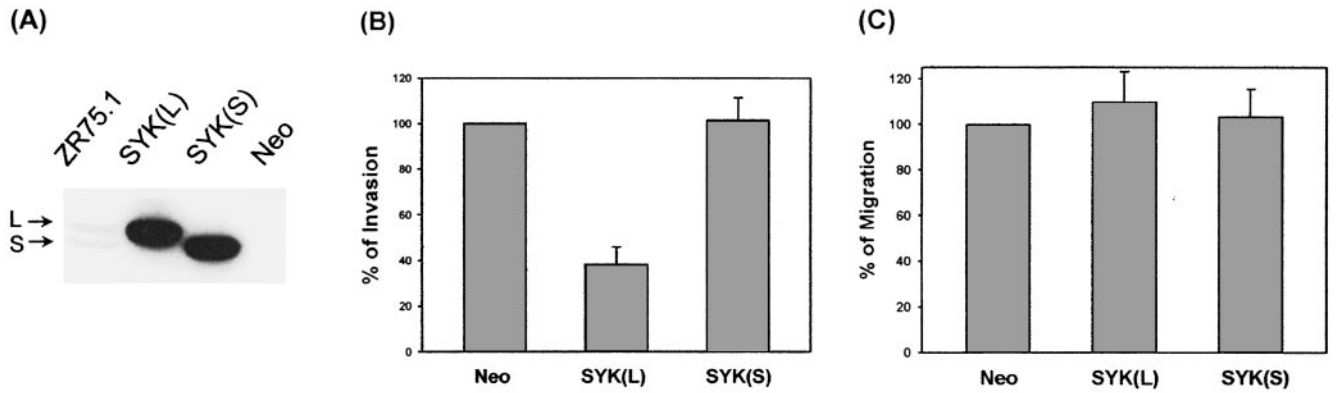

Fig. 3. Expression of Syk(L) but not Syk(S) suppresses breast cancer cell invasiveness. *A*, MDA-MB-435S cells were transfected with pcDNA3.1-SYK(L), pcDNA3.1-SYK(S), or pcDNA3.1 (Neo). G418-resistant clones were pooled and their Syk expression was measured by immunoblotting. ZR75.1 was used as a positive control to verify the mobility of Syk(L) and Syk(S). *B*, the invasion of MDA-MB-435S-SYK stable cells was measured by chemoinvasion assays. The number of cells that had invaded through Matrigel in the Neo group was arbitrarily set as 100%. An average of three independent experiments is plotted. *C*, MDA-MB-435S-SYK stable cells were tested for their migration capacity by chemotaxis assays using transwells without Matrigel coating.

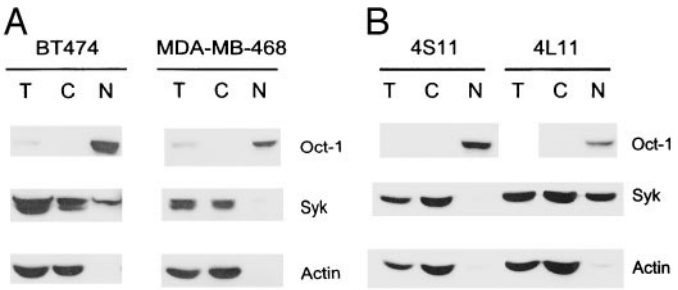

Fig. 4. Differential subcellular distribution of Syk(L) and Syk(S). Subcellular fractionation was carried out to determine the localization of Syk(L) and Syk(S) endogenously expressed in breast cancer lines BT-474 and MDA-MB-468 (*A*), or stably expressed by transfected *SYK(S)* (4S11) or *SYK(L)* (4L11) cDNA in MDA-MB-435S cells (*B*). Cells were harvested and subjected to nuclear and cytosolic fractionation (see "Materials and Methods"). Cytosolic (C) and nuclear (N) fractions and total cell lysates (T) were resolved on SDS-PAGE, followed by immunoblotting with Syk, Oct-1, and actin antibodies. Oct-1 and actin were used as markers for nuclear and cytosolic fractions, respectively.

Syk(S), we measured the level of Syk proteins in nuclear and cytosolic fractions in breast cancer cell lines. We chose BT474 and MDA-MB-468, both of which express endogenous Syk(L) and Syk(S) (Fig. 2*A*). In the cytosolic fraction, both Syk(L) and Syk(S) were present. However, only Syk(L) was present in the nuclear fraction (Fig. 4*A*). This indicated that unlike Syk(S), Syk(L) was able to translocate to the nucleus.

We additionally analyzed the Syk differential subcellular localization by using MDA-MB-435S stable clones that expressed only Syk(L) or Syk(S). In a Syk(S)-expressing clone, 4S11, Syk(S) was present exclusively in the cytoplasm. However, in 4L11, a Syk(L) stable clone, Syk(L) was present in both nuclear and cytosolic fractions (Fig. 4*B*). Experiments using both SYK native expressors and SYK stable lines clearly indicated the nuclear localization of Syk(L) but not Syk(S). This was additionally confirmed by our immunohistochemical studies. As shown in Fig. 5*A*, stable cells that expressed Syk(L) demonstrated both cytoplasmic and nuclear staining, whereas cytoplasmic staining predominated in Syk(S) expressors.

Basic Residues in DEL Are Required for Syk(L) Nuclear Localization. To locate the sequences within DEL that are critical for Syk(L) nuclear translocation, we first generated *SYK(L)* cDNA constructs with limited truncation of DEL (residues 283–305). Mutant constructs harboring DEL of residues 283–296, 287–301, and 293–305 were prepared and transfected into MDA-MB-435S cells. Cells that stably expressed these DEL mutants exhibited dramatically lower nuclear Syk level compared with that of wild-type Syk(L) (data not shown), which suggested that critical residues responsible for Syk(L) nuclear transport are distributed throughout DEL. DEL (TWSAGGI-IS**RIK**SYSFP**K**PGH**RK**) contains five basic residues (R292, K294, K300, R304, and K305). They exist as two clusters (**RIK** and

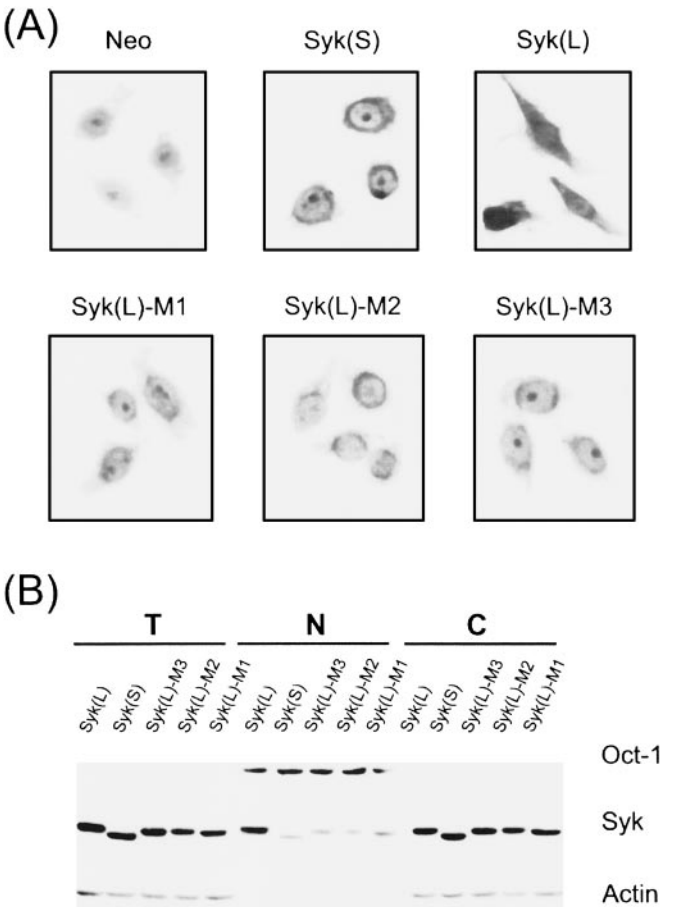

Fig. 5. Basic residues within DEL are required for Syk(L) nuclear localization. *A*, immunohistochemical examination of Syk(L) and Syk(S) subcellular localization. Pooled MDA-MB-435S stable lines that expressed cDNAs of *SYK(L)*, *SYK(S)*, or *SYK(L)* with replaced basic residues (M1, M2, and M3) were fixed. Syk-immunoreactive proteins were examined by N-19 polyclonal antibody as described in "Materials and Methods." After immunodetection, cells were counterstained with hematoxylin. Neo control was used to verify the specificity of Syk immunostaining (background). *B*, the above MDA-MB-435S stable lines were subjected to nuclear (N) and cytosolic (C) fractionation followed by SDS-PAGE and immunoblotting with anti-Syk, Oct-1, or actin antisera. Total cell lysates (T) were run in parallel.

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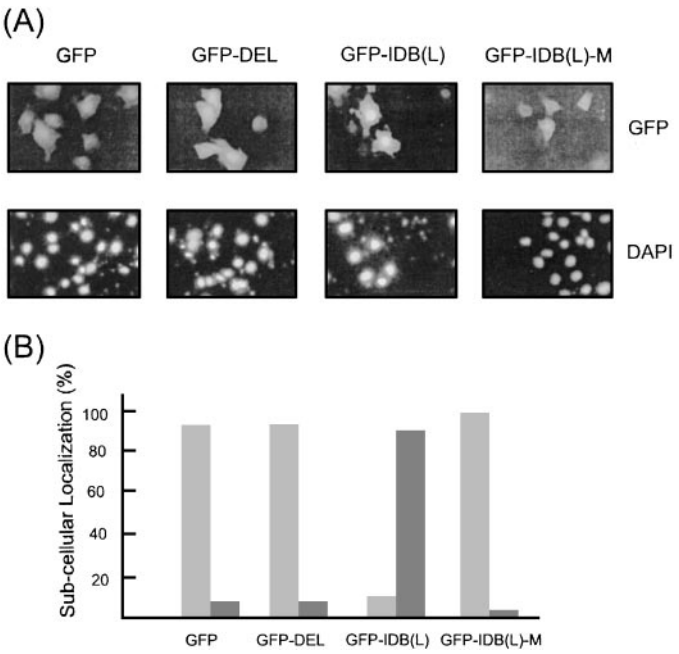

Fig. 6. Subcellular localization of GFP fusion proteins. *A*, COS7 cells were transfected with the parental vector, pEGFP-DEL, pEGFP-IDB(L), or pEGFP-IDB(L)-M. Cells were fixed and the nucleus stained with DAPI. The localization of GFP (*top panel*) and DAPI-stained nucleus (*bottom panel*) was examined by fluorescence microscopy. *B*, localization of GFP fusion protein was scored, and an average of three independent experiments was plotted. Diffuse GFP distribution throughout cells was scored as cytoplasmic (*blue bars*). Nuclear localization was determined when nuclear GFP fusion protein was prominent (*red bars*).

KPGH**RK**) that are separated by a five-residue spacer. This is reminiscent of a bipartite NLS motif (32) that is believed to diverge from the one of SV40 T antigen. To examine whether these basic residues contribute to Syk(L) nuclear localization, we used site-directed mutagenesis to replace either R292 and K294 (M1) or R304 and K305 (M2) with alanines. Nuclear and cytosolic fractionation experiments indicated that M1 and M2 demonstrated markedly decreased nuclear Syk (Fig. 5*B*). Consistently, mutation of all four basic residues (M3) was able to block Syk(L) nuclear localization. In agreement with these observations, immunohistochemical staining indicated that replacements of basic residues in DEL were able to block Syk(L) from entering the nucleus [Fig. 5*A*, compare Syk(L) with Syk(L)-M1, Syk(L)-M2, or Syk(L)-M3]. These data indicated that basic residues are required for the efficient nuclear translocation of Syk(L).

IDB Contains Sequences that Facilitate Nuclear Translocation. Sequences within DEL, especially the basic residues, are required for Syk(L) nuclear translocation. Hypothesizing that the sequences responsible for nuclear translocation may be sufficient to take a heterologous protein into the nucleus, we generated constructs that fused GFP to DEL or IDB(L). COS7 cells were transfected with these constructs, and the subcellular localization of GFP was examined by direct fluorescence microscopy. As shown in Fig. 6*A*, GFP displayed a diffuse localization pattern in both the cytoplasm and nucleus of cells transfected with parental vector, with background levels ($<$ 8% of population) of cells with nuclear GFP. However, when IDB(L) was fused, GFP fusion protein was accumulated predominantly in the nucleus, with nuclear GFP cells reaching 90% (Fig. 6, *A* and *B*), suggesting that the IDB of Syk(L) can function as an autonomous NLS. Interestingly, DEL itself was unable to transport GFP into the nucleus (Fig. 6), indicating that sequences within IDB provide critical domain structure that assists Syk nuclear translocation. Nevertheless, the basic residues at DEL are required for nuclear transport. GFP-IDB(L)-M was constructed in which K294, K300, and K305 were replaced. When these basic residues were mutated, the GFP-IDB fusion protein lost its nuclear localization (Fig. 6, *A* and *B*), indicating the critical role of the basic residues of this bipartite NLS in nuclear translocation of target proteins.

Nuclear Transport-deficient Syk(L) Lacks Invasion Suppression Properties. A differing invasion suppression activity between Syk(L) and Syk(S) may be associated with their differing nuclear localization. To examine whether nuclear localization is required for Syk(L)-mediated invasion suppression in breast cells, we performed chemoinvasion assays to compare the invasion capacities of cells expressing Syk(L) or Syk(L) with mutated NLS. Similar to the results shown in Fig. 3, Syk(L) expression inhibited invasion [Fig. 7, compare Neo and Syk(L)]. However, in contrast to wild-type Syk(L), all three Syk(L) NLS mutants (M1, M2, or M3) did not display inhibited invasiveness, indicating that the nuclear translocation process of Syk(L) is required for its invasion suppression activity (Fig. 7). This requirement elucidates the lack of anti-invasion activity of Syk(S).

DISCUSSION

SYK loss of expression is associated with increased mammary tumor invasiveness and metastasis (25). It was recently reported that *SYK* mRNA as measured by quantitative reverse transcriptase-PCR was down-regulated at least 2-fold in 30% of breast tumors, and the down-regulation was associated with poorer prognosis (26). Our previous studies, which indicated the epigenetic inactivation of the *SYK* gene in breast tumors, supported its roles of tumor or metastasis suppression (29). In this study, we found another level of deregulated SYK expression in breast cancer, *i.e.*, aberrant expression of Syk(S). The Syk expression pattern indicated that Syk deregulation does not necessarily occur at the mRNA level; some cases showed an elevated total SYK (Fig. 2*C*). The high frequency ($\sim$50% of the cases) of Syk(S) expression also explained some paradoxical observations in which some breast tumors cases were found to have up-regulated *SYK* mRNA (26).

The alternatively spliced SYK variant was expressed specifically in primary breast tumors but not in pathologically normal mammary tissues. This consistent switching of isoform expression during breast tumorigenesis suggested an important contributory role of Syk(S). Isoform switching has been observed in cancers of varied sites and is beginning to be recognized as a frequent molecular event that is associated with malignancy (33, 34). For example, an alternative

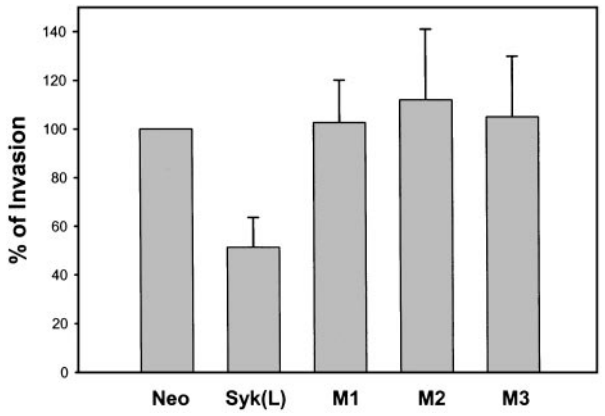

Fig. 7. Mutations that interfered Syk(L) nuclear localization blocked Syk(L)-inducible invasion suppression. Chemoinvasion assays were used to compare the invasiveness of pooled MDA-MB-435S stable lines that expressed Syk(L) or Syk(L)-M1, Syk(L)-M2, or Syk(L)-M3 and Neo control. The number of cells that had invaded through Matrigel in the Neo group was arbitrarily set as 100%. An average of three independent experiments is plotted.

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splicing variant of fibroblast growth factor receptor-1 was identified in glioblastoma (35). Exclusion of a single exon (α) in this variant generated a receptor of higher affinity and promiscuous ligand specificity. During glioblastoma progression, fibroblast growth factor receptor-1 expression gradually switched from the long to the short form, which is believed to enforce growth-stimulating signals. Isoform switching may also generate gene products that interfere with the normal function of the wild-type protein. For instance, an alternatively spliced variant of human MDM2 is frequently present in lung tumors. The variant acts as a dominant negative, and its expression increases p53 activity by inhibiting binding of the wild-type MDM2 to p53 (36). The exact mechanism of tumor-specific alternative splicing remains unclear, but recent studies suggested that interplays between repressors and *trans*-activators of RNA splicing are deregulated in tumors (37). Whatever the mechanism, the end result of isoform switching is believed to generate a protein isoform that aids in neoplastic transformation. The tumor-specific expression of Syk(S) also predicts a biological function different from that of Syk(L). Indeed, unlike Syk(L), Syk(S) was unable to suppress breast cancer cell invasiveness (Fig. 3). Syk(S) by itself may have oncogenic functions, or it may interfere with the biological activity of Syk(L). These alternate mechanisms need to be investigated.

The 23-residue DEL is the sequence difference between Syk(L) and Syk(S). We identified a bipartite NLS within DEL that is required for Syk(L) nuclear translocation. Our results indicated that DEL alone was not sufficient for active nuclear transport. Rather, the IDB(L) contains a motif that is required for nuclear import of a heterologous protein (Fig. 6). We have not determined other residues within IDB necessary for Syk(L) nuclear translocation. Nine additional basic residues are located within IDB(L), and it is plausible that they cooperate in Syk(L) nuclear transport. Moreover, the phosphorylation of flanking sequences shown to modulate NLS activity (32, 38) may also be important for Syk, considering that Syk is highly autophosphorylated when activated. A tyrosine residue within DEL, Y296, was found to be an autophosphorylation site (39) and a critical residue for Syk-mediated immunoreceptor signaling (40). Yet, phosphorylation of this tyrosine site seems not to be involved in Syk(L) nuclear transport because mutation of Y296 to alanine did not affect subcellular distribution of the mutant Syk (L. Wang and J. L. Dai, unpublished data). Nevertheless, the basic residues within DEL are primarily required for Syk nuclear transport.

Differential subcellular distribution of Syk(L) and Syk(S) is accompanied by their apparently different biological activities. *In vitro* kinase assays and COS cell transfection experiments showed that Syk(L) and Syk(S) had similar intrinsic kinase activity to phosphorylate some Syk substrates such as Cbl and Plcγ1 (9). Despite these similar enzymatic activities, Syk(S) was found to display inert biological functions (40). For example, in contrast to Syk(L), Syk(S) was unable to couple the stimulation of FcϵRI on basophils or antigen receptor on T cells to cellular activation because of its inability to phosphorylate downstream targets (40). Given that Syk(L) and Syk(S) have the same catalytic domain, it is intriguing that Syk(S) was unable to deliver this biological response. It has been proposed that the DEL at IDB may interfere with the intramolecular interaction, which may minimize its kinase activity (40). Our results showed that interference with the nuclear localization of Syk(L) was able to abrogate its invasion suppression activity in breast cancer cells (Fig. 7), indicating that a differing subcellular distribution contributes to some of their functional differences. The different subcellular localization may also explain the discrepancy between their *in vivo* and *in vitro* kinase activities.

Syk is believed to be an exclusively cytoplasmic tyrosine kinase. The known Syk signaling pathway involves relays of stimulatory signals from immunoreceptor engagement to cytoplasmic target phosphorylation. To our knowledge, this is the first report demonstrating the nuclear localization of Syk. The presence of Syk(L) in the nucleus opens a new direction of research. In our model, nuclear translocation of Syk(L) is required for its biological activity (Fig. 7). Interestingly, the nuclear localization of Syk(L) is not limited to mammary epithelial cells; Syk(L) expressed in hematopoietic cells is also present in the nucleus (L. Wang and J. L. Dai, unpublished data). Whether Syk has nuclear activities such as gene transcription regulation will be an interesting topic for future studies of breast cancer as well as other systems of Syk signaling.

ACKNOWLEDGMENTS

We thank Drs. Shintaro Yagi and Brigitte Müller for providing *SYK* cDNAs. We also thank Lore Feldman for editorial assistance.

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[CANCER RESEARCH 63, 7003–7004, October 15, 2003]

Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR's series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR's website (http://www.aacr.org).

NEW DIRECTIONS IN TUMOR ANGIOGENESIS

October 15–19, 2003
Sheraton Chicago, Chicago, IL

Chairpersons
Judah Folkman, Boston, MA
Zena Werb, San Francisco, CA
Peter Carmeliet, Leuven, Belgium

SECOND ANNUAL INTERNATIONAL CONFERENCE ON FRONTIERS IN CANCER PREVENTION RESEARCH

October 26–30, 2003
JW Marriott Desert Ridge Resort, Phoenix, AZ

Chairperson
Raymond N. DuBois, Nashville, TN

AACR-NCI-EORTC INTERNATIONAL CONFERENCE ON MOLECULAR TARGETS AND CANCER THERAPEUTICS

November 17–21, 2003
Hynes Center, Boston, MA

Chairpersons
Charles L. Sawyers, Los Angeles, CA
Edward A. Sausville, Bethesda, MD
Jaap Verweij, Rotterdam, The Netherlands

SIXTH JOINT CONFERENCE OF THE AACR AND JCA, ADVANCES IN CANCER RESEARCH

January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii

Chairpersons
Waun Ki Hong, Houston, TX
Takahashi Tsuruo, Tokyo, Japan

CALENDAR OF EVENTS

International Society for Biological Therapy of Cancer Workshop on Cancer Biometrics: Identifying Biomarkers and Surrogates of Tumor in Patients: Primer on Tumor Immunology and Biological Therapy of Cancer, October 30–November 2, 2003, Hyatt Regency, Bethesda, MD. For more information go to www.isbtc.org.

Lung Cancer Awareness Week, November 17–21, 2003. The Great American Smokeout is Thursday, November 20. Toll-free patient support information line: 1-877-646-LUNG (5864). Website: www.lungcancer.org.

10th Hong Kong International Cancer Congress, November 19–21, 2003, Faculty of Medicine Building, The University of Hong Kong, Hong Kong. Contact: 10th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232 or 852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@hku.hk; Website: www.hkicc.org.

Third International Conference and 9th Annual Meeting of the International Society of Cancer Chemoprevention (ISCaC): Controversies in Tumor Prevention and Genetics, February 12–14, 2004, University of St. Gallen, Switzerland. E-mail: info@oncoconferences.ch; website: www.oncoconferences.ch.

The National Comprehensive Cancer Network's 9th Annual Conference: Clinical Practice Guidelines and Outcomes Data in Oncology, March 10–14, 2004. Westin Diplomat Resort and Spa, Hollywood, Florida. Website: www.nccn.org.

The UK Radiological Congress, run by The British Institute of Radiology, The Royal College of Radiologists, The Society and College of Radiographers and The Institute of Physics and Engineering in Medicine, June 6–8, 2004, GMEX Et MICC, Manchester, UK. Abstract deadline: Feb. 2, 2004. Website: www.ukrc.org.uk.

6th International Conference on Head and Neck Cancer, August 7–11, 2004, Marriott Wardman Park, Washington, DC. Contact: Concepts in Meeting & Events, 1805 Ardmore Boulevard, Pittsburgh, PA 15221. Phone: 412.243.5156; Fax: 412.243.5160; E-mail: ssteighnercme@aol.com.

Molecular Targets for Cancer Therapy: 3rd Biennial Meeting, October 1–5, 2004, Don Cesar Beach Resort & Spa, St. Petersburg Beach, FL. Contact: Ann Gordon. Phone: 813.903.4975; E-mail: gordonac@moffitt.usf.edu.

Correction

In the article by Lei Wang *et al.*, titled "Alternative Splicing Disrupts a Nuclear Localization Signal in Spleen Tyrosine Kinase That Is Required for Invasion Suppression in Breast Cancer," which appeared in the August 1, 2003 issue of *Cancer Research* (pp. 4724–4730), figures 5 and 6 should have appeared in color. Below are the correct figures.

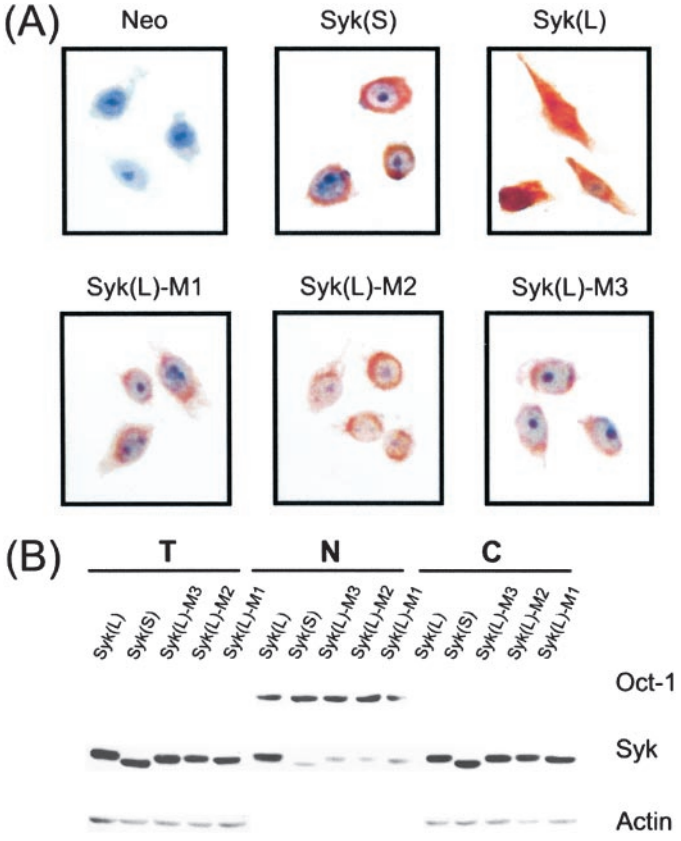

Fig. 5. Basic residues within DEL are required for Syk(L) nuclear localization. *A*, immunohistochemical examination of Syk(L) and Syk(S) subcellular localization. Pooled MDA-MB-435S stable lines that expressed cDNAs of *SYK(L)*, *SYK(S)*, or *SYK(L)* with replaced basic residues (M1, M2, and M3) were fixed. Syk-immunoreactive proteins were examined by N-19 polyclonal antibody as described in "Materials and Methods." After immunodetection, cells were counterstained with hematoxylin. Neo control was used to verify the specificity of Syk immunostaining (background). *B*, the above MDA-MB-435S stable lines were subjected to nuclear (N) and cytosolic (C) fractionation followed by SDS-PAGE and immunoblotting with anti-Syk, Oct-1, or actin antisera. Total cell lysates (T) were run in parallel.

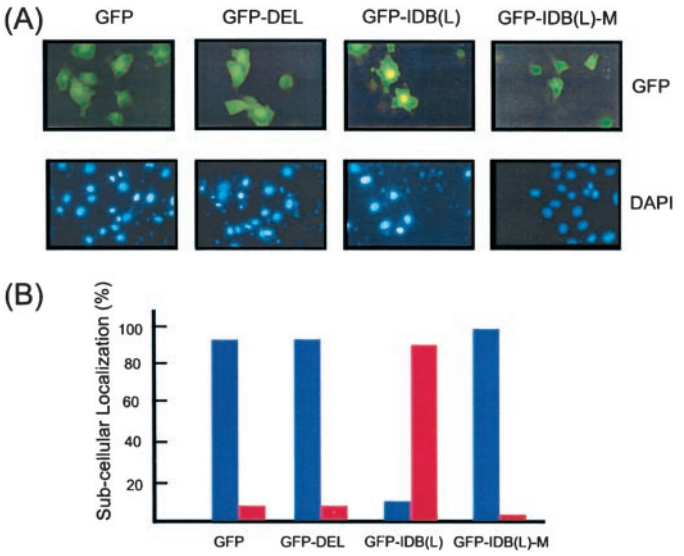

Fig. 6. Subcellular localization of GFP fusion proteins. *A*, COS7 cells were transfected with the parental vector, pEGFP-DEL, pEGFP-IDB(L), or pEGFP-IDB(L)-M. Cells were fixed and the nucleus stained with DAPI. The localization of GFP (*top panel*) and DAPI-stained nucleus (*bottom panel*) was examined by fluorescence microscopy. *B*, localization of GFP fusion protein was scored, and an average of three independent experiments was plotted. Diffuse GFP distribution throughout cells was scored as cytoplasmic (*blue bars*). Nuclear localization was determined when nuclear GFP fusion protein was prominent (*red bars*).

The article by Yanhua Wang *et al.*, titled "A Novel Folate Transport Activity in Human Mesothelioma Cell Lines with High Affinity and Specificity for the New-Generation Antifolate Pemetrexed," which appeared in the November 15, 2002 issue of Cancer Research (pp. 6434–6437) described a novel folate transport activity in human mesothelioma cell lines. Subsequent studies have clarified that this transport activity is associated with infection of the cell culture by *M. hyorhinis* and *M. Arginini* as determined with the ATCC Mycoplasma Detection Kit. When cells were treated with antibiotics (10 μg/ml ciprofloxacin and 0.5 μg/ml Mycoplasma Removal Agent, ICN Biochemicals) for two weeks this activity was lost. In addition, mycoplasma-free cells regained this transport activity within three days after growth in sterile-filtered (0.45 micron) medium derived from the infected cells. NCI-H28 mesothelioma cells newly purchased from ATCC do not exhibit this activity. This activity is not detected using methotrexate as the radiolabelled uptake species (commonly used to characterize folate transport) because of the very low affinity of the transporter for this drug ($K_i \sim 125$ μM). Rather, it was detected fortuitously when pemetrexed was employed because of the very high affinity for this antifolate ($K_t \sim 30$ nM). Indeed, a high ratio of pemetrexed to methotrexate initial uptake rates (when the extracellular concentration is low ~50 nM) suggests the presence of mycoplasma associated with mammalian cells. Recent studies indicate high affinities of this transporter for the active stereoisomer of 5-formyltetrahydrofolate ($K_t \sim 40$ nM) and racemic 5-methyltetrahydrofolate ($K_t \sim 75$ nM). This transport activity was also present, although at far lower levels (Hela and HepG2 cells) or, not detected at all, in other cells that harbored mycoplasma. Hence, this phenomenon appears to be especially prominent for mesothelioma cells.

Y. Wang
R. Zhao
S. Chattopadhyay
I. D. Goldman

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The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Alternative Splicing Disrupts a Nuclear Localization Signal in Spleen Tyrosine Kinase That Is Required for Invasion Suppression in Breast Cancer

Lei Wang, Lindsay Duke, Peter S. Zhang, et al.

Cancer Res 2003;63:4724-4730.

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