Identification and Characterization of a Nuclear Interacting Partner of Anaplastic Lymphoma Kinase (NIPA)*

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Anaplastic large-cell lymphoma is a subtype of non-Hodgkin lymphomas characterized by the expression of CD30. More than half of these lymphomas carry a chromosomal translocation t(2;5) leading to expression of the oncogenic tyrosine kinase nucleophosmin-anaplastic lymphoma kinase (NPM-ALK). NPM-ALK is capable of transforming fibroblasts and lymphocytes in vitro and of causing lymphomas in mice. Previously, we and others demonstrated phospholipase C-γ and phosphatidylinositol-3-kinase as crucial downstream signaling mediators of NPM-ALK-induced oncogenicity. In this study, we used an ALK fusion protein as bait in a yeast two-hybrid screen identifying NIPA (nuclear interacting partner of ALK) as a novel downstream target of NPM-ALK. NIPA encodes a 60-kDa protein that is expressed in a broad range of human tissues and contains a classical nuclear translocation signal in its C terminus, which directs its nuclear localization. NIPA interacts with NPM-ALK and other ALK fusions in a tyrosine kinase-dependent manner and is phosphorylated in NPM-ALK-expressing cells on tyrosine and serine residues with serine 354 as a major phosphorylation site. Overexpression of NIPA in Ba/F3 cells was able to protect from apoptosis induced by IL-3 withdrawal. Mutations of the nuclear translocation signal or the Ser-354 phosphorylation site impaired the antiapoptotic function of NIPA. Overexpression of putative dominant-negative NIPA mutants. These results implicate an antiapoptotic role for NIPA in NPM-ALK-mediated signaling events.

Receptor tyrosine kinases play an important role in the control of cell proliferation, differentiation, and malignant transformation. Receptor-specific ligands lead to their dimerization and activation with resultant auto- and cross-phosphorylation (1). By mutation, truncation, or recombination, receptor tyrosine kinases may escape their strict ligand-mediated control and exhibit unregulated kinase activity, leading to constitutive activation of intracellular downstream signaling molecules and causing cell transformation and aberrant proliferation.

Anaplastic large-cell lymphoma (ALCL)1 constitutes a subgroup of non-Hodgkin lymphomas that express the membrane antigen CD30 (2–4). Since the initial discovery of the chromosomal translocation t(2;5)(p23;q35) that fuses nucleophosmin (NPM) on chromosome 5 to anaplastic lymphoma kinase (ALK) on chromosome 2, a number of oncogenic fusions involving ALK in ALCL have been identified, such as TPM3-ALK, TFG-ALK, ATIC-ALK, and CLTC-ALK among others (5–12). Oncogenic ALK fusion proteins can be detected in about 60% of ALCL cases (13). The various fusion partners of ALK serve as oligomerization domains that lead to constitutive activity of the kinase. NPM-ALK, the most common and best characterized ALK fusion protein, has been shown to transform fibroblasts and lymphocytes and to induce a lymphoma-like disease in mice (14–17).

Recent studies revealed a number of downstream targets of NPM-ALK possibly involved in mediating its oncogenicity, including Shc, IRS-1, Grb2, phospholipase C-γ, PI-3-kinase, and Stat3/5 (15, 16, 18–22). Previously, we have described a role for phospholipase C-γ in the promitogenic function of NPM-ALK. We and others have also shown an important role of the PI-3-kinase/AKT and the Stat3/5 pathway for the antiapoptotic function of NPM-ALK (16, 18–20, 22). Despite the studies of these signaling mediators that are commonly found in association also with other receptor tyrosine kinases and non-receptor tyrosine kinases, the molecular mechanisms contributing to the distinct oncogenic features of NPM-ALK remain incompletely understood. In this study, we used an ALK fusion protein as bait in a yeast two-hybrid screen to find additional interacting partners of NPM-ALK that may be involved in mediating oncogenesis.

MATERIALS AND METHODS

Cloning of Human and Mouse NIPA—The complete hNIPA cDNA was isolated from a yeast two-hybrid screen with LexA-TBP-ALK as

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† The abbreviations used are: ALCL, anaplastic large-cell lymphoma; ALK, anaplastic lymphoma kinase; NPM, nucleophosmin; NIPA, nucleic acid transferase-mediated deoxyribonucleotidyltransferase; NLS, nuclear localization signal; PI, propidium iodine; PI3-kinase, phosphatidylinositol 3-kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; ATIC, 5-amino-4-imidazolecarboxamide ribonucleotide transformylase/inosine monophosphate cychoylodrolase; TPR, tricistriptide repeat; KD, kinase-deficient; h, human; m, mouse.

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bait using a human CML K562 cDNA library (Clontech). LexA fusion proteins were created in pEG202 modified by adding the Trp selection gene. Interactions were tested in the yeast strain L40 by assaying activation of the HIS3 reporter, as described previously (23). 5' upstream stop codons were confirmed by rapid amplification for cDNA ends using a human testis cDNA library (Clontech). The mNIPA cDNA was cloned from a mouse cDNA library derived from embryos at 17 days (Clontech), based on expressed sequence tag fragments homologous to hNIPA.

Accession Numbers—Homo sapiens mRNA for nuclear interacting partner of ALK (NIPA gene) was given the GenBank™ accession number AJ537494. Mus musculus mRNA for nuclear interacting partner of ALK (NIPA gene) was given the GenBank™ accession number AY537495.

**Northern Blot Analysis**—Northern blot analysis was carried out using a human mRNA tissue membrane (Clontech) with a probe spanning nucleotides 362–1248 of the hNIPA cDNA, according to the procedure of the ExpressHyb system (Clontech).

**Cell Lines**—Human kidney 293 cells and monkey kidney Cos7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine albumin (FBS). Murine pro-B lymphocyte Ba/F3 cells were cultured in RPMI 1640 medium with 5% FBS and 2 ng/ml mIL-3. NPM-ALK-transformed Ba/F3 cells were maintained in culture containing 5% FBS without mIL-3.

**Construction of Expression Plasmids and Site-directed Mutagenesis**—The NIPA cDNA was cloned into pcDNA3 (Invitrogen). The NIPA constructs were cloned in the pcDNA3.1-Zeo vector. Site-directed mutagenesis was performed with Pfu DNA polymerase (Stratagene, Heidelberg, Germany), as described previously (23). All mutated DNAs were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (PerkinElmer Life Sciences).

**GST Fusion Proteins, Pull-down Assays, and Phosphotyrosine Competition**—The CDNAs of NIPA wild type or NIPA 306–402 were cloned into the pGEX-KG vector and expressed in Escherichia coli BL21 cells. Affinity purification using glutathione-Sepharose (Amersham Biosciences) was carried out as described previously (23). For GST pull-down assays, 1 × 10^7 cells were solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of phenanthroline, aprotinin, leupeptin, and pepstatin), precleared with glutathione beads and incubated with 5 μg of GST fusion proteins for 1 h at 4 °C. Protein complexes were collected on glutathione-Sepharose beads and subjected to SDS-PAGE. For competition with phosphotyrosine, lysates were incubated with the relevant GST fusion protein with free phosphotyrosine (Sigma) at a final concentration of 2 μM.

**Immunoprecipitation and Immunoblotting**—Cells were suspended in lysis buffer and kept on ice for 30 min. Lysates were preclarified by centrifugation and subjected to SDS-PAGE. Alternatively, the lysates were incubated for 2–12 h with 1–2 μg of antibody at 4 °C. Immune complexes were precipitated with 30 μl of protein A-Sepharose (Amersham Biosciences) for 1 h, washed three times with lysis buffer, and then boiled in 2% SDS sample buffer followed by separation on SDS-polyacrylamide gels. Immunoblotting was done with nitrocellulose membranes, and proteins were visualized by chemiluminescence, as recommended by the manufacturer (Fierce).

**Immunofluorescence**—Cos7 cells were transfected using the 1,2-di-oleoyl-3-trimethylammonium propane reagent (Roche Applied Science). After 48 h, cells were fixed and stained as described (24) and then visualized by fluorescence microscopy (Zeiss Axioskop) and an imaging system from TILL Photonics (Munich, Germany) as described previously (Jahn et al. [42]).

**In Vivo Labeling and Phosphoamino Acid Analysis**—293 cells transfected with the indicated Myc-tagged NIPA constructs were cultured in phosphate-free medium containing 1% FBS and 0.2 mM/ml [³²P]orthophosphate for 2 h at 37 °C. Anti-Myc immunoprecipitation was performed, and the immune complexes were subjected to SDS-PAGE and anti-Myc immunoblotting. [³²P]Myc-NIPA was visualized by autoradiography. In a subsequent procedure of two-dimensional chromatography, the radioactivity has been described previously (25). Briefly, the localized radioactive signal on the membrane was cut out and hydrolyzed in 6 N HCl for 1 h at 110 °C. Together with the mixture of O-phospho-D-serine, -threonine, and -tyrosine serving as standards, the sample was loaded onto a thin-layer cellulose plate (Merek). Chromatography was carried out in a solvent containing ethanol/glacial acetic acid/water (1:1:1, v/v/v) and subsequently in a solvent made of 2-methyl-1-propanol/formic acid/water (8:3:4, v/v/v). Apoptosis analysis was performed by flow cytometry.

Parental Ba/F3 cells stably transfected with various NIPA constructs were cultured with murine IL-3 as growth factor. After IL-3 withdrawal, apoptosis was measured by TUNEL or propidium iodine (PI) staining. TUNEL was performed by using a Fluorescein-FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA), with the exposed 3'-OH ends of DNA fragments generated in response to apoptosis fluorescein isothiocyanate-fluorescein-labeled by terminal deoxynucleotidyl transferase. Results were evaluated on a flow cytometer at 488 nm. In the studies investigating wortmannin-induced apoptosis, NPM-ALK-transformed Ba/F3 cells overexpressing the indicated NIPA constructs were incubated with 300 nm wortmannin for 3 days with daily addition of wortmannin. Every 24 h, samples were collected and analyzed by PI staining and flow cytometry.

**RESULTS**

**Identification of NIPA**—Yeast two-hybrid screens have been utilized as a powerful method in searching for novel interacting partners of a particular protein. An essential part of such screening with a tyrosine kinase as bait is to ensure its sufficient autophosphorylation in yeast, enabling the docking of phosphorylation-dependent binding partners. A LexA-NPM-ALK fusion protein failed to be efficiently autophosphorylated in yeast (data not shown). Previous studies revealed that an artificially constructed TPR-ALK hybrid protein in which NPM was substituted with the TPR oligomerization domain displayed transforming activity similar to NPM-ALK (14). A LexA-TPR-ALK fusion protein was shown to be efficiently autophosphorylated in yeast (data not shown), and this protein was therefore used as bait to screen a library prepared from K562, a human chronic myeloid leukemia (CML) cell line.

Among the ~400 clones isolated, more than 90% encoded an unknown protein of 60 kDa, which we have termed nuclear interacting partner of ALK (NIPA). The mouse homologue of NIPA (mNIPA) was cloned from a cDNA library prepared from mouse embryos at day 17 and was shown to share 85% amino acid identity with the human protein (Fig. 1A). NIPA was broadly expressed in the human tissues tested by Northern blot, with the highest expression in heart, skeletal muscle, and testis. In leukocytes, NIPA was detectable only at very low levels (Fig. 1B). The expression of NIPA in ALCL cells was demonstrated by identification of the gene in a cDNA library prepared from the cell line Karpas 299, which was derived from an ALK-positive ALCL patient (data not shown).

**NIPA Binds Only Kinase-active NPM-ALK—**GST pull-down experiments showed that NIPA binds specifically to kinase-active NPM-ALK but not to the kinase-defective NPM-ALK-KD protein (Fig. 2A). Co-immunoprecipitation experiments confirmed that NIPA forms a complex only with kinase-active NPM-ALK (Fig. 2, B and C). Consistent with these findings, NIPA interacted with kinase-active TPR-ALK in yeast but not with a kinase-defective TPR-ALK (data not shown). Thus, NIPA interacts with ALK in a kinase-dependent manner.

Sequence analysis of NIPA failed to show any significant homology to known proteins. Phosphotyrosine-binding motifs such as SH2 or PTB domains are absent in NIPA. The only functional motif identified in the protein is a classical NLS sequence characterized by the predominant presence of the positively charged residues Arg and Lys (Fig. 1A, the NLS is underlined). Two mutants of NIPA targeting the NLS (NIPA-KP, -RKK) were generated. In comparison with wild-type NIPA, both NLS mutants of NIPA appeared to form even more abundant complexes with NPM-ALK (Fig. 2D, lower portion). Deletion mapping delineated the binding region for NPM-ALK to amino acids 306–402 of NIPA. Deletion of these NIPA residues abolished its complex formation with NPM-ALK completely (Fig. 2D, lower portion, right lane). Likewise, a GST fusion construct of NIPA comprising amino acids 306–402 mediated binding to NPM-ALK (Fig. 2E). The association of
both NIPA wild type and NIPA 306–402 to NPM-ALK was displaced upon competition with free phosphotyrosine, indicating dependence on tyrosine phosphorylation (Fig. 2E).

**NIPA Is a Nuclear Protein**—FLAG-tagged NIPA was transiently expressed in Cos1 cells and stained by indirect immunofluorescence. The wild-type NIPA protein was found to be strictly confined to the nucleus (Fig. 3A). Exchange of 1 residue of the NLS (K399P) resulted in partial cytoplasmic redistribution of NIPA, and exchange of 3 residues (R398A, K399A, K401A) in complete cytoplasmic redistribution of NIPA (Fig. 3, B and C).

NPM/B23 is a 38-kDa nuclear protein containing two NLSs that is involved in cytoplasmic/nuclear trafficking and in the control of centrosome duplication (26–28). The truncated NPM portion of NPM-ALK does not contain an NLS. However, NPM undergoes self-oligomerization (29), as well as hetero-oligomerization with NPM-ALK, which leads to the partial nuclear localization of NPM-ALK due to shuttling by normal NPM (14). When overexpressed in Cos1 cells, NPM-ALK is distributed mainly in the cytoplasm with a slight nuclear presence (Fig. 3D). In cells cotransfected with NPM-ALK and wild-type NIPA, both molecules retained their original patterns of subcellular localization (Fig. 3E). The NIPA-KP mutant was expressed partially in the cytoplasm and displayed co-localization with NPM-ALK also in the cytoplasm (Fig. 3F). Co-localization both in the nucleus and in the cytoplasm was also observed in cells expressing NPM-ALK and NIPA-RKK mutant (Fig. 3G). These immunofluorescence data are in agreement with our co-immunoprecipitation experiments, in which the NIPA-KP and NIPA-RKK mutants appeared to form more abundant complexes with NPM-ALK than the wild-type NIPA (Fig. 2D).

A number of chromosomal translocations involving ALK...
have been identified in ALCL in recent years and shown to fuse ALK with a variety of N-terminal partners that mediate oligomerization and kinase activation. For example, ATIC encodes an enzyme responsible for the final steps of de novo purine nucleotide biosynthesis. In ATIC-ALK, the N-terminal 229 residues of ATIC are fused to the truncated ALK, resulting in homo-oligomerization and constitutive activation of the ATIC-ALK chimeric kinase (9, 11, 30). All ALK fusion proteins other then NPM lack a dimerization partner in the nucleus. Therefore all alternative ALK fusions are localized mainly in the cytoplasm (14, 31). We used our artificially constructed TPR-ALK (a form of dimeric ALK yet to be described in ALK positive malignancies) as representative of the ALK fusion proteins that are expressed exclusively in the cytoplasm (Fig. 3H). Co-transfection of NIPA led to a nuclear redistribution of TPR-ALK (Fig. 3D). A similar redistribution of ATIC-ALK into the nucleus was observed when ATIC-ALK and NIPA were coexpressed (data not shown). Thus, under these conditions of overexpression, nuclear-located NIPA is able to relocalize ALK fusion proteins into the nucleus.

**NIPA Is Phosphorylated on Serine and Tyrosine Residues**

To test whether the complex formation of NIPA with kinase-active NPM-ALK leads to NIPA phosphorylation, NPM-ALK, kinase-defective NPM-ALK-KD, ATIC-ALK, or TPR-ALK were co-transfected with NIPA (Fig. 4A). All kinase-active ALK fusion proteins induced a retarded migration (shift) of NIPA on blots (Fig. 2A). Deletion of NIPA amino acids 306–402 eliminates binding to NPM-ALK. Deletion analysis narrowed the binding region of NIPA with NPM-ALK to amino acids 306–402, termed the ALK-binding domain (ABD). A classic NLS is present within the ABD. Various FLAG-tagged NIPA constructs as indicated were transfected together with NPM-ALK in 293 cells, and anti-FLAG immunoprecipitations were performed. Immunoprecipitations were analyzed by anti-ALK immunoblot (upper panel). The expression of the FLAG-tagged NIPA constructs and the cotransfected NPM-ALK was examined by anti-FLAG (middle panel) and anti-ALK blotting (lower panel), respectively. E, phosphotyrosine dependent binding of NIPA amino acids 306–402 to NPM-ALK. Flag-tagged NPM-ALK was transiently transfected in 293 cells. Cell lysates were incubated with GST-NIPA wild type (WT) or GST-NIPA amino acids 306–402 and left either untreated or supplemented with phosphotyrosine.
SDS-PAGE (Fig. 4A). Treatment of these cell lysates with phosphatases eliminated the shift, which was however maintained in the presence of additional phosphatase inhibitors (Fig. 4B). These results suggested that the shift is caused by the phosphorylation of NIPA. In vivo labeling of Myc-tagged NIPA with [32P]orthophosphate confirmed these results. A strong 32P signal of NIPA was observed in cells coexpressing NPM-ALK (Fig. 4C). Interestingly, phosphoamino acid analysis revealed that mainly serine residues were phosphorylated (Fig. 4D). By systematic mutation analysis, we found that alteration of serine residue 354 significantly reduced the NPM-ALK-induced gel shift of NIPA (Fig. 4E, lower panel) and F). In addition, significantly less [32P] incorporation was observed into NIPA-S354A as compared with wild-type NIPA (Fig. 4E, upper panel). The NPM-ALK-induced shift could be partially restored by substituting serine 354 with aspartic acid (NIPA-S354D), which imitates a constitutively phosphorylated serine residue (Fig. 4F, left blot). Notably, NIPA-S354D was not shifted without cotransfected NPM-ALK (Fig. 4F, left blot, compare lanes 3 and 5). Together, these data suggest that phosphorylation of NIPA at serine 354 might be necessary for the concomitant phosphorylation at additional sites, which together contribute to the retarded migration on SDS-PAGE.

A weak tyrosine phosphorylation signal was also detectable by phosphoamino acid analysis (Fig. 4G). In contrast to the many serine residues in hNIPA (13.7% of the total residues), only two tyrosine residues (Tyr-105, Tyr-137) are present. Both of these tyrosine residues were mutated (NIPA-YF). No alteration of the migration pattern on SDS-PAGE of this mutant was detectable (Fig. 4F, right blot). Thus, since NPM-ALK is a tyrosine kinase, the major phosphorylation of NIPA must be carried out by a yet unidentified serine kinase, which is activated in NPM-ALK expressing cells.

**NIPA Delays Apoptosis in Ba/F3 Cells**—An important mechanism of transformation is the constitutive activation of anti-apoptotic signaling pathways. We therefore investigated whether NIPA might be involved in the regulation of apoptosis. Ba/F3 cells are IL-3-dependent pro-B lymphocytes that undergo apoptosis after IL-3 withdrawal within several hours. This response is in large part a result of abrogation of the...
IL-3-activated antiapoptotic signaling pathway mediated by PI3-kinase/AKT (32, 33). Measuring apoptosis by TUNEL assay, 95% of parental Ba/F3 cells were apoptotic following 24 h of IL-3 withdrawal (Fig. 5A, filled squares). In marked contrast, overexpression of wild-type NIPA significantly suppressed apoptosis down to 20% of the assayed cells (Fig. 5A, filled circle). The NIPA-KP mutant, whose nuclear localization signal is partially disrupted, showed a reduced protective function against apoptosis as compared with cells expressing wild-type NIPA (Fig. 5A, filled triangle). Thus, NIPA is able to protect Ba/F3 cells from apoptosis induced by IL-3 withdrawal, and the correct nuclear localization of NIPA is required for this antiapoptotic function. Histograms of the flow cytometry at 0 and 24 h after IL-3 withdrawal are shown on the right of Fig. 5A.

In a second experiment with independently generated Ba/F3 cells stably transfected with various FLAG-tagged NIPA mutants, DNA was stained with PI. After 24 h of IL-3 deprivation, 75% of the parental Ba/F3 cells underwent apoptosis, in contrast to 36% found in cells expressing wild-type NIPA (Fig. 5B). NIPA-KP and NIPA-S354A demonstrated reduced antiapoptotic function, whereas the NIPA-RKK mutant, whose NLS is completely disabled, showed nearly no effect in preventing apoptosis.

Thus, nuclear localization and an intact phosphorylation site Ser-354 seem to be essential for the antiapoptotic function of NIPA in Ba/F3 cells. Although NIPA exhibits remarkable antiapoptotic effects in Ba/F3 cells, it did in fact only delay apoptosis, not enable cells to completely escape apoptosis induced by IL-3 deprivation; 72–96 h after IL-3 withdrawal, no cells survived (data not shown).

NPM-ALK transforms Ba/F3 cells and leads to IL-3-independent proliferation. It has been shown that the PI3-kinase/AKT antiapoptotic signaling pathway is activated by NPM-ALK and that incubation with the PI3-kinase inhibitor wortmannin can induce apoptosis in ALCL cells (20, 21). To further explore the possible role of NIPA in NPM-ALK-mediated malignancy, various NIPA constructs were introduced into Ba/F3 cells transformed by NPM-ALK (Ba/F3-NPM-ALK). Treatment with 300 nM wortmannin resulted in 17% apoptosis in Ba/F3-NPM-ALK transformed cells after 72 h (Fig. 5C). Coexpression of either wild-type NIPA or NIPA-S354A with NPM-ALK produced only a marginal effect in preventing apoptosis triggered by wortmannin. However, cells coexpress-
ing NPM-ALK with NIPA with a disrupted NLS (NIPA-KP and -RKK) showed increased apoptosis (Fig. 5C). These data are consistent with a dominant-negative function of these mutants and are in agreement with an antiapoptotic role of NIPA as perceived from the results illustrated in Fig. 5, A and B. As demonstrated by co-immunoprecipitation and immunofluorescence experiments, the NLS-disrupted NIPA mutants tend to form a stronger complex with NPM-ALK. A dominant-negative function may take place, as the non-physiologically located NIPA mutants are successfully competing with endogenous NIPA for binding to NPM-ALK.

An even more pronounced increase in apoptosis was achieved by coexpressing NPM-ALK and NIPA-YF, in which the potential phosphorylated tyrosine residues (Tyr-105, Tyr-137) are mutated to phenylalanine. Seventy percent of the NIPA-YF-expressing Ba/F3-ALK cells stably transfected with various FLAG-NIPA constructs as indicated and cultured in IL-3-free medium. Cells were treated with 300 nM wortmannin added every 24 h to induce apoptosis. Apoptosis was measured by PI staining and flow cytometry as in panel B. The expression of the FLAG-NIPA constructs and NPM-ALK was demonstrated by anti-FLAG or anti-ALK immunoblotting (right panel). IP, immunoprecipitation. As shown in D, proposed functional model of the interaction of NIPA with NPM-ALK.

**DISCUSSION**

In this study, we describe the isolation of NIPA (nuclear interacting partner of ALK) as an interacting partner specifically for kinase-active ALK fusion proteins only. From previous screenings with the K562 library used, we expected to isolate substrate proteins containing classical phosphotyrosine-binding domains such as SH2 or PTB domains that bind to specific autophosphorylation sites in ALK (34). However, NIPA lacks either any classical SH2 or PTB domains. We were able to map the region in NIPA responsible for kinase-dependent binding to ALK to amino acids 306–402 of the protein. This domain may represent a new type of phosphotyrosine-binding domain.

NIPA is phosphorylated in cells expressing ALK fusion proteins such as NPM-ALK, TPR-ALK, or ATIC-ALK. This phosphorylation leads to a shift of the protein on SDS-PAGE. In vivo labeling of NIPA with [32P]orthophosphate revealed that mainly serine residues are phosphorylated, with some phosphorylation also detectable on tyrosine residues. A point mutation at Ser-354 to alanine significantly reduced phosphorylation of NIPA. Thus, Ser-354 could be the major phosphorylation site in NIPA or may function as a regulatory site, the phosphorylation of which allows or initiates the phosphorylation of other residues in the protein. By substituting Ser-354 with aspartic acid, the negatively charged side chain mimics a phosphate.

**FIG. 5.** Antiapoptotic function of NIPA in Ba/F3 cells. As shown in A, overexpression of wild-type NIPA delays apoptosis induced by IL-3 deprivation in Ba/F3 cells. IL-3-dependent Ba/F3 cells were transfected with the indicated Myc-NIPA constructs and stably selected by Zeocin. After IL-3 withdrawal, apoptosis was measured by the TUNEL method as described under “Materials and Methods.” The expression levels of NIPA and NIPA-KP were examined by anti-Myc immunoprecipitation and immunoblotting. In the right panel, the histograms of flow cytometry comparing the staining at 0 and 24 h after IL-3 withdrawal are presented. As shown in A, apoptosis in Ba/F3 cells stably transfected with the indicated constructs was analyzed by PI staining. In the right panel, the histograms of PI-staining by flow cytometry are shown comparing Ba/F3 cells, Ba/F3 cells transfected with NIPA-KP, and Ba/F3 cells transfected with NIPA after 24 h of IL-3 deprivation. As shown in C, NPM-ALK-transformed Ba/F3 cells were stably transfected with various FLAG-NIPA constructs as indicated and cultured in IL-3-free medium. Cells were treated with 300 nM wortmannin added every 24 h to induce apoptosis. Apoptosis was measured by PI staining and flow cytometry as in panel D. The expression of the FLAG-NIPA constructs and NPM-ALK was demonstrated by anti-FLAG or anti-ALK immunoblotting (right panel). IP, immunoprecipitation.
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group, the typical phosphorylation-induced gel shift pattern of NIPA was partially restored. Interestingly, a complete shift of SS54D is induced in cells expressing NPM-ALK. These data imply that Ser-354 is not the sole phosphorylation site in NIPA but is required for further phosphorylation of additional serine residues.

NIPA binds only to kinase-active ALK fusion proteins and is phosphorylated in cells expressing kinase-active ALK. Since ALK is a tyrosine kinase, direct phosphorylation of NIPA by ALK fusions on serine residues would not be expected. By sequence analysis and autokinase assays (data not shown), we were unable to obtain any evidence that NIPA itself is a serine kinase. Thus, NIPA is phosphorylated by yet unidentified serine kinases induced in kinase-active ALK-expressing cells.

An important feature of NIPA is its nuclear localization. Nuclear localization of proteins is mediated by the activity of importins that recognize NLS sequences (35). Nuclear transport is believed to be a constant process that actively moves proteins into and out of the nucleus. We were able to identify a NLS signal in NIPA that is responsible for its nuclear localization. Mutation of this NLS led to a complete cytoplasmic redistribution of the protein. In contrast, NPM-ALK does not contain an NLS. Its partially nuclear localization is due to dimerization with the predominantly nuclear NPM shuttle protein. All other ALK fusion proteins appear to be mainly distributed in the cytoplasm based on immunostaining in paraffin-embedded ALCL tissues (11, 14, 31). This could be confirmed by immunostaining of Cos7 cells artificially overexpressing TPR-ALK. Interestingly, the presence of overexpressed NIPA was able to partially relocate TPR-ALK into the nucleus. This finding raises the possibility that the other ALK fusion proteins, similarly to NPM-ALK, can be relocated to the nucleus by interacting with a nuclear protein. Further studies are required to determine whether alternative ALK fusion proteins can also be detected in the nucleus by using sensitive methods or perhaps by looking at cells arrested at specific points in the cell cycle.

Overexpression of wild-type NIPA was able to protect Ba/F3 cells from IL-3 withdrawal-induced apoptosis. The proper nuclear localization of NIPA is essential for its antiapoptotic function since the NLS mutants NIPA-KP and NIPA-RKK failed to prevent apoptosis. The mechanism for the antiapoptotic function of NIPA is unclear. The apoptotic cascade is triggered by the loss of integrity of the outer mitochondrial membrane accompanied by release of cytochrome c that activates protease-activating factors and subsequently the caspase cascade (36). In Ba/F3 cells, AKT was shown to phosphorylate the Bcl-2 family member Bad, thus preventing the activation of caspases by maintaining mitochondrial integrity (32, 37). Akt is also involved in the activation of NF-κB, inhibition of caspase-9, and reduction of the transcription of Fas ligand (FasL), all of which contribute to its antiapoptotic function (38, 39). One of the major upstream regulators of Akt is PI3-kinase, which phosphorylates the 3′-OH position of phosphatidylinositol. PI3-kinase activates Akt by recruiting Akt to the cytoplasmic membrane, where regulatory residues of Akt are phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (39).

As a nuclear protein, NIPA could regulate the transcription of antiapoptotic proteins. A number of transcriptionally regulated proteins involved in apoptosis have been described recently. One target of Akt is the forkhead transcription factors (FKHRs) (40). FKHRs may up-regulate Fas ligand and thereby induce apoptosis. Another example is the Wnt-1 signaling pathway, which has been found to inhibit apoptosis by activating β-catenin/T cell factor-mediated transcription (41). In yeast, NIPA does not exhibit transcriptional activity (data not shown). Also, in a Gal4 reporter assay, Gal4-NIPA displayed no transcriptional activity (data not shown). Based on these observations, NIPA does not seem to function directly as a transcriptional factor. However, as a nuclear protein, NIPA may be involved by other mechanisms in the regulation of transcriptional events. In Ba/F3 cells, NIPA delayed but did not prevent apoptosis. Preliminary data indicate that NIPA is phosphorylated in a cell-cycle dependent manner. The antiapoptotic function of NIPA may therefore be mediated in part by its effects on the cell cycle.

Previous studies from our laboratory and others found that NPM-ALK recruits the p85 subunit of PI3-kinase and activates the AKT antiapoptotic signaling pathway (20, 21). Blocking the PI3-kinase/AKT pathway in NPM-ALK-transformed cells with the PI3 kinase inhibitor wortmannin results in apoptosis (20). Overexpression of wild-type NIPA showed only marginal effects on wortmannin-induced apoptosis. We also investigated the possibility of direct activation of AKT by NIPA. Neither NIPA wild type nor any of the discussed NIPA mutants directly affected the activation of AKT in overexpression experiments (data not shown). However, the NLS mutants NIPA-RKK and NIPA-KP further enhanced wortmannin-induced apoptosis in NPM-ALK-expressing Ba/F3 cells. NIPA-KP and NIPA-RKK are capable of forming more abundant complexes with NPM-ALK, as demonstrated by co-immunoprecipitation and immunofluorescence studies. The increased apoptosis could thus be explained by a dominant-negative function of these NLS NIPA mutants due to efficient competition with endogenous NIPA for interaction with NPM-ALK.

Intriguingly, the mutation of two potential phosphorylation sites, Tyr-105 and Tyr-137 (NIPA-YF), significantly enhanced the apoptosis induced by wortmannin. This double mutation does not affect the nuclear localization of NIPA (data not shown), and the mechanism underlying this biological effect needs to be elucidated in future studies.

Interestingly, besides ALK fusion proteins, we have found that other tyrosine kinases also lead to the phosphorylation of NIPA. Given the broad expression of NIPA and the antiapoptotic effect of NIPA in Ba/F3 cells, an antiapoptotic role of NIPA in tumorigenesis in general seems plausible.

In summary, we have characterized a novel nuclear protein that interacts with kinase-active ALK fusion proteins. The antiapoptotic function of NIPA suggests a role in the pathogenesis of ALK-positive malignancy.

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