The Tyrosine Phosphatase Shp2 Interacts with NPM-ALK and Regulates Anaplastic Lymphoma Cell Growth and Migration

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Abstract

Anaplastic large cell lymphomas (ALCL) are mainly characterized by the reciprocal translocation t(2;5)(p23;q35) that involves the anaplastic lymphoma kinase (ALK) gene and generates the fusion protein NPM-ALK with intrinsic tyrosine kinase activity. NPM-ALK triggers several signaling cascades, leading to increased cell growth, resistance to apoptosis, and changes in morphology and migration of transformed cells. To search for new NPM-ALK interacting molecules, we developed a mass spectrometry–based proteomic approach in HEK293 cells expressing an inducible NPM-ALK and identified the tyrosine phosphatase Shp2 as a candidate substrate. We found that NPM-ALK was able to bind Shp2 in coprecipitation experiments and to induce its phosphorylation in the tyrosine residues Y542 and Y580 both in HEK293 cells and ALCL cell lines. In primary lymphomas, antibodies against the phosphorylated tyrosine Y542 of Shp2 mainly stained ALK-positive cells. In ALCL cell lines, Shp2-constitutive phosphorylation was dependent on NPM-ALK, as it significantly decreased after short hairpin RNA (shRNA)–mediated NPM-ALK knock down. In addition, only the constitutively active NPM-ALK, but not the kinase dead NPM-ALK (K210R), formed a complex with Shp2, Gab2, and growth factor receptor binding protein 2 (Grb2), where Grb2 bound to the phosphorylated Shp2 through its SH2 domain. Shp2 knock down by specific shRNA decreased the phosphorylation of extracellular signal-regulated kinase 1/2 and of the tyrosine residue Y416 in the activation loop of Src, resulting in impaired ALCL proliferation and growth disadvantage. Finally, migration of ALCL cells was reduced by Shp2 shRNA. These findings show a direct involvement of Shp2 in NPM-ALK lymphomagenesis, highlighting its critical role in lymphoma cell proliferation and migration. [Cancer Res 2007;67(9):4278–86]

Introduction

Anaplastic large cell lymphomas (ALCL) represent a subgroup of non–Hodgkin’s lymphomas characterized by the frequent chromosomal translocation t(2;5)(p23;q35). As a consequence, the anaplastic lymphoma kinase (ALK) gene fuses to nucleophosmin (NPM) gene, resulting in the aberrant expression of the chimeric protein NPM-ALK (1). The NPM-ALK fusion protein is composed of the entire catalytic domain of ALK fused to the NPM oligodimerization motif that leads to the dimerization of the protein and to its constitutive autophosphorylation (2). NPM-ALK plays a key role in ALCL lymphomagenesis and it has been shown to cause lymphocyte and fibroblast transformation in vitro and in vivo, to induce cellular proliferation, to inhibit apoptosis, to modify the cytoskeleton, and to deregulate cell-cell adhesion and migration (3–6). NPM-ALK kinase is essential for the survival and growth of ALK-positive ALCLs in vitro and in vivo (7, 8) and exerts its transforming capacity through phosphorylation and activation of several cellular pathways such as Ras/extracellular signal-regulated kinase (Erk), PLC-γ, phosphatidylinositol 3-kinase (PI3K)/Akt and the Jak3/signal transducers and activators of transcription 3 (Stat3). refs. 9–13. Activation of Ras/Erk pathway through the recruitment of different adaptor proteins such as SHC and IRS1 leads to increased ALK-positive ALCL cell growth (14), whereas phosphorylation of Stat3 and stimulation of PI3K pathway prevent cell death and result in increased cell survival (10, 11). As the molecular mechanisms mediating the oncogenic properties of NPM-ALK remain in part unclear, the identification of new interacting molecules and substrates could further elucidate NPM-ALK–mediated transformation and, eventually, reveal new potential targets for drug therapy in lymphomas. Mass spectrometry (MS)–based proteomics is a powerful tool to study protein-protein interactions and, in general, to identify molecules involved in intracellular signaling (15–17). We used a proteomic approach based on immunoaffinity purification followed by nanoflow reverse-phase liquid chromatography-tandem MS (LC-MS/MS) to identify NPM-ALK interacting molecules and substrates. The same approach has allowed the identification of p130Cas as a new NPM-ALK downstream molecule required for NPM-ALK cytoskeleton reorganization and transformation (6). In this study, we report the identification of the SH2 domain–containing tyrosine phosphatase Shp2 and elucidate its role in NPM-ALK–mediated transformation. We show that Shp2 undergoes phosphorylation after association with NPM-ALK in human ALCL cells and that its activation is dependent on ALK tyrosine kinase activity. More importantly, our findings identify Shp2 as a mediator of NPM-ALK oncogenic properties by promoting ALCL cell proliferation and migration.

Materials and Methods

Cell lines, cell cultures, and reagents. For the in vitro experiments, the following cell lines were used: Karpas 299, SU-DHL-1, and TS (a subclone of SUP-M2; NPM-ALK–positive human lymphoid cells), K562 (chronic myeloid leukemia cell line), CEM, and Jurkat (T lymphoblastic cell lines), all obtained from the American Type Culture Collection (ATCC). The cell lines were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Heat-killed Mycoplasma tests were performed once a month by the ATCC Mycoplasma Detection Kit (ATCC, Manassas, VA). The mediums were changed every 3 days. Confluent cells were harvested using 0.05% trypsin and 0.02% EDTA. All experiments involving radiation were performed in a Faraday cage. Four different recombinant and wild-type NPM-ALK were produced by In vitro transfection of cDNA encoding the entire catalytic domain of human ALK fused to the NPM oligodimerization motif into 293T cells and purified by affinity chromatography and size exclusion chromatography. In vitro transfection and affinity chromatography were performed on a preparative scale for the production of recombinant fusion proteins. The purity of the recombinant fusion proteins was assessed by SDS-PAGE and Western blot analysis.

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from New York University and maintained in RPMI 1640 (BioWhittaker) containing 10% FCS (7).

Human embryonal kidney (HEK) cells 293T and 293T-Rex Tet-On (Invitrogen) were cultured in DMEM supplemented with 10% FCS. In 293 Tet systems, the working concentration of trypsin in the medium was 1 µg/mL.

Inducible ALK short hairpin (shRNA) SU-DHL-1 and TS (SU-DHL-1 TTA A5 and TS TTA A5, respectively) cells were obtained by cotransduction with pLV-IVTRKB8 (TTA) vector (18) and pLVTHM vector containing the H1 promoter–shRNA (A5) cassette (7). These cells undergo NPM-ALK silencing when 1 µg/mL doxycycline is added to the medium for at least 72 h.

Immunoprecipitation, trypsin digestion, and MS analysis. Anti-phosphotyrosine immunoprecipitation before MS analysis and protein identification was done as previously described (6). Briefly, HEK 293T-Rex Tet-On cells transfected with NPM-ALK or a kinase dead mutant control NPM-ALKK210R were grown to confluency and induced with 1 µg/mL of tetracycline for 24 h. Cells were lysed in 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 in the presence of protease and phosphatase inhibitors. Ten milligrams of cleared cell lysate were immunoprecipitated with 50 µg of agarose-conjugated monoclonal 4G10 (Upstate Biotechnology) and 10 µg monoclonal PY20 (Transduction Laboratories) and incubated rotating at 4°C overnight. Immunoprecipitated samples were resolved on SDS-PAGE and silver stained. Bands of interest were excised from the gel and subjected to in-gel reduction, alklylation, and digestion with trypsin (Promega). Samples were analyzed by LC-MS/MS using a nanoflow high performance liquid chromatography system (Ultimate; LC Packings) interfaced to electrospray quadrupole time-of-flight (Q-TOF) tandem mass spectrometers (QTOF Ultima or QTOF Micro, Waters/Micromass). Protein identification was achieved by analyzing MS/MS spectra using the Mascot software for searching the National Center for Biotechnology Information nonredundant protein database.

Cell lysis, coimmunoprecipitation, and Western blot analysis. Total cellular proteins were extracted with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride, 10 mM Na3VO4, and a phosphatase inhibitors cocktail (Roche). Samples were resuspended in the lysis solution and incubated at 4°C for 30 min. Cell lysates were clarified by centrifugation at 10,000 × g (13,000 rpm) for 10 min at 4°C and the supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method. Thirty micrograms of proteins were run on SDS-PAGE under reducing conditions.

For immunoprecipitation experiments, 0.5 to 2 mg whole-cell lysates were precleared with 30 µL protein G-Sepharose beads (Amersham) for 1 h at 4°C, then incubated with 1 to 5 µg of monoclonal antibody or 5 to 10 µg of polyclonal antibody at 4°C for 1 h or overnight. Immune complexes were collected on 40 µL protein A-Sepharose beads, and the beads were washed thrice with lysis buffer. Bound proteins were recovered by boiling in Laemmli sample buffer and resolved on SDS-PAGE.

For immunoblotting, proteins were transferred to nitrocellulose, incubated with the specific antibody, and detected with peroxidase-conjugated secondary antibodies and enhanced chemiluminescent reagent (Amersham). The following antibodies were used: anti-ALK (1:2,000: Zymed), monoclonal anti-ALK (1:4,000: Zymed), anti–phospho-Tyr (PY20; 1:1,000; Transduction Laboratories), monoclonal anti–growth factor receptor binding protein 2 (Grb2; 1:2,000; Santa Cruz Biotechnology), anti–phospho-ALK Y1604 (1:1,000; Cell Signaling Technology), anti–phospho-SHP-2 Y542 (1:1,000; Cell Signaling Technology), anti–phospho-Grb2 Y524 (1:1,000; Cell Signaling Technology), anti-Gab2 (1:2,000; Upstate Biotechnology), anti–phospho-Gab2 (1:1,000; Cell Signaling Technology), anti–phospho-Stat3 (1:1,000; Cell Signaling Technology), anti–phospho-Stat5 (1:3,000; Cell Signaling Technology), anti–phospho-STAT3 (1:3,000; Cell Signaling Technology), anti–phospho-ERK1/2 (1:1,000; Cell Signaling Technology), anti–phospho-Erk1/2 (1:1,000; Cell Signaling Technology), anti–phospho-MAPK (1:1,000; Cell Signaling Technology), anti–phospho-MAPK (1:1,000; Cell Signaling Technology), anti–phospho-Src (1:1,000; Santa Cruz Biotechnology), and anti–tubulin (1:10,000; Santa Cruz Biotechnology).

Immunohistochemical and immunofluorescence stainings. Three primary cases of ALK-positive ALCLs were retrieved from the archives of the Department of Pathology of the University of Torino. Immunohistochemical stainings were done on formalin-fixed, paraffin-embedded or frozen tissues of ALCLs. All diagnosis were confirmed by CD30 and ALK stainings. Sections were incubated with primary antibodies anti-ALK (1:1,000; Zymed), anti–CD30 (clone BerH2, 1:100, DAKO), and against the phosphotyrosine Y542 of Shp2 (1:100, Cell Signaling Technology). Bound complexes were revealed by the DAKO’s Envision+ system on a semi-automated immunostainer. For immunofluorescence stainings, 293T cells were grown for 12 h on glass coverslips and transiently transfected with GFP-NPM-ALK or the kinase dead mutant GFP-NPM-ALKK210R for 24 h. Samples were fixed in PBS containing 4% paraformaldehyde at room temperature for 10 min and permeabilized with PBS containing 0.3% Triton X-100 for 5 min. Coverslips were incubated with PBS containing 3% bovine serum albumin for 1 h at room temperature and stained with primary antibody for 1 h followed by tetramethyl rhodamine isothiocyanate–conjugated secondary antibody (1:400; Sigma) for 1 h at room temperature. Primary antibodies anti-ALK (Zymed), anti–phospho-ALK Y1604 (Cell Signaling Technology), anti-Shp2 (Cell Signaling Technology), and anti–phospho-Shp2 Y542 (Cell Signaling Technology) were diluted 1:50 before use. Nuclei were stained 10 min at room temperature with HOECHST 300 (ng/mL; Sigma). Coverslips were mounted on slides and images were acquired on a Leica DM LA upright microscope equipped with a DC300F camera and were analyzed with IM 50 software.

shRNA sequences, DNA constructs, and mutagenesis. NPM-ALK or the kinase dead mutant NPM-ALKK210R were cloned into pcDNA3TO vector (Invitrogen) into HindIII/XhoI sites and stably transduced into 293T-Rex Tet-On cells using Effectene reagents (Qiagen) as previously described (6). To obtain the Shp2 construct, total cDNA from ALCL cell lines was amplified using specific primers flanking the Shp2 coding region. Primers were as follows: Shp2 forward 5′-CGGAGCCTGACAGAGCAG-3′ and Shp2 reverse 5′-CTGAACTTGGTTGCCAGTTTCT-3′. PCR products were cloned into pCI vector using the TA-cloning system (Invitrogen) and sequenced. For transient transfection in HEK 293T cells, Shp2 was digested to obtain a BamHI/EcoRV fragment and subcloned in the plasmid vector pcDNA3 at the same cloning sites (Invitrogen). For lentiviral vectors, Shp2 construct were digested to obtain a BamHI/EcoRV fragment, blunted with Klenow enzyme (Roche), and cloned into the SulI-Smal–blunted sites of the bidirectional lentivector pCCL.snr.GFP.polY.AC2E. (described in ref. 19) and kindly provided by Dr. Luigi Naldini (San Raffaele Scientific Institute, Milan, Italy).

Grb2mut and the dominant-negative Grb2mut (mutated in the NH2-terminal SH3 domain) and Grb2mk (mutated in the SH2 domain) were kindly provided by Dr. A. Pellicer (New York University, New York, NY).

Human Shp2-specific shRNA sequences were designed and cloned into plVTHM vector containing the H1 promoter kindly provided by Dr. Trono [School of Life Sciences, National Center for Competence in Research, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; ref. 18] as previously described (7). The sense strand of Shp2-shRNA that we used is 5′-GGGCCAGAGCAGTGCACTTAA-3′.

Transfection, lentivirus production, and cell infection. shRNA Shp2 lentiviruses were obtained by cotransfection of HEK 293T with pLVT vector and pMD1-2SV-G, pCMV.8.74 and pRSV.REV plasmid vectors (18) into human 293T cell line (Invitrogen). Transfections were done using Effectene reagent (Qiagen). High-titer lentiviral vector stocks were produced in HEK 293T as previously described (7). Briefly, aliquots of virus, supplemented with 8 µg/mL polybrene (Sigma), were used to infect 10 × 10^6 TS and SU-DHL-1 cells and after 72 h the percentages of transduced cells were analyzed for GFP expression by fluorescence-activated cell sorting (FACS).

Inducible Shp2-shRNA interference SU-DHL-1 and TS cells were obtained by cotransduction with pLVTHM vector containing the H1 promoter Shp2-shRNA cassette and pLV-IVTRKB8 vector, as described above.

For cell sorting, cells were resuspended at a concentration of 10 × 10^6/mL in basic sorting buffer and then sorted for enhanced green fluorescent protein (EGFP) expression on a MoFlo High-Performance Cell Sorter (DAKO Cytomation) as described (6).

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Cell cycle analysis and cell apoptosis assay. Cell cycle analysis was carried out by flow cytometry. Cells were washed in PBS and fixed for 1 h in 70% ethanol at 4°C. After washing, cells were treated with RNase (0.25 mg/mL) and stained with propidium iodide (50 µg/mL). The G1-G0 phase fraction was calculated using the CellQuest program (BD Pharmingen Biosciences; ref. 7).

Apoptosis was measured by flow cytometry after staining with the mitochondrion-permeable, voltage-sensitive dye tetramethylrhodamine methyl ester (TMRM; Molecular Probes). Cells were washed once in PBS and incubated for 15 min at 37°C. Cells were washed in PBS and fixed for 1 h in 70% ethanol at 4°C. After washing, cells were treated with RNase (0.25 mg/mL) and stained with propidium iodide (50 µg/mL). The G1-G0 phase fraction was calculated using the CellQuest program (BD Pharmingen Biosciences; ref. 7).

Cell proliferation assay.

For cell proliferation assay, 5 × 10⁴ cells/mL were grown in triplicate in 24-well plates in complete RPMI. The number of viable cells was determined each day up to 6 days by trypan blue exclusion.

Chemotaxis assay. Cell migration was evaluated using a 24-well, 8-µm pore size Transwell system plates (Costar). Purified cells were washed once in RPMI 1640 (BioWhittaker) containing 0.1% FCS and then adjusted to 9 × 10⁵ cells in 100 µL in the same medium. Cells (9 × 10⁵) in 100 µL medium were placed in triplicate on the top of the Transwell. Stromal-derived factor-1α (SDF-1α; 100 ng/mL; R&D Systems) in 600 µL of the same RPMI medium was added to the bottom of the Transwell system. After 3-h incubation at 37°C in a 5% CO₂ atmosphere, the inserts of the transwell chambers were removed and the cells that had migrated to the bottom well were collected and counted on a hemacytometer (6).

Coculture and flow cytometry analysis. For coculture experiments, ALCL cells and ALCL cells infected with Shp2 shRNA and control shRNA, respectively, were mixed in a 1:1 ratio and cultured in standard conditions for 3 weeks. The same experiment was carried out for control cells CEM and K562 infected with Shp2 shRNA. GFP expression was checked over time by FACS and normalized against the initial time point (day 0).

Results

Identification of Shp2 by MS. We developed a MS-based proteomic approach to identify new substrates of NPM-ALK. We stably transfected NPM-ALK or the kinase dead mutant NPM-ALKK210R as a control into human 293-Tet-On cell line. NPM-ALK expression was induced by the addition of tetracycline to the medium (Fig. 1A). We did immunoprecipitations with a mixture of anti-phosphotyrosine antibodies and compared the band patterns of NPM-ALK and NPM-ALKK210R samples. The bands present only in the NPM-ALK sample were digested and analyzed by LC-MS/MS. LC-MS/MS analysis allowed the identification of previously known NPM-ALK interacting molecules (PLC-γ, PI3K, Stat3, and p130Cas) and new potential substrates (Fig. 1B). The most abundant protein at 76 kDa corresponded to NPM-ALK as confirmed by MS analysis. Duplicate MS/MS analysis of the digested bands resulted in the identification of 46 unique proteins belonging to different Gene Ontology functional groups (data not shown). Among phosphatases, we identified four peptides corresponding to the SH2 domain–containing phosphatase Shp2 with a 6% sequence coverage.
Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity. A, Shp2 is phosphorylated in the presence of NPM-ALK but not NPM-ALKK210R. T-Rex-293 Tet-On cells were induced with 1 μg/mL tetracycline for 24 h. Total cell lysates were immunoprecipitated (IP) with a mixture of monoclonal anti-phosphotyrosine antibodies and blotted with anti-Shp2 antibody. B, Shp2 phosphorylation was clearly detectable in ALK-positive lymphoma cell lines (Karpas 299, SU-DHL-1, and TS) compared with controls (CEM and Jurkat). Lymphoid cell lines were immunoprecipitated as described in A. C and D, NPM-ALK silencing by shRNA decreases Shp2 phosphorylation. TS TTA A5 and SU-DHL-1 TTA A5 were grown in the presence of 1 μg/mL doxycycline and harvested after 72 h. Total cell lysates were immunoprecipitated with a mixture of monoclonal anti-phosphotyrosine antibodies and blotted with an anti-Shp2 and anti-ALK antibodies (C), and were immunoblotted with specific anti–phospho-Shp2 antibodies (Y542 and Y580; D).

Accordingly, the phosphorylation of both the C-tail tyrosine residues (Y542 and Y580) of Shp2 was significantly reduced after ALK silencing, as detected by immunoblotting with specific antibodies (Fig. 2D). We concluded that in ALCL cell lines, Shp2 phosphorylation was strictly correlated to NPM-ALK kinase activity.

To confirm the findings obtained on ALCL cell lines in primary tissues, we, then, analyzed ALK-positive lymphomas and found that Shp2 was strongly phosphorylated in anaplastic cells also expressing ALK and CD30, whereas surrounding reactive lymphocytes showed a weak to negative staining (Fig. 3B).

NPM-ALK forms a complex with Shp2, Gab2, and Grb2. To explore whether Shp2 associates with NPM-ALK, we did NPM-ALK immunoprecipitations in 293T-Rex cells. As shown in Fig. 4A, Shp2 associated only with active NPM-ALK, as the binding did not occur in the presence of the kinase dead mutant NPM-ALKK210R. Significantly, the association between the two molecules was also confirmed in ALK-positive cell lines (Karpas 299, SU-DHL-1, and TS; Fig. 4A). ALK-negative Jurkat and CEM cells were used as controls. The same results were obtained with anti-Shp2 immunoprecipitations (data not shown).

Previous studies reported that the association of Shp2 to the scaffolding adapter protein Gab2 is a frequent event in response to coverage. MS/MS spectrum of the peptide 221-INAAEIESR-229 is shown in Fig. 1C. As Shp2 has been described to be involved in the transformation of other hematologic malignancies (20, 21), we decided to further elucidate its role in NPM-ALK–mediated lymphomagenesis.

Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity. Because Shp2 is mutated in a subset of patients with hematologic malignancies (22, 23), we first ruled out the presence of activating mutations of Shp2 in ALCL cell lines by cDNA cloning and sequencing (data not shown). Immunoblotting total cell lysates from 293T cells with anti-Shp2 antibody showed the presence of two protein species, suggesting posttranscriptional modified forms (Fig. 2A). Immunoprecipitation assays with anti-phosphotyrosine antibody confirmed that Shp2 was phosphorylated only in the presence of NPM-ALK and not in cells expressing the kinase dead mutant NPM-ALKK210R, indicating that an active kinase domain is required for a functional activation (Fig. 2A). Moreover, Shp2 phosphorylation was detectable in ALCL cells compared with ALK-negative cell lines (Fig. 2B).

To investigate whether Shp2 phosphorylation was dependent on NPM-ALK tyrosine kinase activity in ALK-positive ALCL cells, we generated a doxycycline-dependent anti-ALK shRNA inducible system to abrogate NPM-ALK expression. TS TTA A5 and SU-DHL-1 TTA A5 cells were cultured in the presence of doxycycline for 72 h to knock down NPM-ALK expression and anti-phosphotyrosine immunoprecipitations showed a marked reduction of Shp2 phosphorylation in both cell lines (Fig. 2C).
growth factor stimulation (20, 21, 24) and that Gab2 precipitated with NPM-ALK in ALCL cell lines (11). Therefore, we investigated whether Gab2 is phosphorylated in a complex with NPM-ALK. In total cell lysates from 293T-Rex cells, Gab2 was phosphorylated only in the presence of NPM-ALK (Fig. 4B). Anti-Gab2 immunoprecipitation in 293T-Rex cells showed that Gab2 precipitated only with the active NPM-ALK, but not with the kinase dead mutant (Fig. 4C). The same results were observed in ALCL cell lines (Fig. 4C). Reciprocal anti-ALK immunoprecipitation in both HEK293 and ALCL cells confirmed the above-described association (data not shown).

Because Grb2 has been described to interact with NPM-ALK (14) and to mediate the recruitment of Shp2 through Gab2 in Bcr-Abl–transformed leukemia cells (20), we hypothesized that Grb2 was involved in the interaction of Shp2 with NPM-ALK. Transient cotransfection experiments in 293T cells showed that Grb2 was able to bind Shp2 only in the presence of active NPM-ALK (Fig. 4D). In addition, the use of two dominant-negative forms of Grb2, mutated, respectively, in the NH2-terminal SH3 domain (P49L) and in the SH2 domain (R86K), showed that the Shp2 binding to Grb2 was mediated by the SH2 domain of Grb2 as Shp2 did not precipitate in the presence of R86K Grb2-mutant, whereas the binding of Shp2 to NPM-ALK was not abrogated (Fig. 4D). This finding suggests that Grb2 is involved as a downstream molecule of Shp2 rather than a mediator of the binding to NPM-ALK.

**Shp2 down-modulation affects lymphoma cell proliferation but not apoptosis.** To further study the involvement of Shp2 on ALCL cell proliferation, we generated cell lines carrying a doxycycline-dependent inducible Shp2 shRNA silencing. TS and SU-DHL-1 were cotransduced with pLV-tTRKRAB (TTA) and pLVTHM Shp2 shRNA. Cotransduced cells grown in the presence of doxycycline efficiently down-regulated Shp2 expression as shown in Fig. 5C. TS and SU-DHL-1 transduced with the pLV-tTRKRAB vector only were used as control. The abrogation of Shp2 in both cell lines significantly impaired cellular proliferation over time compared with cells grown without doxycycline (Fig. 5D) or to the control cell lines (with or without doxycycline; data not shown). To understand whether the observed low proliferation rate was due to cell cycle arrest and/or increased apoptosis, we did cell cycle

**Figure 3.** Shp2 is phosphorylated in NPM-ALK–expressing 293T cells and in primary ALK-positive human lymphomas. A, immunofluorescence stainings of 293T cells transfected with NPM-ALK (left) or NPM-ALKK210R (right) were done by the indicated specific antibodies (red fluorescence). Green fluorescence, EGFP reporter protein encoded by the transfection vectors. All images were taken with a ×100 oil-immersion objective. B, formalin-fixed, paraffin-embedded sections from a representative case of NPM-ALK–positive primary ALCL were stained with H&E (top left) and with antibodies against CD30 (top right) and ALK (lower left) by immunohistochemistry. A corresponding frozen section from the same case was stained with the phosphospecific antibody that recognizes the Y542 phosphotyrosine of Shp2 (lower right). All images were taken with a ×40 objective.
analysis with propidium iodide staining and TMRM apoptosis assay. Cell cycle analysis revealed that Shp2 knock down led to a significant increase in the percentage of TS cells in the G1 phase and a corresponding decrease in the S and G2-M phases, without changes in the apoptotic fraction as indicated by the sub-G0-G1 populations (Fig. 6A). Furthermore, the absence of apoptosis was confirmed by TMRM staining (data not shown). We showed that, in TS cells, Shp2 knock down led to a decrease in Erk1/2 phosphorylation (Fig. 6B). A similar decrease was observed in the SU-DHL-1 cell line (Supplementary Fig. S1). Concomitantly, we observed a decrease in the phosphorylation of the tyrosine residue Y416 in the activation loop of Src, whereas Stat3 and Akt phosphorylations were not affected (Fig. 6B). Notably, NPM-ALK total amount and phosphorylation did not change after Shp2 silencing, thus excluding major effects of Shp2 on NPM-ALK stability, contrary to what has been described for Bcr-Abl (Fig. 6B; ref. 27). These findings indicate that the activation of Shp2 in ALCL cells regulates the Ras/Erk signaling pathway and the activation of Src, thus influencing ALCL cell proliferation.

Shp2 knock down reduces spontaneous and SDF-1α–induced migration of ALCL cells. Our group recently described that NPM-ALK increases ALCL spontaneous and SDF-1α–induced cell migration (6). As Shp2 is required for integrin-driven cell spreading and migration (28, 29) and participates in α-chemokine receptor CXCR4–mediated cell signaling in T lymphocytes (30), we investigated its role in ALCL cell migration. We did a chemotaxis assay in the inducible TS TTA shShp2 shRNA cells. Shp2 knock down reduced both the spontaneous migration and the SDF-1α–induced migration of lymphoma cells, suggesting its role in ALCL cell migration (Fig. 6C).

Discussion

In this study, we used a functional proteomic approach to identify new NPM-ALK phosphorylated and/or interacting proteins by immunoaffinity purification and MS by using 293T-Rex cells ectopically expressing NPM-ALK under an inducible tetracycline promoter. In addition to known NPM-ALK substrates, our analysis

Figure 4. NPM-ALK coprecipitates with Shp2, Gab2, and Grb2. A, Shp2 coprecipitates with NPM-ALK in T-REx-293 and in lymphoma cell lines (Karpas 299, SU-DHL-1, and TS). Total cell lysates from T-REx-293 (left) and lymphoid cell lines (right) were immunoprecipitated with anti-ALK antibody and blotted with anti-Shp2. B, Gab2 is phosphorylated only in the presence of NPM-ALK. Total cell lysates from T-REx-293 were immunoblotted with a specific anti-phospho-Gab2 antibody and anti-Gab2 antibody as control. C, Gab2 coprecipitates with NPM-ALK in T-REx-293 and in ALK-positive ALCL cells. Total cell lysates from T-REx-293 (top) and lymphoid cell lines (bottom) were immunoprecipitated with anti-Gab2 antibody and blotted with the indicated antibodies. D, HEK 293T cells were cotransfected with Shp2wt and the indicated constructs. As detected by anti-Grb2 immunoprecipitation and anti-Shp2 immunoblotting (top left), Grb2 was able to bind Shp2 only in the presence of NPM-ALK, and the binding was disrupted in the presence of Grb2 dominant-negative mutants (right).
revealed novel potential targets. Among the identified proteins, we found the ubiquitously expressed SH2 domain–containing tyrosine phosphatase Shp2. We showed that Shp2 forms a complex with NPM-ALK both in 293T-Rex cells and in ALK-positive ALCI cells TS, SU-DHL-1, and Karpas 299. In ALCI cells, Shp2 phosphorylation was higher compared with other cell lines, such as K562 and Jurkat (data not shown), in which it is reported to be overexpressed and constitutively phosphorylated (25). More importantly, in ALCI cells, Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity as it is almost completely abrogated when NPM-ALK expression is knocked down by shRNA. As it is reported for other transforming tyrosine kinases, this phosphorylation status is indicative of an activation state of Shp2 (20).

It has been shown that Shp2 can bind to receptor tyrosine kinases (RTK) either directly or through adaptor/scaffolding molecules such as IRS1 and Gab family molecules (31, 32), and here we report that NPM-ALK precipitates both Gab2 and Shp2. Therefore, we can suppose that NPM-ALK forms a multimeric complex with Gab2 and Shp2 as described for Bcr-Abl, although we cannot exclude that Shp2 binds directly to NPM-ALK. The Gab2-Shp2 complex with other tyrosine kinases such as Bcr-Abl very often involves the adaptor protein Grb2 although it is controversial whether this association promotes the full Erk activation (33, 35). In fact, Shp2 is commonly required for the activation of the Ras/Erk signaling pathway in response to several cytokines and RTKs (31, 32). Our results reveal that Shp2 knocked down by specific shRNA in ALCI cells results in ~50% reduction of Erk phosphorylation compared with ALCI cells still expressing the phosphatase. Thus, in ALCI cells, our findings suggest that Shp2 acts upstream of the Ras pathway, possibly by recruiting a Grb2/Sos complex. Also, Shp2 could regulate the Ras pathway activation through the Src family protein-tyrosine kinases as we observed a strong decrease in the phosphorylation of the SH3 domain to Gab2 or its SH2 domain to the phosphotyrosine Y542 of Shp2 (20, 33–35). Previous studies showed that Grb2 coprecipitates with NPM-ALK (14), and we show that Shp2-Grb2 interaction is dependent on the catalytic activity of NPM-ALK as there is no association in the presence of the kinase dead mutant NPM-ALKK210R. Moreover, the association is mediated by the Grb2-SH2 domain as its dominant-negative SH2 mutant R86K disrupts the binding. On the contrary, Shp2 association to NPM-ALK is not abrogated. As previously described in fibroblasts stimulated with platelet-derived growth factor or fibroblast growth factor (FGF), tyrosyl-phosphorylated Shp2 functions as an adaptor protein for Grb2 although it is controversial whether this association promotes the full Erk activation (33, 35).

Figure 5. Shp2 regulates cell growth of ALCI cells. A, Shp2 expression is abrogated by specific shRNA. TS and SU-DHL-1 cells were transduced with Shp2 shRNA and control shRNA lentiviral vectors. After 72 h, protein expression was knocked down as verified by Western blot. No up-regulation of nonspecific IFN-mediated responses was observed as shown by blotting with anti–P-eIF2a. B, SU-DHL-1 and TS transduced with Shp2 shRNA and cocultured with the untransduced counterpart showed a significant growth disadvantage according to Student’s t test (P = 0.001 and P = 0.0036, respectively). TS and SU-DHL-1 cells infected with a control shRNA and the T lymphoblastoid control cell line CEM infected with Shp2 shRNA did not change over time. Results are representative of three different experiments. C, TS TTA Shp2 shRNA and SU-DHL-1 TTA Shp2 shRNA were grown in the presence of 1 μg/mL doxycycline for at least 72 h to knock down Shp2 expression as shown in the Western blot with anti-Shp2 antibody. TS TTA and SU-DHL-1 TTA transduced with pLV-TRKRAB were only used as controls. D, Shp2 silencing by shRNA affects lymphoma cell proliferation. TS TTA and SU-DHL-1 TTA infected with Shp2 shRNA were induced with 1 μg/mL doxycycline (Dox) for 96 h and then plated at 5 × 10^4/mL in a 24-well plate. Cells were counted each day for 6 d by trypan blue exclusion. Points, mean of triplicates; bars, SE. One representative experiment from two independent assays.
activating tyrosine Y416 of Src after Shp2 silencing by specific shRNA. This is a conceivable parallel pathway given that upon RTK stimulation Shp2 regulates PAG/Cbp tyrosyl phosphorylation, thereby controlling Csk recruitment to Src (36), and Src is activated by NPM-ALK in ALCL cells (37).

Shp2 has been previously described to be involved in the transformation of some hematologic malignancies, either acting as an oncogene or as an effector molecule (20, 21, 25, 26). Moreover, Shp2 is implicated both in growth factor receptor and integrin signaling usually with a positive regulatory role, thus promoting mitogenic and proliferative signals (31, 32) and its phosphatase activity seems to mediate the transforming capacity of constitutively active forms of some tyrosine kinase receptors such as epidermal growth factor receptor, FGF receptor 3, and RET (38–40). ALCL cells transduced with Shp2 shRNA showed a significant growth disadvantage and a lower proliferation rate compared with control cell lines. These effects are not related to a decrease in the NPM-ALK stability in ALCL cells, in contrast to what it has been described for Bcr-Abl–transformed cells in which Shp2 stabilizes the protein through an interaction with HSP90 (27). The impaired proliferation coincided with the reduced activation of Src and of the Ras/Erk pathway. Interestingly, in ALCL cell lines, knocking down Erk1/2 by specific shRNA leads to a similar impairment of cell proliferation as the knock down of Shp2 (41). Taken together, our results show that in ALCL cells Shp2 is a critical mediator of NPM-ALK–driven cell growth.

Shp2 has been described to transduce promigratory signals from various type of receptors (31, 42), including a CXCR4-mediated signaling pathways in T cells (30). In ALCL cells, our group recently showed that NPM-ALK is able to influence both spontaneous and SDF-1α/CXCR4–induced migration (6). Here, we found that Shp2 down-modulation by shRNA decreased significantly the migration

**Figure 6.** Shp2 knock down leads to ALCL cell accumulation in G1 by reducing Ras/Erk pathway activation and decreases lymphoma cell migration. A, flow cytometry profiles of propidium iodide–stained TS TTA Shp2 shRNA not induced or induced with 1 μg/mL doxycycline for 96 h. In the absence of Shp2, ALCL cells showed a significant cell cycle arrest in G1, but not increased sub–G0–G1 populations. One of four independent experiments. B, TS TTA Shp2 shRNA were induced with 1 μg/mL doxycycline for 96 h. In Shp2 knocked down cells, the phosphorylation of Erk1/2 and of the tyrosine residue Y416 of Src were decreased, whereas Stat3 and Akt phosphorylations were not affected. Samples were incubated with the indicated antibodies. Phospho–Src Y416, phospho–Stat3 Y705, Akt, and Erk1/2 phosphorylations were measured by normalizing the absorbance of the phosphorylated bands to the total amount of the indicated proteins (right). These findings are representative of two independent experiments. C, TS TTA Shp2 shRNA cells were induced with 1 μg/mL doxycycline for 96 h followed by a Transwell migration assay in the presence or absence of the chemokine SDF-1α (100 ng/mL). Columns, mean of triplicates for each experiment; bars, SE. One representative experiment from two independent assays. Right, Western blot analysis of Shp2 knock down by shRNA in the same cell line.
rate of ALK-positive ALCI cells. It is likely that NPM-ALK might promote ALCI cell migration through Shp2 engagement, although further studies are required to better elucidate the mechanism.

In conclusion, our work provides another clue in the molecular mechanisms underlying NPM-ALK transformation. Our findings show that Shp2 associates to NPM-ALK and forms a multimeric complex with Gab2 and Grb2, resulting in the activation of multiple downstream effectors such as the Ras/Erk pathway and show that Shp2 plays a critical role in ALK-evoked cell proliferation and migration.

References

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Correction: The Tyrosine Phosphatase Shp2 Interacts with NPM-ALK and Regulates Anaplastic Lymphoma Cell Growth and Migration

In this article (Cancer Res 2007;67:4278–86), which appeared in the May 1, 2007 issue of Cancer Research (1), two individual lanes in the Western Blot panels in Fig. 5C were inadvertently duplicated during the assembly of the figure. The authors reviewed the original scans and found the correct Western Blots for panels Shp2 and NPM-ALK in SU-DHL-1 TTA cell line, Shp2 shRNA experiment. The corrected Fig. 5C appears below.

This correction does not affect in any way the article’s conclusions as the results are fully consistent with those shown in the original figure. The authors apologize for these errors.

Reference

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The Tyrosine Phosphatase Shp2 Interacts with NPM-ALK and Regulates Anaplastic Lymphoma Cell Growth and Migration

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