Mutations in the PTPN11 gene product, Shp2, are associated with several human diseases (for review, see Ref. 1), including multiple lentigines/LEOPARD syndrome (LS) (2, 3), Noonan syndrome (NS) (4), and various malignancies (5–8). LS (MIM 151100) and NS (MIM 163950) are developmental disorders with many shared features. NS is characterized by facial dysmorphia, typically ocular hypertelorism, cardiac defects, most commonly pulmonary valve stenosis, and proportionate short stature. Cryptorchidism and deafness are also reported in NS patients (9). However, lentigines, a hallmark of LS (10), are uncommon in NS, and less penetrant NS abnormalities, such as webbed neck, skeletal defects, and bleeding/coagulation abnormalities, are typically absent in LS (10–12). Both LS and NS are associated with increased risk of malignancy. However, acute myelogenous leukemia and neuroblastoma are associated with LS (12, 13), whereas various childhood hematological disorders, most notably juvenile myelomonocytic leukemia and possibly acute lymphoblastic leukemia, are found at increased incidence in NS (1). Somatic PTPN11 mutations are found in sporadic juvenile myelomonocytic leukemia (~35%), B cell acute lymphoblastic leukemia (~7%), other childhood myeloproliferative or myelodysplastic disorders (1–5%), adult acute myelogenous leukemia (~5%), and occasionally in neuroblastoma and other solid tumors (5–8).

Shp2 is a ubiquitously expressed, non-receptor protein-tyrosine phosphatase (PTP) comprising two N-terminal SH2 domains, a catalytic domain and a proline-rich stretch (14). Via its SH2 domains, Shp2 binds directly to PDGF receptor (PDGF) receptor, as well as to several scaffolding adapters, including IRS, FGF receptor substrate (FRS), and Gab proteins. Formation of such complexes is required for full activation of the Ras/Erk cascade in most, if not all receptor-tyrosine kinase, cytokine receptor, and integrin signaling pathways. Consequently, Shp2 plays an important role in mediating multiple downstream biological responses, such as proliferation and/or survival, adhesion, and migration.

All known biological functions of Shp2 require its catalytic activity (14). Shp2 activity is tightly regulated by an elegant “molecular switch” mechanism that couples activation with recruitment of Shp2 to its binding proteins (14–16). In the basal state (i.e. in unstimulated cells when Shp2 is found primarily in the cytoplasm), the “backside loop” of the N-Sh2 domain (the side opposite to its phosphotyrosyl peptide binding pocket) interacts with the PTP domain, preventing substrate access to the active site (15). Binding to a phosphotyrosyl (Tyr(P)) protein ligand (e.g. in a receptor-tyrosine kinase or scaffolding adapter) alters the conformation of the N-Sh2 domain, rendering it unable to bind the PTP domain and activating the enzyme (Fig. 1A). Earlier studies showed that mutating key contacts between the N-Sh2 and the PTP domains led to biochemical and biologically “activated mutants” of Shp2 (17).
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by PTPN11 mutations, they have been viewed as overlap syndromes (1). We examined the enzymatic properties of LS mutants and their effects on receptor-tyrosine kinase signaling. Surprisingly, despite its phenotypic and genetic similarities to NS, we found that LS is caused by catalytically defective, loss-of-function mutations in PTPN11.

EXPERIMENTAL PROCEDURES

Structural Interpretation and Molecular Modeling—The Shp2 structure 2SHP (Protein Data Bank), comprising the two SH2 domains plus the PTP domain and corresponding to the basal, inactive form of the enzyme in the absence of Tyr(P) peptide (15), and the predicted effects of various mutants were visualized using the PyMol Molecular Graphic System, Version 0.97 (DeLano Scientific).

Expression Constructs and Protein Purification—Point mutations were introduced using the QuikChange kit (Stratagene). Wild type Shp2 (WT) and mutant Shp2 in pGEX-4T-2F, a modified version of pGEX-4T (Amersham Biosciences) that generates proteins with N-terminal GST- and C-terminal FLAG tags, were produced and purified on glutathione-Sepharose (Amersham Biosciences), as described previously (20). Protein concentrations were determined by densitometric analysis of Coomassie-stained SDS-PAGE gels using bovine serum albumin as the standard. Purified enzymes were stored in the presence of 33% glycerol at −80 °C.

PTP Assays—PTP assays using 32P-labeled carboxamido-methylated and -maleylated lysozyme (RCML) (typical specific activity ∼4000 cpm/pmol) as substrate were conducted in assay buffer (50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1 mg/ml bovine serum albumin, 10 mM dithiothreitol, 5 mM EDTA, 2 mM DCM, and 5 mM GST-enzyme) with or without IRS-1 Tyr(P)-1172 (SLNpYIDLDLVK; pY is Tyr(P)) or Tyr(P)-1222 (LSTpYASINFQK) peptides as described (20). Assays using para-nitrophenyl phosphate (pNPP, obtained from Sigma) as substrate were carried out in 30 mM Hepes (pH 7.4), 120 mM NaCl, 5 mM dithiothreitol, 10 mM pNPP, and 5 mM enzyme with or without various concentrations of Tyr(P)-peptide at 30 °C for 10 min and terminated with 0.2 N NaOH. Phosphate release was determined by measuring 0.1% SDS-PAGE sample buffer (1.0% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM NaVO₃ plus a protease mixture (5 µg/ml leupeptin, 5 µg/ml aprotinin; 1 µg/ml pepstatin A, 1 µm phenylmethylsulfonyl fluoride, 1 mM benzamidine) and clarified in a microcentrifuge. Protein concentrations were measured by using Coomassie protein reagent. Proteins were resolved by SDS-PAGE. Immunoblotting onto Immobilon P and detection by enhanced chemiluminescence (ECL) were performed essentially as described (21). Rabbit polyclonal anti-Shp2 and anti-total Erk antibodies (Santa Cruz Biotechnology, Inc.), anti-Gab-1 antibodies (Upstate Biotechnology, Inc.), anti-phospho-Erk1/2 antibodies (Cell Signaling Technology), and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Amersham Biosciences) were all used according to their manufacturer’s instructions.

Stable Cell Lines—Retroviruses expressing WT or mutant Shp2 were generated by transient co-transfection of 293T cells with pBABE-puro constructs and AmphoPac (Invitrogen). Viral supernatants were collected 48 h post-transfection, passed through a 0.45-µm filter, and used to infect fresh 293T cells in the presence of 4 µg/ml Polybrene (Sigma). Forty-eight hours later cells were selected in 1 µg/ml puromycin (Sigma). Cell stimulations and analyses were performed as described above.

RESULTS

LS Mutations Are Catalytically Inactive—In contrast to NS mutations, which are scattered throughout the Shp2 molecule, LS mutants are confined to the PTP domain (11, 12, 23) (Figs. 1, B–D). Based on structural and enzymologic studies of other PTPs (e.g. PTP1B (24, 25)), the six mutations specifically linked to LS (Y279(C/S), T468M, A461T, Q506P, Q510P) involve residues predicted to affect catalysis (26). Three (Ala-461, Gly-464, and Thr-468) reside within the “signature motif” ((I/V)HCAGXGR(S/T)GT) that defines the PTP family (26) and comprises the “PTP loop” in PTP structures (24, 25). The conserved cysteinyl residue within this motif carries out a nucleophilic attack on the substrate phosphotyrosyl residue, generating a thiophosphate intermediate that subsequently undergoes hydrolysis by a bound water molecule. Other signature motif residues are important for lowering the $K_{i}$p (i.e. enhancing the nucleophilicity) of the catalytic cysteine, orienting the substrate for nucleophilic attack, maintaining the structural integrity of the catalytic pocket, and/or neutralizing the charge of the phosphotyrosine in the substrate (26). Shp2 Tyr-279 is cognate to Tyr-46 in PTP1B, which sets the depth of the catalytic cleft and, thus, confers specificity for phosphotyrosine-containing substrates (24). Gln-506 or possibly Gln-510 is the Shp2 analog of Gln-262 in PTP1B (24, 25) and comprises the “PTP loop” in PTP structures (24, 25). The conserved cysteinyl residue within this motif carries out a nucleophilic attack on the substrate phosphotyrosyl residue, generating a thiophosphate intermediate that subsequently undergoes hydrolysis by a bound water molecule. Other signature motif residues are important...

Cell Culture—293T cells were cultured at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 1% sodium pyruvate, and 1% penicillin-streptomycin.

Transient Transfections—WT or mutant Shp2 cloned in the mammalian expression vector pBABE-puro (Invitrogen) were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). For some experiments, WT or mutant Shp2 (5 µg) were co-transfected with HA-Erk (0.5 µg) and/or HA-Gab-1 (2.0 µg). Twenty-four hours post-transfection cells were serum-starved for an additional 24 h and then either left unstimulated or stimulated with EGF (50 ng/ml), fibroblast growth factor (FGF)-2 (20 ng/ml), or PDGF (100 ng/ml) for various times. All
N-SH2 domain (Fig. 2A). D61G had increased basal activity (compared with WT) that was further enhanced upon the addition of Tyr(P)-1172, whereas E76K was maximally activated even in the absence of Tyr(P) peptide. In contrast, none of the LS mutants exhibited detectable PTP activity against either substrate, even in the presence of saturating amounts of Tyr(P)-1172 (Fig. 2A) or over a wide range of doses of another activating phosphopeptide, Tyr(P)-1222, of IRS-1 (Fig. 2B).

To exclude the possibility that the recombinant LS proteins produced in bacteria folded inaccurately and/or were unstable, mammalian expression constructs for (untagged) WT Shp2 and the indicated mutants were transiently transfected into 293T cells, which were then starved and either left unstimulated or stimulated with EGF. Endogenous (i.e. empty vector-transfected) Shp2 activity, as measured by immune complex PTP assay, was enhanced slightly upon growth factor stimulation (Fig. 2C). Cells transfected with WT Shp2 showed slightly higher basal and stimulated Shp2 activity, whereas those expressing the leukemia-associated E76K mutant showed markedly enhanced and constitutive Shp2 activity. However, transient expression of LS mutants led to no increase in PTP activity even though these mutants were expressed at levels comparable with WT Shp2 or E76K (Fig. 2C, bottom panel). Instead, LS mutants tended to cause decreased EGF-stimulated Shp2 activity (as measured by immune complex assay), raising the possibility that such mutants have dominant negative effects.

**LS Mutations Disrupt the Catalytic Pocket of Shp2**—Examination of the Shp2 crystal structure (Fig. 1D) provided potential explanations for these observations. Because Tyr-279 sets the depth of the catalytic cleft, substitution by cysteine (or serine as in another LS patient (12)) should alter the depth of the cleft and might also perturb the orientation of the catalytic cysteine, Tyr(P)-1222, of IRS-1 (Fig. 2B).

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LS Mutants Are Dominant Negative—We next examined the effects of LS mutants on receptor-tyrosine kinase signaling. Expression constructs for WT or mutant Shp2, Gab1, and HA epitope-tagged Erk1 were co-transfected into 293T cells, as described previously (18). Transiently transfected cells were starved or stimulated with EGF, and total Tyr(P) (data not shown) and Erk activation was monitored by antiphospho-Erk immunoblotting (Fig. 4A). As expected, EGF stimulation activated Erk in WT-expressing cells, and Erk activation was enhanced

FIGURE 2. LS mutants are catalytically impaired. A, activities of the indicated GST-Shp2-FLAG proteins were measured using the artificial substrate 32P-labeled carboxamidomethylated and -maleylated lysozyme in the absence (closed bars) or presence (open bars) of the IRS-1-derived peptide Tyr(P)-1172 (100 μM). Phosphate release was quantified by charcoal binding assay. Compared with WT Shp2, the NS mutant D61G and the leukemia-associated mutant E76K show increased activity basally and in the presence of Tyr(P)-1172. In contrast, LS mutants are inactive.

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A transient assays. 293T cells were transiently transfected with HA-Erk1, WT or mutant Shp2, and Gab-1 expression constructs as indicated and then starved and either stimulated with EGF (50 ng/ml) or left unstimulated. Erk activation was assessed by anti-phospho-Erk immunoblotting of total cell lysates. The lower panels show reprobes for total Erk-1 and Shp2 levels. In contrast to the enhanced Erk activation evoked by the NS mutant D61G and the leukemia mutant E76K, LS mutations, like the catalytically inactive mutant C459S, impair Erk activation. B, stable expression assays. Retrovirally transduced pools of 293T cells stably expressing WT Shp2 or the indicated mutants were either starved or stimulated with EGF (50 ng/ml), lysed, and immunoblotted with phospho-Erk1/2 antibodies. The lower panels depict the same lysates blotted for total Erk1/2 and Shp2. C, time course of Erk activation in cells expressing Shp2 mutants. 293T cells stably expressing vector control (Vec), WT Shp2, or the indicated mutants were either starved or stimulated with EGF (50 ng/ml) for various times, as indicated, then lysed and immunoblotted with anti-phospho-Erk1/2 antibodies. The lower panels show equivalent loading. Note that LS mutants preferentially impair sustained Erk activation. D and E, Erk activation in response to other growth factors is also inhibited by LS mutations. 293T cells stably expressing WT Shp2 or the indicated mutants were either starved or stimulated with EGF (50 ng/ml) for various times, as indicated, then lysed and immunoblotted with anti-phospho-Erk1/2 antibodies. The lower panels show equivalent loading. Note that LS mutants preferentially impair sustained Erk activation.

LS Mutants Preferentially Associate with Gab1—At first glance, expression of one normal and one catalytically inactive version of Shp2 might be expected to have the same effect as loss of a normal Shp2 allele, yet hemizygous Shp2 mutant mice have no apparent cardiac, facial, or size abnormalities (29). However, complexes between Shp2 and a relevant binding protein are the active signaling entities, because Shp2 must to a much greater extent in cells expressing E76K or D61G. Expression of a mutant of the essential catalytic cysteinyl residue (C459S) inhibited Erk activation, as reported (28). Notably, the two most common LS mutants, Y279C and T468M, also had dominant negative effects, strongly inhibiting EGF-evoked Erk activation (Fig. 4A). Likewise, in stable cell pools expressing each mutant, D61G enhanced, whereas LS mutants strongly impaired, EGF-evoked Erk activation (Fig. 4B). Similar to the effects of Shp2 deficiency in fibroblasts (29–31), the major effect of LS mutants was to inhibit sustained EGF-evoked Erk activation (Fig. 4C). LS mutants also impaired Erk activation in response to FGF or PDGF stimulation (Fig. 4, D and E), consistent with a general ability of these mutants to impair receptor-tyrosine kinase signaling.

FIGURE 3. Shp2 structure reveals that LS mutants disrupt the catalytic cleft. Representations of relevant views of WT Shp2 structure (A, C, E, and G) are shown on the left side, and hypothetical structures that might be expected in the indicated LS mutants (B, D, F, and H) are presented on the right. Residues mutated in LS are represented in green, and catalytic loop residues are in red. A and B, Y279C results in substitution of the smaller cysteinyl side chain for the more hydrophobic and larger tyrosyl group. This removes the Tyr(P) substrate recognition residue and disrupts a hydrogen bond to Ser-460 of the PTP catalytic loop.

FIGURE 4. LS mutants are dominant negative ex vivo. A transient assays. 293T cells were transiently transfected with HA-Erk1, WT or mutant Shp2, and Gab-1 expression constructs as indicated and then starved and either stimulated with EGF (50 ng/ml) or left unstimulated. Erk activation was assessed by anti-phospho-Erk immunoblotting of total cell lysates. The lower panels show reprobes for total Erk-1 and Shp2 levels. In contrast to the enhanced Erk activation evoked by the NS mutant D61G and the leukemia mutant E76K, LS mutations, like the catalytically inactive mutant C459S, impair Erk activation. B, stable expression assays. Retrovirally transduced pools of 293T cells stably expressing WT Shp2 or the indicated mutants were either starved or stimulated with EGF (50 ng/ml), lysed, and immunoblotted with phospho-Erk1/2 antibodies. The lower panels depict the same lysates blotted for total Erk1/2 and Shp2. C, time course of Erk activation in cells expressing Shp2 mutants. 293T cells stably expressing vector control (Vec), WT Shp2, or the indicated mutants were either starved or stimulated with EGF (50 ng/ml) for various times, as indicated, then lysed and immunoblotted with anti-phospho-Erk1/2 antibodies. The lower panels show equivalent loading. Note that LS mutants preferentially impair sustained Erk activation. D and E, Erk activation in response to other growth factors is also inhibited by LS mutations. 293T cells stably expressing WT Shp2 or the indicated mutants were either starved or stimulated with EGF (50 ng/ml) for various times, as indicated, then lysed and immunoblotted with anti-phospho-Erk1/2 antibodies. The lower panels show equivalent loading. Note that LS mutants preferentially impair sustained Erk activation.
**DISCUSSION**

Several lines of evidence show that whereas NS and neoplasia-associated PTPN11 mutants exhibit gain-of-function, LS mutants are, unexpectedly, catalytically impaired and dominant negative. Bacterially produced LS mutant proteins have markedly decreased PTP activity when assayed against standard artificial substrates. The same is true when these mutants are expressed transiently in mammalian cells and subjected to immune complex PTP assays. Under the same conditions, NS and neoplasia-associated mutants exhibit substantially increased catalytic activity. These *in vitro* biochemical differences in Shp2 properties are accompanied by (and presumably cause) diagnostically opposite effects of NS/neoplasia-associated and LS mutants, respectively, on receptor-tyrosine kinase signaling. Transient or stable expression of NS/leukemia-associated mutants enhances, but LS mutants markedly impair Erk activation in response to multiple growth factors, including EGF, FGF, and PDGF. Consistent with their impaired catalytic activity observed *in vitro* and their dominant negative effects on receptor-tyrosine kinase signaling *in vivo*, molecular modeling studies predict that LS mutants should markedly alter the catalytic cleft of Shp2 and disrupt the basal inhibitory interaction between the N-SH2 and PTP domains, thereby giving rise to open, inactive forms of the enzyme. Mathematical modeling predicts that these open, inactive mutants should have more potent dominant negative effects (supplemental material).

Our findings raise an obvious conundrum; how do gain-of-function and dominant negative mutants cause similar disease phenotypes? Although the precise explanation for this apparent paradox remains to be determined experimentally, there are several possible explanations. Shp2 plays important roles downstream of multiple receptors and acts at several different times during development (14). We suspect that the NS and LS phenotypes result from differential effects of mutant Shp2 on different receptor-tyrosine kinase pathways at distinct developmental times. For example, previous studies suggest a possible explanation for the similar cardiac defects in LS and NS. Valves develop from specialized cushion mesenchymal cells in embryos lacking the RasGap NF1, the
product of the neurofibromatosis type I gene, NFI (34, 35). In contrast, heparin binding epidermal growth factor activates the EGF receptor (ErbB1) at ~E13.5, which transmits signals that help terminate mesenchymal cell proliferation and promotes morphogenesis by inhibiting Smad family transcription factors (36, 37). D61G/+ “knock-in” embryos exhibit excess cushion mesenchymal proliferation by E12.5 (21), a phenotype very similar to the effects of NFI deficiency. Likewise, adenoviral expression of the NS mutant Q79R in chicken cardiac cushion explants increased Erk activation and to an Erk-dependent increase in cell proliferation (38). By contrast, Shp2 hemizygosity enhances the effects of EGFR hypomorphism on cardiac valvulogenesis (36). Although other explanations remain possible, we suspect that the predominant effect of NS mutants is to enhance epithelial-mesenchymal transformation/mesenchymal cell proliferation by increasing ErbB2/3 (and/or ErbB3/4) signaling, whereas LS mutants antagonize HB-EGF/ErbB1 signaling at later times. The differential effects of NS and LS mutants on these two signaling pathways likely reflect distinct signaling thresholds for activating the Ras/Erk (or other) pathways downstream of ErbB2/3 and ErbB1, respectively, which could result from different expression levels of Shp2 binding proteins (e.g. Gab proteins) and/or other pathway regulators.

Other phenotypes common to NS and LS (e.g. facial abnormalities and short stature) might involve defective migration and/or differentiation, which might result from increased or decreased signaling involving the same pathway (e.g. either abnormally strong or weak cell adhesion could impair migration) (39). In this regard, the altered ability of Shp2 to control activation of the small G protein Rho (17, 40, 41) may play an important role in the pathogenesis of NS and LS. We also cannot exclude the possibility that as yet undetermined or poorly understood functions of Shp2 underlie pathogenesis of one or both of these disorders. For example, a recent study reported that a small percentage of Shp2 resides within mitochondria (42). The function(s) of mitochondrial Shp2 and the effects of disease-associated mutants on this function(s) remain to be determined.

Resolving the detailed cellular basis for the similar defects in NS and LS will require mouse modeling of LS, which is currently under way in our laboratory. However, our data argue strongly that instead of classifying diseases caused by PTPN11 mutations by current clinical criteria, characterization should be based on the biochemical effects of a given PTPN11 mutation on Shp2 activity and signaling.

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Note Added in Proof—Preliminary limited proteolysis experiments also support the notion that, under basal conditions, LS mutants are in a more “open” conformation than the WT-Shp2.

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