Diversity and Functional Consequences of Germline and Somatic *PTPN11* Mutations in Human Disease

Marco Tartaglia,[1] Simone Martinelli,[1] Lorenzo Stella,[2] Gianfranco Bocchinfuso,[2] Elisabetta Flex,[1] Viviana Cordeddu,[1] Giuseppe Zampino,[3] Ineke van der Burgt,[5] Antonio Palleschi,[2] Tamara C. Petrucci,[1] Mariella Sorcini,[1] Claudia Schoch,[6] Robin Foà,[4] Peter D. Emanuel,[7] and Bruce D. Gelb[8]

[1]Dipartimento di Biologia Cellulare e Neuroscienze, Istituto Superiore di Sanità, [2]Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, [3]Istituto di Clinica Pediatrica, Università Cattolica del Sacro Cuore, and [4]Dipartimento di Biotecnologie Cellulari ed Ematologia, Università La Sapienza, Rome; [5]Department of Human Genetics, University Medical Centre, Nijmegen, The Netherlands; [6]Department of Internal Medicine III, Ludwig-Maximilians-University, Munich; [7]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham; and [8]Departments of Pediatrics and Human Genetics, Mount Sinai School of Medicine, New York

Germline mutations in *PTPN11*, the gene encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome (NS) and the clinically related LEOPARD syndrome (LS), whereas somatic mutations in the same gene contribute to leukemogenesis. On the basis of our previously gathered genetic and biochemical data, we proposed a model that splits NS- and leukemia-associated *PTPN11* mutations into two major classes of activating lesions with differential perturbing effects on development and hematopoiesis. To test this model, we investigated further the diversity of germline and somatic *PTPN11* mutations, delineated the association of those mutations with disease, characterized biochemically a panel of mutant SHP-2 proteins recurring in NS, LS, and leukemia, and performed molecular dynamics simulations to determine the structural effects of selected mutations. Our results document a strict correlation between the identity of the lesion and disease and demonstrate that NS-causative mutations have less potency for promoting SHP-2 gain of function than do leukemia-associated ones. Furthermore, we show that the recurrent LS-causing Y279C and T468M amino acid substitutions engender loss of SHP-2 catalytic activity, identifying a previously unrecognized behavior for this class of missense *PTPN11* mutations.

The *PTPN11* gene (MIM 176876) encodes SHP-2, a cytoplasmic protein tyrosine phosphatase (PTP) characterized by two tandemly arranged Src homology 2 (SH2) domains at the N-terminus, a catalytic domain, and a C-terminal tail containing a proline-rich region and two tyrosyl residues that undergo reversible phosphorylation (Neel et al. 2003). SHP-2 is a critical component of signal transduction for several growth factor–, hormone-, and cytokine-signaling pathways controlling developmental processes (Tang et al. 1995; Saxton et al. 1997, 2000; Qu et al. 1998; Chen et al. 2000) and hematopoiesis (Qu et al. 1997, 1998, 2001), as well as energy balance and metabolism (Zhang et al. 2004). Consistent with the crucial role of SHP-2 in development, germline missense mutations in *PTPN11* cause Noonan syndrome (NS [MIM 163950]) (Tartaglia et al. 2001), a developmental disorder characterized by short stature, facial dysmorphisms, skeletal and hematological defects, and cardiovascular abnormalities (Noonan 1968; Allanson 1987). Accumulating genetic, modeling, and biochemical data indicate that *PTPN11* mutations account for ~50% of affected individuals and promote SHP-2 gain of function (Tartaglia and Gelb 2005*b*). Germline lesions in *PTPN11* have also been identified in the clinically related LEOPARD syndrome (LS [MIM 151100]) (Digilio et al. 2002; Legius et al. 2002), with two amino acid substitutions (Y279C and T468M) occurring in the vast majority of subjects (Tartaglia and Gelb 2005*b*). Before the study described in this article, there was no information regarding the consequences of LS-causing *PTPN11* mutations on the function of SHP-2.

Children with NS are prone to develop malignancies—most commonly, juvenile myelomonocytic leukemia (JMML [MIM 607785]), a myeloproliferative disorder of childhood (Emanuel 2004). Since excessive signaling through the RAS/MAPK pathway has been implicated in a wide variety of cancers and because SHP-2 is a positive modulator of RAS signaling (Neel et al. 2003; Tartaglia et al. 2004*b*), it was consistent that germline *PTPN11* mutations were present in children with NS and JMML (NS/JMML) (Tartaglia et al. 2003). Moreover, somatic missense mutations in *PTPN11* have been documented with variable prevalence in a heterogeneous group of hematologic malignancies (Tartaglia et al. 2003, 2004*a*, 2005; Loh et al. 2004*a*, 2004*b*, 2005) and

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Address for correspondence and reprints: Dr. Marco Tartaglia, Dipartimento di Biologia Cellulare e Neuroscienze, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy. E-mail: mtartaglia@iss.it

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solid tumors (Bentires-Alj et al. 2004). Similar to what was observed for NS, genetic, biochemical, and functional data support the view that acquired lesions contributing to leukemia promote SHP-2 gain of function (Tartaglia et al. 2003; Chan et al. 2005; Keilhack et al. 2005; Mohi et al. 2005; Shubbert et al. 2005), providing the first evidence of a mutated PTP acting as an oncoprotein in cancer.

Our initial studies indicated that mutations identified in NS (germline origin) and leukemia (somatic origin) rarely overlap (Tartaglia et al. 2003). On the basis of those results, we proposed a model requiring at least two classes of activating mutations of *PTPN11* with different roles in development and leukemogenesis. A third class, as-yet functionally uncharacterized, would include a few mutations, including the recurrent LS-causing lesions, which affect residues clustering within or in proximity to the active site of the protein and are predicted to impair catalysis. To test this model, we investigated the diversity and structural consequences of germline and somatic *PTPN11* mutations, delineated their association with disease, and characterized biochemically and structurally a panel of amino acid changes recurring in NS, LS, and leukemia that affect distinct functional domains of the protein.

Material and Methods

Subjects

Two large cohorts containing subjects with NS or LS who might have germline *PTPN11* mutations ($N = 425$) and those with hematologic malignancies ($N = 303$) who might have somatic ones were included in the study. For the NS/LS cohort, there were two groups. For one ($N = 116$), subjects were enrolled in research protocols. Clinical features for the majority of these individuals satisfied the diagnostic criteria reported by van der Burgt et al. (1994) or Voron et al. (1976), but individuals who lacked sufficient features to receive a definitive diagnosis were also included in the study. The second group ($N = 309$) comprised mutation data from anonymous samples from individuals with phenotypes suggestive of NS or LS and prompting commercial DNA diagnostic testing at GeneDx (Gaithersburg, MD); no phenotype data were available for these persons. In the leukemia cohort, which included 63 children with JMML, 147 adult subjects with acute myeloid leukemia (AML), and 93 adult subjects with acute lymphoblastic leukemia (ALL), diagnosis was established according to standard morphologic, cytochemical, and immunological criteria (Bene et al. 1995; Emanuel 2004) and was centrally reviewed (R.F., C.S., and P.D.E.). All nonanonymous samples were collected under institutional review board–approved protocols and with informed consent.

Molecular Analysis and Data Collection

Genomic DNA was isolated from peripheral blood lymphocytes (NS/LS) or from bone marrow aspirates (leukemias) obtained at the time of diagnosis, before therapy, as well as during follow-up. *PTPN11* mutational screening was performed by denaturing high-performance liquid chromatography analysis with the use of the Wave 2100 System (Transgenomics) at column temperatures recommended by the Navigator version 1.5.4.23 software (Transgenomics), as described elsewhere (Tartaglia et al. 2002, 2004*a*). Amplimers having abnormal denaturing profiles were purified (Microcon PCR [Millipore]) and were sequenced bidirectionally with the use of the ABI BigDye Terminator Sequencing Kit v.1.1 (Applied Biosystems) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Length and dinucleotide mutations were determined by cloning purified PCR products in a pCR 2.1 TOPO vector (Invitrogen) and sequencing purified clones (Plasmid Mini Kit, Qiagen). The entire *PTPN11* coding sequence was screened in the NS/LS cohort. On the basis of our previously generated data, exons 1, 2, 3, 4, 7, 8, 12, 13, and 14—encompassing all the *PTPN11* lesions identified in NS, LS, and leukemias—were screened in the leukemia cohort.

Germline and somatic *PTPN11* mutations reported in studies published (or available online as preprint versions) before September 2005 were collected. In the resulting database, germline mutations referred to lesions identified in subjects with a developmental disorder (NS, LS, or a related condition), whereas somatic mutations referred to defects identified in subjects with isolated hematologic malignancies, preleukemic disorders, or solid tumors. The database and relative references are reported in table 1.

Biochemical Studies

Full-length human His-tagged *PTPN11* cDNA cloned in pET-26b (Novagen) was a kind gift from Antonio Pizzuti (Department of Experimental Medicine and Pathology, University La Sapienza, Rome). The single nucleotide changes resulting in T42A, A72S, A72V, T73I, E76D, E76K, E139D, Y279C, I282V, N308D, T468M, M504V, and C459G were introduced by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit [Stratagene]). Recombinant SHP-2 proteins were expressed in *Escherichia coli* Rosetta 2 (DE3) competent cells (Novagen). After induction, harvesting, and cell lysis, proteins were purified by chromatography with the use of Ni-NTA magnetic agarose beads (Qiagen) (fig. 1). In vitro phosphatase assays were performed using 20 pmol of purified recombinant SHP-2 proteins in 200 μl of PTP buffer (25 mM Hepes, pH 7.4; 50 mM NaCl; 2.5 mM EDTA; 60 μg/ml BSA; and 5 mM dithiothreitol) supplemented with 20 mM of paranitrophenyl phosphate (pNPP) (Sigma) as substrate, either in basal condition or with the activating PTP nonreceptor type substrate 1 (PTPNS1) (DITpYADLNLPKGKKPAPQAAEPNNHTEpYASIQTS-NH2) (Primm) bisphosphoryl tyrosine-based activation motif (BTAM) peptide (10 μM), and incubated for 30 min

Table 1

SHP-2 Affected Residues and Amino Acid Changes Germinally Transmitted or Somatically Acquired

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 1 Coomassie-stained SDS polyacrylamide gel showing purity of the wild-type and mutated SHP-2 proteins expressed in *E. coli* Rosetta 2 (DE3) cells and utilized in phosphatase assay experiments. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

at 30°C. Reactions were stopped by the addition of 800 μl of 0.1N NaOH. pNPP dephosphorylation was evaluated by measuring absorbance at 410 nm. Amount, purity, and integrity of recombinant SHP-2 proteins were evaluated using the Protein Assay Kit (Bio-Rad), Coomassie staining, and immunoblot analysis with anti–SHP-2 monoclonal antibody (Santa Cruz Biotechnology).

Transfections in COS1 cells and immunoprecipitations were performed as described elsewhere (Tartaglia et al. 2003; Fragale et al. 2004). Phosphatase assays using immunocomplexes were performed using pNPP as substrate, basally or after BTAM peptide stimulation.

Molecular Dynamics Simulations

Initial coordinates of human SHP-2 were taken from the x-ray crystal structure (Hof et al. 1998) (PDB entry 2shp, chain A). The program DeepView 3.7 (Guex and Peitsch 1997) was used to determine the conformation of missing loops in SHP-2 crystal structure by homology modeling and to introduce residues T2, F41, and F513 and the disease-causing amino acid substitutions after an analysis of all possible rotamers of the side chains. The same program was used to add polar and aromatic hydrogen atoms, providing the initial coordinates of the complete chain from residue 2 to 525.

Molecular dynamics (MD) simulations were performed using GROMACS 3.1.4, with the *ffgmx* force field (Lindhal et al. 2001). The simulations were performed as described elsewhere (Stella et al. 1999), except for the following details. After energy minimization in vacuo, the protein was centered in a triclinic box (6.4 × 8.5 × 8.7 nm) and was hydrated using the simple point charge water model (Berendsen et al. 1981), maintaining the water molecules included in the crystallographic structure and located within 0.5 nm of the protein. The final fully hydrated system contained >13,000 water molecules. Initial strains in the system were released by a two-step energy minimization and a 100-ps MD simulation with protein atoms (except those belonging to mutated or modeled residues) position restrained. The system was kept at a constant temperature (300 K) and pressure (1 bar) by the Berendsen weak-coupling method (Berendsen et al. 1984), with the use of separate temperature baths for protein and solvent and with a relaxation time of 0.1 ps for temperature and 1 ps for pressure. Nonbonded interactions were treated in accordance with the twin-range method (cut-off radii of 1 nm and 1.5 nm).

The simulated trajectory was 5.3 ns long in the case of mutant E76K and 9.8 ns long for mutants E76D, A72V, and A72S. Two simulations were performed for the wild-type protein, differing for the random initial velocities: 5.3 ns long (indicated as WT_s) and 9.8 ns (WT_l). These calculations were performed on the computer cluster of the E. Fermi Research Center of Rome (32 Pentium IV nodes interconnected through Myrinet 2000).

Molecular graphics and solvent-accessible surface calculations were performed with MOLMOL software (Koradi et al. 1996). H-bonds were assigned in accordance with standard GROMACS criteria. Root mean square positional deviations (RMSD) were calculated according to the following definition:

$$\mathrm{RMSD}(t) = \sqrt{\frac{1}{N}\sum_{i=1}^{N}[r_i(t) - r_i(0)]^2}\ ,$$

where the summation runs over the N atoms of the N-SH2 loop (residues 58–62), $r_i(t)$ is the positional vector of atom i at time t, and $r_i(0)$ is its position in the initial structure (Stella and Melchionna 1998). Translational and rotational motions of the protein were removed by fitting the positions of the PTP signature motif atoms (residues 457–467) to their coordinates in the initial structure. In this way, the differences in the RMSD values were due to both the internal conformational changes of the N-SH2 loop and the relative motions between this loop and the PTP active site.

Results

Diversity of Germline and Somatic PTPN11 Mutations

Heterozygous germline *PTPN11* mutations were identified in 204 individuals with NS or LS and constituted 47 molecular lesions, including six novel defects (table 2). None of the mutations was observed in 300 unaffected individuals of European descent. Parental DNA specimens were available for 47 families with both unaffected parents and for 17 families transmitting the disorder. In these cases, testing of DNAs demonstrated either the de novo origin of the mutation (including the previously unreported 179delGTGAinsT and 184T→A) or cosegregation with disease (including the novel 802G→A and 854T→G). Parental DNA specimens were not available to confirm the de novo origin of the novel 172A→C and 802G→T changes, but both of the affected residues (N58 and G268) are mutated in other NS cases, supporting a pathogenic role for the variants. Consistent with our previous data, the vast majority of NS-causative mutations were missense and preferentially affected exons 3 and 8.

The present data and available published records (updated to August 2005) were utilized to analyze the diversity of germline mutations and to classify them on the basis of their predicted effect on protein function. SHP-2 switches between inactive and active conformations, depending on its binding to phosphotyrosyl (pY)–containing signaling partners. In the unliganded inactive conformation, the N-SH2 domain interacts extensively

Table 2

List of Germline *PTPN11* Mutations Identified in 204 Subjects with NS or LS

Predicted Amino Acid Change	No. of Cases	Nucleotide Substitution	Exon	Domain
T2I	1	5C→T	1	N-SH2
T42A	3	124A→G	2	N-SH2
N58H	1	172A→C[a]	3	N-SH2
N58D	1	172A→G	3	N-SH2
N58K	1	174C→G	3	N-SH2
G60A	4	179G→C	3	N-SH2
G60V+delD61	1	179delGTGAinsT[a]	3	N-SH2
D61N	5	181G→A	3	N-SH2
D61G	9	182A→G	3	N-SH2
Y62N	1	184T→A[a]	3	N-SH2
Y62D	4	184T→G	3	N-SH2
Y63C	12	188A→G	3	N-SH2
A72S	9	214G→T	3	N-SH2
A72G	6	215C→G	3	N-SH2
T73I	3	218C→T	3	N-SH2
E76D	7	228G→C	3	N-SH2
E76D	1	228G→T	3	N-SH2
Q79R	9	236A→G	3	N-SH2
D106A	2	317A→C	3	Linker
E110A	1	329G→C	3	Linker
E139D	8	417G→C	4	C-SH2
E139D	2	417G→T	4	C-SH2
Q256R	2	767A→G	7	PTP
G268S	1	802G→A[a]	7	PTP
G268C	1	802G→T[a]	7	PTP
Y279C	10	836A→G	7	PTP
Y279S	1	836A→C	7	PTP
I282V	4	844A→G	7	PTP
F285C	1	854T→G[a]	8	PTP
F285S	2	854T→C	8	PTP
N308D	40	922A→G	8	PTP
N308S	13	923A→G	8	PTP
N308T	2	923A→C	8	PTP
I309V	1	925A→G	8	PTP
G464A	1	1391G→C	12	PTP
T468M	14	1403C→T	12	PTP
P491S	2	1471C→T	13	PTP
P491L	2	1472C→T	13	PTP
S502A	1	1504T→G	13	PTP
S502T	2	1504T→A	13	PTP
S502L	1	1505C→T	13	PTP
G503R	2	1507G→A	13	PTP
G503R	3	1507G→C	13	PTP
M504V	2	1510A→G	13	PTP
Q506P	2	1517A→C	13	PTP
Q510E	2	1528C→G	13	PTP
Q510P	1	1529A→C	13	PTP

[a] Novel mutation.

with the PTP domain, blocking the active site (Hof et al. 1998). Binding of an exposed pocket of the N-SH2 to a pY-containing peptide promotes a conformational change of the domain, leading to the disruption of the N–SH2/PTP interaction and to the activation of the phosphatase. According to this allosteric mechanism of activation and the crystallographic structure of the protein, and taking into account conserved structural and functional features of SH2 (Bradshaw and Waksman 2002) and PTP (Andersen et al. 2001) domains, the *PTPN11* germline mutations (62 distinct amino acid changes or small inframe deletions documented in 573 subjects; see table 1) were classified into six major groups (table 3 and fig. 2*A*). Group I included lesions affecting residues located in or close to the N–SH2/PTP–interacting surface with no direct role in catalysis (T2, N58, G60, D61, Y62, Y63, E69, F71, A72, T73, E76, Q79, and Q256). Such lesions are predicted to perturb the equilibrium between the inactive and active conformation of the protein. The next two groups included changes affecting surface-exposed PTP residues contributing to the stability of the catalytically inactive conformation but also participating in catalysis (group II: Y279, I282, A461, and G464 at the pY-recognition site; Q506 and Q510 in the Q loop) or controlling substrate specificity (group III: G502 and G503). These mutations are predicted to perturb SHP-2 switching between its catalytically inactive and active conformation and/or its catalytic activity/substrate specificity. Group IV included mutations affecting residues not contributing significantly to the N–SH2/PTP interaction nor to the catalytic function directly but either with a role in maintaining the overall PTP structure (F285, N308, I309, and P491) or interacting with residues participating in catalysis (G268, T468, R498, R501, and M504). Group V included mutations affecting residues located at the phosphopeptide-binding cleft of each SH2 domain (A42, L43, and E139), which are implicated in the intermolecular interactions of the protein with its signaling partners and which control SHP-2 translocation and activation. Mutations affecting these pockets are predicted to perturb phosphopeptide-binding specificity and/or affinity. Finally, group VI included lesions affecting residues located in the linker stretch connecting the N-SH2 and C-SH2 domains (D106 and E110). These residues interact with residues of both SH2 domains and are pre-

Table 3

Classification and Relative Distribution of Germline and Somatic *PTPN11* Mutations

Mutation Group	Predicted Effect on SHP-2 Function[a]	Germline Origin ($N = 573$)		Somatic Origin ($N = 256$)	
		N	%	*N*	%
I	A/I switching	243	*42.4*	217	*84.8*
II	A/I switching and catalysis	66	*11.5*	3	*1.2*
III	A/I switching and specificity	27	*4.7*	27	*10.5*
IV	A/I switching and/or catalysis	195	*34.0*	4	*1.6*
V	SH2 pY-binding	28	*4.9*	5	*1.9*
VI	SH2 orientation or mobility	12	*2.1*	…	…
Others	…	2	*.4*	…	…

[a] A/I = Active/inactive conformation.

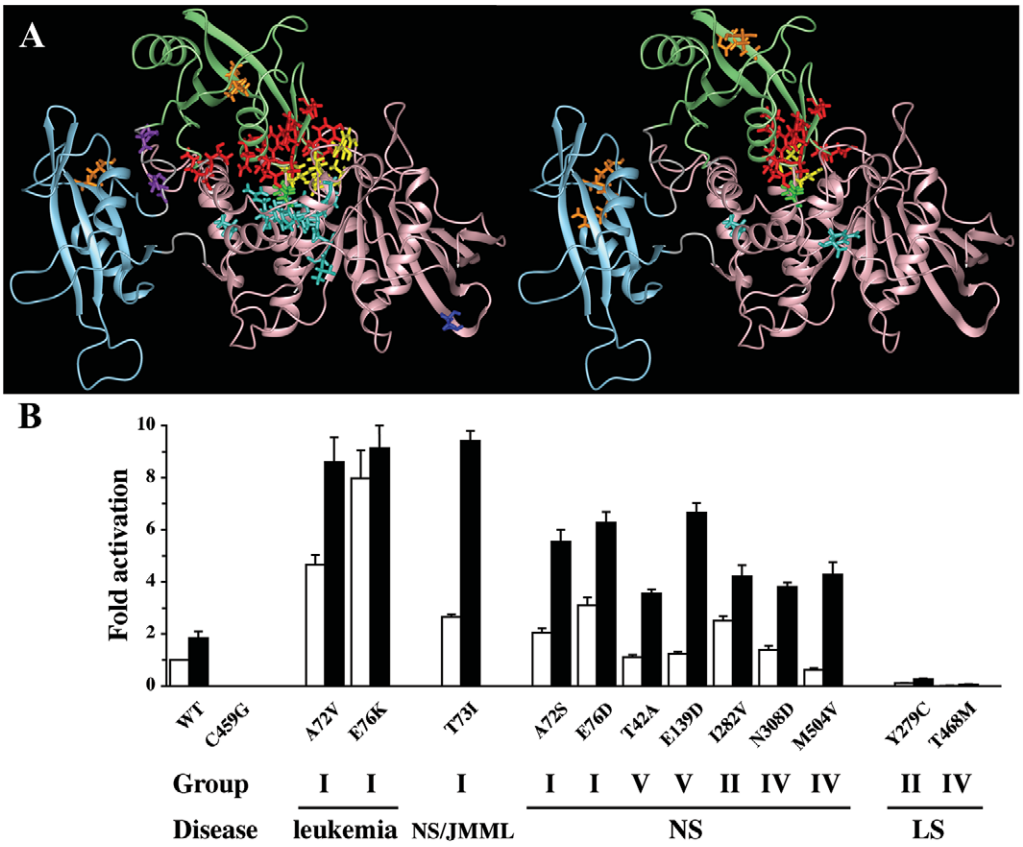

Figure 2 Germline and somatic *PTPN11* mutations in human disease. *A*, Location of mutated residues in the three-dimensional structure of SHP-2 in its catalytically inactive conformation (*green*, N-SH2 domain; *cyan*, C-SH2 domain; *pink*, PTP domain). Residues affected by germline (*left*) or somatically acquired (*right*) mutations are shown with their lateral chains colored according to the proposed classification (*red*, group I; *yellow*, group II; *green*, group III; *cyan*, group IV; *orange*, group V; *violet*, group VI; *blue*, unclassified). *B*, In vitro phosphatase assay of wild-type and mutated SHP-2 proteins. The C459G mutant is a catalytically inactive protein used as negative control. Activity was measured as picomoles of phosphate released, with the use of pNPP as substrate in basal (*white bars*) and BTAM peptide-stimulated (*black bars*) conditions. Values are means ± SDs of at least four independent experiments and are normalized to unstimulated wild-type SHP-2.

dicted to alter their relative orientation or mobility. Two mutations, T411M and L560F, did not fit rationally into any of the groups. T411 maps onto the PTP surface but resides far from the N–SH2/PTP–interacting region and active site. No structural data are available for residue L560, which maps within the proline-rich domain in the C-terminal tail.

Mutational screening of bone marrow specimens from 303 pediatric or adult subjects with hematologic malignancies identified 36 subjects with *PTPN11* mutations constituting 16 distinct lesions, including 2 that were previously unreported (table 4). All mutations affected exons 3 or 13 and were missense defects. With the exception of a 1507GG→CT dinucleotide change identified in a single AML case, all mutations were single-nucleotide substitutions. Analysis of available DNAs from bone marrow samples obtained during disease remission demonstrated absence of the mutated allele in all cases, providing evidence that mutations were somatic events acquired in the leukemic clones. None of these defects was observed in control DNAs.

The vast majority of somatic mutations associated with malignancies (see table 1) altered group I residues (N58, G60, D61, Y63, E69, F71, A72, T73, E76, and L77) (table 3 and fig. 2*A*). Among the remaining lesions, nine different changes affected group III residues (S502 and G503), whereas only a few uncommon amino acid substitutions involved group II (T507 and Q510), group IV (R289 and P491), or group V (T52, H53, R138, and E139) residues.

Genotype-Phenotype Correlation

The present data and available published records (see table 1) were utilized to explore genotype/phenotype relationships. Indeed, a correlation between individual amino acid substitution and disease was observed. Specifically, two-thirds of the residues altered by one or

Table 4

List of Somatic *PTPN11* Mutations Identified in 36 Subjects with Hematologic Malignancies

Predicted Amino Acid Change	No. of Cases	Nucleotide Substitution	Exon	Domain
D61Y	4	181G→T	3	N-SH2
D61V	3	182A→T	3	N-SH2
A72T	4	214G→A	3	N-SH2
A72V	2	215C→T	3	N-SH2
T73I	2	218C→T	3	N-SH2
E76K	6	226G→A	3	N-SH2
E76Q	2	226G→C	3	N-SH2
E76G	5	227A→G	3	N-SH2
E76A	1	227A→C	3	N-SH2
E76V	1	227A→T	3	N-SH2
L77V	1	229T→G[a]	3	N-SH2
S502T	1	1504T→A	13	PTP
G503R	1	1507G→A	13	PTP
G503L	1	1507GG→CT[a]	13	PTP
G503E	1	1508G→A	13	PTP
G503A	1	1508G→C	13	PTP

[a] Novel mutation.

more *PTPN11* mutations were associated only with germline (NS or LS) or somatically acquired (malignancies) disorders. Among these amino acid residues, a relative few (T42, Y62, Q79, D106, Y279, I282, F285, N308, T468, and M504) constituted ~50% of the total germline events. Among the one-third of residues for which substitutions were associated with both classes of disorders, there was almost complete specificity between the precise amino acid substitution and disease. Exceptions to this were the G60A, D61N/G, Y63C, E139D, S502T, and G503R substitutions, which showed an unambiguous predominance of germline origin (135/573 vs. 9/256; $\chi^2 = 49.53$, $P < .001$), and the F71L (1/573 vs. 4/256), T73I (21/573 vs. 6/256), P491S/L (8/573 vs. 3/256), and S502A/L (4/573 vs. 3/256) changes. Significantly, T73I had been identified rarely in subjects with NS without JMML (~2% of cases), while it represented the most common lesion among infants and young children with NS associated with this myeloproliferative disease (~50% of cases) (Kratz et al. 2005). These observations indicate that subjects carrying the T73I substitution are at risk for developing myeloproliferative disorders. Since the myeloproliferative disorder observed in infants and children with NS may regress without treatment (Bader-Meunier et al. 1997; Fukuda et al. 1997; Choong et al. 1999; Yoshida et al. 2004*b*; Jongmans et al. 2005), these data also suggest that a transient myeloproliferative condition might represent an unrecognized feature in young subjects with NS carrying this amino acid change. Overall, the accumulated genetic data strongly support the idea that the germline-transmitted *PTPN11* mutations have different effects on development and hematopoiesis than those acquired somatically.

Biochemical Analysis

To explore the consequences of germline (NS- and LS-associated) and somatic (leukemia-associated) *PTPN11* mutations on SHP-2 function, wild-type and 12 mutant SHP-2 proteins were expressed in bacteria, and their phosphatase activities were compared basally and after stimulation with BTAM peptide (fig. 2*B*). The T42A, A72S, E76D, E139D, I282V, N308D, and M504V mutations were selected to represent germline defects recurrent in NS and belonging to different mutation groups (see above). The A72V and E76K changes are among the most common lesions specifically associated with leukemia and were selected for direct comparison with the NS-causing A72S and E76D lesions, respectively. Y279C and T468M were included as representative for LS, since they account for the majority of those cases. Finally, the T73I change was included because of its specific association with NS/JMML (see above). Overall, these mutants can be considered as representative of the majority of germline and somatic SHP-2 defects identified in human disease thus far.

Under basal conditions, recombinant wild-type SHP-2 exhibited a relatively low catalytic activity. Addition of SH2 domain-binding peptide (phosphorylated PTPNS1 BTAM motif) (O'Reilly et al. 2000) promoted a twofold increase in substrate dephosphorylation. The leukemia-associated mutants, A72V and E76K, exhibited highest phosphatase activities in both basal and stimulated conditions. E76K showed the highest basal activation (ninefold higher than wild-type SHP-2), whereas A72V and E76K activities were comparable after stimulation with BTAM peptide. Similarly, the NS-causative mutants from group I (A72S and E76D) and group II (I282V) were basally activated. A lower but statistically significant higher basal phosphatase activity was also documented for the N308D mutant (group IV) compared with the wild-type protein (unpaired *t* test, $P < .05$). All of these mutants also exhibited a statistically significant higher phosphatase activity after stimulation (in all comparisons, $P < .001$). Of note, the phosphatase activities of these four NS-causative mutants were significantly lower than those of the leukemia-associated mutants basally ($P < .01$, in all comparisons) as well as after activation with BTAM peptide ($P < .05$, in all comparisons). The NS/JMML mutant, T73I, showed a basal catalytic activity within the range of those observed for the four NS mutants but after stimulation displayed a dramatic increase in phosphatase activity that was comparable to that observed in leukemia-associated mutants. Both of the group V mutants, T42A and E139D, exhibited basal activities comparable to those observed for wild-type SHP-2 but were more activated after BTAM stimulation (approximately twofold and fourfold higher, respectively, than the wild-type protein). Increased activation after stimulation was also

observed for the M504V mutant (group IV), which, however, exhibited lower basal catalytic activity than wild-type protein ($P < .01$ for both comparisons). Remarkably, extremely low phosphatase activities were documented for the LS-associated Y279C (group II) and T468M (group IV) mutants in both basal and stimulated conditions. Of note, both the Y279C and T468M SHP-2 proteins appeared to be responsive to BTAM stimulation ($P < .001$ for both comparisons). A similar behavior for these mutants was also observed in phosphatase assays using SHP-2 immune complexes obtained from transiently transfected COS1 cells (fig. 3).

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 3 Impaired phosphatase activity of the Y279C and T468M SHP-2 mutants expressed in COS1 cells. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

MD Simulations

To investigate the effects of the A72S, A72V, E76D, and E76K changes on SHP-2 structure, we performed MD simulations. The N-SH2 loop (residues 58–62) has a major role in controlling SHP-2 catalytic activation, by blocking the active site (Hof et al. 1998). Remarkably, both the leukemia-associated A72V and E76K mutants displayed a pronounced displacement of this loop from its initial position in the crystallographic structure of the inactive conformation (fig. 4*A*). A quantitative measure of this motion is provided by the RMSD of N-SH2 loop atoms from their starting coordinates (fig. 4*B*). In contrast, simulations relating with the NS-causing A72S and E76D mutants did not show any significant difference from the trajectories observed in the wild-type protein during the 10-ns time range accessible to our simulations. The N-SH2 loop displacement observed for the leukemia-associated mutants was mainly due to a collective motion of the regulatory domain with respect to the active site rather than due to a local rearrangement of the N-SH2 structure. No effect of the mutations on the overall conformation of this domain nor on the structure of the phosphopeptide-binding pocket was observed (data not shown). In the case of the E76K protein, the mutation changed an attractive electrostatic interaction between this residue and the catalytic domain into a repulsive strain, which slowly relaxed during the simulated trajectory as the N-SH2 and PTP domains drifted apart. This interdomain motion caused a significant increase in solvent-accessible surface of the PTP active site (fig. 4*C*). In the case of the A72V protein, the mutation induced an unfavorable steric repulsion between V72 and residues I282, I463, S502, G503, and Q506 of the PTP domain. Relaxation of this strain caused a rearrangement of the interdomain orientation and a consequent displacement of the N-SH2 loop from its position. This structural change resulted in a modification of the pattern of interactions between this loop and the PTP domain, illustrated in figure 4*D* by the loss of the hydrogen bonds connecting residues N58-Q506, G60-Q510, and A72-Q506 during the simulated trajectory. Remarkably, neither the E76K nor the A72V mutation induced any intradomain stress (data not shown), which explains the lack of significant structural perturbations in the N-SH2 domain.

Discussion

In the present article, we provided a more-complete assessment of the diversity of germline and somatically acquired *PTPN11* lesions, developed a classification of mutations based on the predicted role of affected residues, evaluated their associations with disease, and investigated their structural and functional consequences on SHP-2 function. In combination with data from previous surveys, our findings indicate that oncogenic and NS/LS–causing *PTPN11* mutations are almost always missense changes, although small inframe deletions affecting N-SH2 residues exposed towards the PTP surface occur in a small number of cases. Available records based on 829 germline and somatic defects revealed a complete absence of nonsense, frameshift, and splicing defects in NS or in contributing to oncogenesis. Moreover, this study strongly indicated that specificity in the amino acid substitution is relevant to the functional deregulation of SHP-2 and disease pathogenesis. Specifically, an invariant amino acid change is observed for several residues (T42, Y63, T73, D106, E139, I282, T468, M504, and Q506; approximately one-fourth of total events), suggesting a specific role for the substituted residue. Similarly, particular amino acid substitutions were specifically or preferentially associated with NS, LS, or malignancy, as observed for lesions involving T42, N58, G60, D61, Y62, Y63, E69, A72, E76, Q79, D106, E139, Q256, Y279, I282, F285, D308, T468, G503, M504, Q506, and Q510. On the other hand, the identity of substitution does not seem to be critical in a few cases, suggesting a crucial role in the function of SHP-2 for the amino acid residue being replaced (Y62, Q79, Q256, G268, Y279, F285, N308, and R498).

Comparison of the molecular spectrum peculiar to the germinally transmitted and somatically acquired *PTPN11* mutations confirmed previous data from our group and from others indicating a clear-cut genotype/phenotype correlation (Tartaglia and Gelb 2005*a*). Indeed, the currently available data indicate that leukemia-associated mutations rarely occur as germline events in NS or LS, suggesting that these lesions might either be

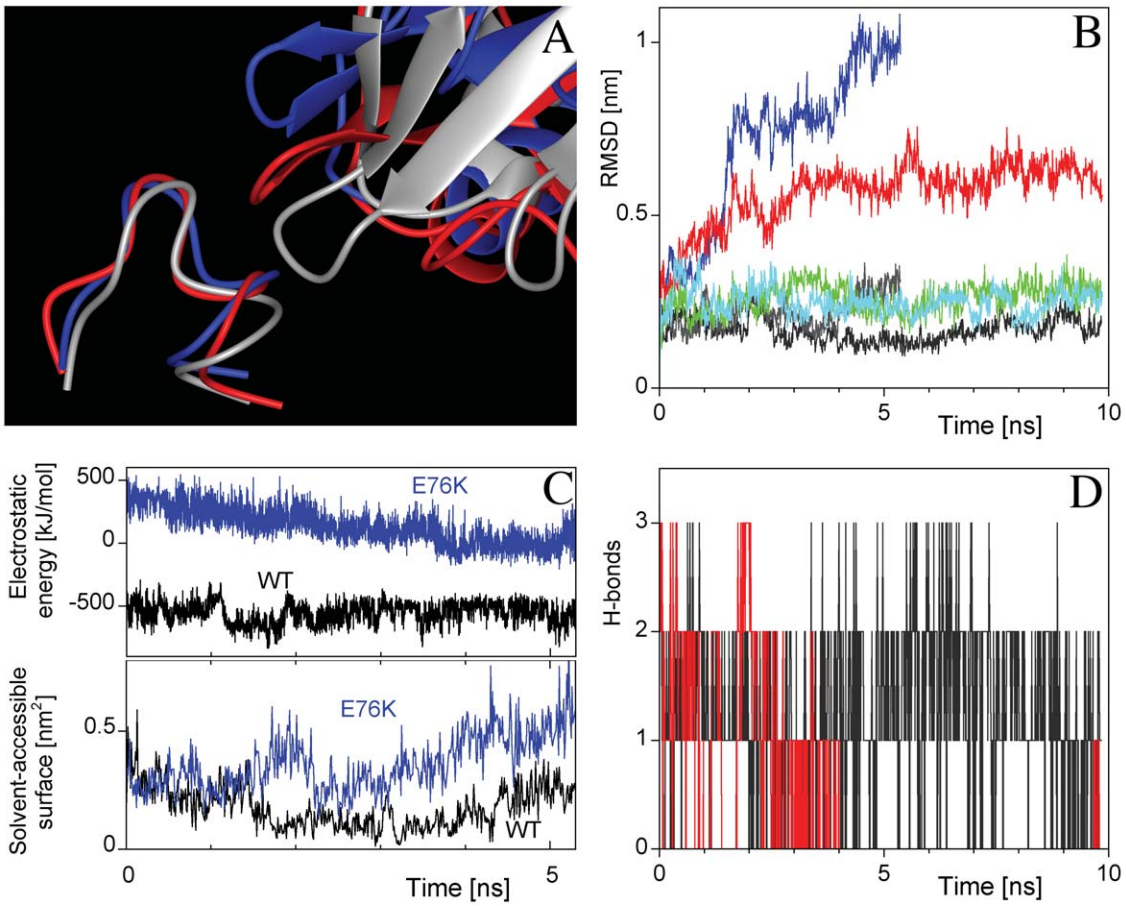

Figure 4 MD simulations. *A,* Comparison between the crystallographic structure of SHP-2 (*gray*) and the conformation attained at the end of simulations by the E76K (*blue*) and A72V (*red*) SHP-2 mutants. The N-SH2 domain and the signature motif of the PTP domain (residues 457–467) are depicted with a ribbon representation. *B,* RMSD of the N-SH2 loop (residues 58-62) from its starting position. Translational and rotational motions of the protein were removed by fitting the positions of the PTP signature motif atoms to their coordinates in the initial structure. A72V (*red*), A72S (*green*), E76K (*blue*), E76D (*cyan*), and wild-type (*light* and *dark gray*) SHP-2 proteins. *C,* Electrostatic energy between residue 76 and the PTP domain (*upper panel*) and solvent-accessible surface area of the PTP signature motif (*lower panel*) during simulations with the E76K (*blue*) and wild-type (*black*) SHP-2 proteins. *D,* Time behavior during simulations of the H-bonds between residues N58-Q506, G60-Q510, and A72-Q506, contributing to stabilize the interaction between the N-SH2 loop and the PTP active site, in the A72V (*red*) and wild-type (*black*) SHP-2 proteins.

associated with clinically distinct developmental disorders or severely affect fetal development. Since fetal lethality is documented in NS, we prefer the latter hypothesis and predict a higher prevalence of leukemia-associated mutations among miscarried fetuses with features suggestive of NS. Moreover, consistent with the observation that JMML, ALL, and AML represent uncommon complications in NS, NS-causative *PTPN11* mutations are rarely observed as somatic events contributing to leukemia. Mutation data also indicate an overlap with respect to the substitutions occurring in subjects with NS or NS/JMML, as recently discussed by Kratz et al. (2005). Of note, the clinical course of the myeloproliferative disorder in NS/JMML tends to be relatively benign compared with isolated JMML (Bader-Meunier et al. 1997; Fukuda et al. 1997; Choong et al. 1999), supporting the hypothesis that these mutations have a milder effect on myeloid precursor cell proliferation. Such a genotype/phenotype correlation supports a model in which distinct gain-of-function thresholds for SHP-2 might be required to induce cell-, tissue-, or developmental-specific phenotypes, each depending on the transduction network context involved in the phenotype (Tartaglia et al. 2003). The accumulated mutation data, however, demonstrate that a few amino acid changes can occur in both NS and isolated hematologic malignancies. These mutations appear to be recurrent among subjects with NS, whereas they are extremely rare in leukemias. Exceptions to this rule are represented by the T73I, P491S/L, and S502A/L changes, which are almost

equally represented within the two groups. Although functional studies are required to understand the biological relevance of these lesions in leukemogenesis, it is possible that these mutations might contribute differentially to distinct malignancies, as observed for the P491S/L changes in ALL (Tartaglia et al. 2004*a*), or that they might occur as a late event in the leukemic clone, conferring merely an additional proliferative advantage in a leukemic subpopulation (Tartaglia et al. 2005).

In both developmental disorders and malignancies, the distribution of the altered amino acid residues in SHP-2 had a nonrandom pattern. According to the crystallographic structure of SHP-2 and the proposed mechanism of activation, affected residues were clustered in six major groups. Phosphatase assays strongly supported a gain-of-function role for a panel of mutations that can be considered representative of the majority of NS-causative and leukemia-associated lesions and confirmed previous biochemical and functional observations indicating a more-activating effect associated with the latter (Tartaglia et al. 2003; Keilhack et al. 2005; Mohi et al. 2005; Shubbert et al. 2005).

MD simulations provided, for the first time, direct evidence supporting the hypothesis that mutations leading to strong basal activation, as observed among the leukemia-associated mutants, perturb the interaction between the N-SH2 and PTP domains, and they described the molecular interactions leading to the displacement of the N-SH2 loop from the PTP active site. Such structural rearrangements were not observed for the NS-causing A72S and E76D mutants. One limitation of this approach was that the simulated trajectories were necessarily much shorter than the time scale of interdomain motions (because of the relatively large size of SHP-2), so that the conformations attained by the mutants during the simulations were not equilibrium structures. Therefore, the absence of observable effects for the A72S and E76D SHP-2 proteins does not necessarily imply a lack of structural effects of these lesions but simply indicates that the N-SH2 loop displacement is less favored, suggesting a lower potency of these lesions, a finding that was in agreement with the activity data.

We predicted a possible negative effect on catalysis for some of the mutations affecting residues located in or close to the active site (groups II and IV). Biochemical data confirm such a prediction for the two common LS-causing Y279C and T468M substitutions. T468 is adjacent to the catalytic PTP-loop (residues 454–467), and it is relatively conserved (40% identity) in human PTP domains (Andersen et al. 2001). Since it is buried in a densely packed region, the steric strain caused by the T468M substitution would cause a rearrangement of the region, including the catalytic loop. In the case of Y279C, the comparison with the I282V mutant is illuminating, since these group II mutations have opposite effects on the activity of SHP-2. Both Y279 and I282 contribute to the hydrophobic region binding the pY-phenyl ring and interact with N-SH2 domain residues. Consequently, loss of these residues would cause loss of affinity for the substrate and perturbation of the interaction between the N-SH2 and PTP domains. Sequence comparison of human PTP domains (Andersen et al. 2001) showed, however, that Y279 is strongly conserved (80% identity), with cysteine never observed in that position. In contrast, isoleucine and valine are almost equally represented in position 282 along the sequence of human PTP domains (40% and 30%, respectively). Therefore, whereas Y279C strongly impaired catalysis, the I282V mutation did not substantially perturb the affinity for the phosphopeptide substrate. In the I282V mutant, protein activation by N-SH2 dissociation prevailed.

By demonstrating that a distinct perturbing role on SHP-2 function must be ascribed to the recurrent Y279C and T468M mutations, the present study identified a previously unrecognized behavior for a SHP-2 mutant. The adverse biochemical consequences on catalytic activity of the Y279C and T468M substitutions would seem to imply loss of function in the pathogenesis of LS. Of note, no developmental defect was observed in mice heterozygous for a Shp-2 mutant unable to bind to intracellular signaling partners (*Shp-2*$^{\Delta 46-110}$) (Saxton et al. 1997) or hemizygous for Shp-2 (*Shp-2*$^{+/-}$) (Arrandale et al. 1996). Moreover, no lesion that would be expected to eliminate SHP-2 (e.g., nonsense or frameshift mutation near the N-terminus) has been observed in LS. This genetic observation, together with the observed responsiveness of the Y279C and T468M mutants to BTAM peptide, suggest that the pathogenesis of LS does not derive simply from haploinsufficiency. Rather, the available data would be more consistent with a dominant negative mechanism. Clearly, additional functional studies and a gene knock-in approach are required to elucidate more fully the effects of these mutations on protein function and to evaluate if these mutants are defective in signaling or interfere with normal SHP-2 function.

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Web Resources

URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/Omim/ (for *PTPN11*, NS, LS, and JMML)

Protein Data Bank (PDB), http://pdbbeta.rcsb.org/pdb/ (for SHP-2 crystal structure [2shp])

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