Diverse Biochemical Properties of Shp2 Mutants

IMPLICATIONS FOR DISEASE PHENOTYPES*

Heike Keilhack‡, Frank S. David‡, Malcolm McGregor**, Lewis C. Cantley‡‡, and Benjamin G. Neel§§

From the †Cancer Biology Program, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, the ‡Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02115, **Serono Reproductive Biology Research Institute, Cancer Biology, Rockland, Massachusetts 02370, and the §§Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

Mutations in the Src homology 2 (SH2)-containing protein-tyrosine phosphatase Shp2 (PTPN11) underlie half of the cases of the autosomal dominant genetic disorder Noonan syndrome, and somatic Shp2 mutations are found in several hematologic and solid malignancies. Earlier studies of small numbers of mutants suggested that disease-associated mutations cause constitutive (SH2 binding-independent) activation and that cancer-associated mutants are more active than those associated with Noonan syndrome. We have characterized a larger panel of Shp2 mutants and find that this “activity-centric” model cannot explain the behaviors of all pathogenic Shp2 mutations. Instead, enzymatic, structural, and mathematical modeling analyses show that these mutants can affect basal activation, SH2 domain-phosphopeptide affinity, and/or substrate specificity to varying degrees. Furthermore, there is no absolute correlation between the mutants’ extents of basal activation and the diseases they induce. We propose that activated mutants of Shp2 modulate signaling from specific stimuli to a subset of effectors and provide a theoretical framework for understanding the complex relationship between Shp2 activation, intracellular signaling, and pathology.

The nonreceptor protein-tyrosine phosphatase Shp2, encoded by the PTPN11 gene, is a signal-enhancing component of growth factor, cytokine, and extracellular matrix receptor signaling and plays an important role in regulating cell proliferation, differentiation, and migration. Biochemical and genetic evidence place Shp2 upstream of Ras; accordingly, in the absence of functional Shp2, Erk activation is impaired or absent (1). Shp2 can also function in several other downstream signaling cascades, although its roles in these pathways are receptor- and/or cell context-dependent (1).

Recent studies implicate Shp2 in human disease (1–5). Shp2 mutations cause ~50% of cases of Noonan syndrome (NS) (3, 6–8), an autosomal dominant disorder characterized by facial abnormalities, proportional short stature, and cardiac defects (9, 10), as well as increased propensity for developing certain types of leukemia (11, 12). Somatic Shp2 mutations are found in ~35% of sporadic juvenile myelomonocytic leukemia (2, 4, 13), 5–10% of childhood myelodysplastic syndrome (2, 4), 7% of B-cell precursor acute lymphoblastic leukemia (14), up to 5% of pediatric and adult acute myelogenous leukemia (4, 15, 16), and some solid tumors (16).

Shp2 contains two SH2 domains at its N terminus (N-SH2 and C-SH2, respectively), a central protein-tyrosine phosphatase (PTP) domain and a C-terminal tail containing tyrosyl phosphorylation sites and a proline-rich motif. PTP activity appears to be required for all biological functions of Shp2, whereas the C-terminal phosphorylation sites play a modulatory role in some signaling pathways (1, 17). Shp2 binds via its SH2 domains to specific tyrosyl phosphorylation sites on some receptor tyrosine kinases, scaffolding adapters, and so-called “inhibitory receptors” (1). Its basal catalytic activity is low, but upon the addition of mono- or bis-Tyr(P) peptides that bind its SH2 domain(s), Shp2 is activated (3–10-fold or 40-fold, respectively) (18, 19). Structural studies reveal that in the basal state, the “backside loop” of the N-SH2 domain (i.e. the side opposite to its Tyr(P) peptide binding site) wedges into and blocks the PTP domain (20). Binding of a Tyr(P) peptide alters the conformation of the N-SH2 (20, 21) and releases the PTP domain from inhibition.

Several Shp2 mutations found in NS and/or sporadic leukemia (e.g. D61G, D61Y, E76D, and E76K) affect residues on the N-SH2/PTP domain interface important for autoinhibition (20) and cause biochemical and biological activation (22). These data, the autosomal dominant inheritance of NS, and the heterozygosity of Shp2 mutations in leukemia have led to the idea that disease-associated Shp2 mutants are “activated.” Although molecular modeling (3) and enzymatic studies of selected Shp2 mutants (4, 23) support this hypothesis, other disease-associated mutations map away from the N-SH2/PTP domain interface, making it unclear how (or if) they activate Shp2. Furthermore, whereas at least some leukemia-associated mutants appear to be more activated than

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§§ To whom correspondence should be addressed: Cancer Biology Program, Dept. of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-2823; Fax: 617-667-0610; E-mail: bneel@bidmc.harvard.edu.

The abbreviations used are: NS, Noonan Syndrome; PTP, protein-tyrosine phosphatase; GST, gluthathione S-transferase; RCML, reduced carboxymethylated lysozyme; WT, wild type; SH2, Src homology 2. This paper is available on line at http://www.jbc.org

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Enzymology of Disease-associated Shp2 Mutants

TABLE I

| Mutant | $K_0$ (derived) $(-$fold change in parentheses$)$ | $K_0$ (calculated) | $K_0$ | $K_i$ | $K_i$ | $K_i/K_0$ $(-$fold change in parentheses$)$ | $R^2$ |
|--------|-------------------------------------------------|--------------------|-------|------|------|---------------------------------|-------|
| WT     | 0.0308 ($1$)                                    | 0.0291             | 0.690 | 2.38 | 10.85 | 3.44 ($1$)                       | 0.994 |
| T42A   | 0.104 ($3.4$)                                   | 0.0689             | 0.198 | 3.09 | 10.85 | 15.6 ($4.3$)                     | 0.994 |
| D61G   | 0.402 ($12.1$)                                  | 0.357              | 0.459 | 1.85 | 10.85 | 4.93 ($1.17$)                    | 0.994 |
| D61Y   | 1.2 ($29.0$)                                    | 1.11               | 0.8238| 0.039 | 10.85 | 1.63 ($0.48$)                    | 0.586 |
| T73I   | 0.29 ($9.4$)                                    | 0.263              | 1.150 | 2.64 | 10.85 | 2.24 ($0.65$)                    | 0.986 |
| E76K   | 17.1 ($555$)                                    | 15.6               | 5.691 | 1.95 | 10.85 | 0.34 ($0.10$)                    | 0.833 |
| D106A  | 0.0406 ($1.3$)                                  | 0.0399             | 0.448 | 3.09 | 10.85 | 6.89 ($2.00$)                    | 0.999 |
| E139D  | 0.0711 ($2.3$)                                  | 0.0623             | 0.293 | 3.79 | 10.85 | 12.94 ($3.76$)                   | 0.999 |

$^a$ Compared with WT.

$^b$ $K_i/K_0$, relative affinity of Tyr(P) peptide for the open and closed states, respectively.

NS mutants (4, 23), it is unclear whether this is a general property of cancer-associated Shp2 mutants. Initial studies failed to reveal strong genotype-phenotype correlations in NS (6, 7, 24, 25), but differences in the biochemical properties of individual NS-associated mutants could lead to distinct phenotypic effects that may have been obscured by the genetic diversity of the human population.

To address these issues, we analyzed the enzymatic properties of 12 disease-associated Shp2 mutants. Our biochemical analysis, together with kinetic and structural modeling studies, demonstrates that individual mutations can affect basal activity of the enzyme, the binding properties of its SH2 domains, and/or its substrate specificity. These results suggest that pathologic mutations in Shp2 exert their effects through multiple mechanisms and provide a framework for understanding how they cause NS and cancer.

MATERIALS AND METHODS

Expression Constructs—The modified pGEX-4T-2 vector was generated by digesting the parental vector with EcoRI and NotI. A synthetic oligonucleotide 5′-AGGAATTCAGATGGCGGCTCAAAGCACGATGCGACGAGGGCGCCGCGCAGACCTAGAGCAGCTACAAAGCAGCAGCAAGTGAGCGGCCGCGCAG-3′ was designed to contain the last two codons for Shp2, an AefI site for fusion with the Shp2 cDNA, codons for a 5′ glycine linker, and a FLAG tag followed by a stop codon. This oligonucleotide was annealed and cloned into pGEX-4T-2, generating pGEX-4T-2-FT. The human Shp2 cDNA (26) was digested with EcoRI and XmnI, which removes its FLAG tag followed by a stop codon. This oligonucleotide was annealed to the Shp2 cDNA and cloned into pGEX-4T-2, generating pGEX-4T-2-FT. Point mutations in Shp2 were introduced by site-directed mutagenesis kit (Bio-Rad) or the QuikChange™ mutagenesis kit (Stratagene). The N−SH2 domain was amplified by PCR using the primers 5′-AGGAATTCAGATGGCGGCTCAAAGCACGATGCGACGAGGGCGCCGCGCAGACCTAGAGCAGCTACAAAGCAGCAGCAAGTGAGCGGCCGCGCAG-3′ and 5′-ATGGCGCGCCGCTCAACAGTTCAGAGGATATTTAAGC-3′. The resultant fragment was cloned into EcoRI and NotI and cloned into pGEX-4T-1.

Protein Purification—Glutathione S-transferase (GST) fusion proteins were produced as described previously (27). All manipulations were done at 4 °C with ice-cold solutions. After elution with free glutathione, the eluate was incubated with M2 anti-FLAG-agarose (Sigma). The beads were washed three times with phosphate-buffered saline and eluted with free FLAG peptide (10 μg/ml) dissolved in PBS. Protein concentrations were determined by densitometric analysis of Coomassie-stained SDS-polyacrylamide gels, using bovine serum albumin as a standard. Purified enzymes were stored in the presence of 33% glycerol at −80 °C. For isothermal calorimetry experiments, isolated N−SH2 domains were purified as described above and dialyzed into 20 mM Hepes, pH 7.4.

PTP Assays—PTP assays using 32P-labeled reduced, carboxymethylated lysozyme (RCML) as substrate were conducted as described previously (19). To generate radiolabeled RCML, a reaction mix (250-μl final volume) consisting of Abl kinase buffer (New England Biolabs) containing 100 mM RCML, 1 mM ATP, and 250 μCi of [32P]ATP, 0.5 mM Na3VO4, and 5 μg of β-insulin receptor kinase catalytic domain (kindly provided by CEPTYR, Inc., Bothell, WA) was incubated at room temperature overnight. Incorporation efficiency (typically greater than 80%) was determined by spotting 1 μl of the reaction on P81 paper, washing three times with 0.5% phosphoric acid, and scintillation counting. Proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 10% for 30 min on ice, followed by centrifugation for 15 min at 4 °C and resuspension of the pellet in 2 mM Tris. The resuspended pellet was applied to a 10-ml Sephadex G50 column equilibrated with 50 mM Hepes, pH 7.4, and 150 mM NaCl, and fractions containing phosopho-RCML were pooled and stored at aliquots at −80 °C. Typical specific activities of 32P-RCML were 4000 cpm/μmol. The Src529 peptide (TSTEEP(YQFGQEN) was synthesized using Fmoc (9-9-fluorenylmethoxycarbonyl) chemistry and radiolabeled to a specific activity of 3000 cpm/μmol in a reaction ml Biore containers containing 1 μl of 10 μg/ml peptide in Me2SO, 1 mM ATP, 1 mM Na3VO4, 50 μCi of [32P]ATP, and 2 μg BHK II in Abl kinase buffer (30-μl final volume) for 4 h at 30 °C. The mixture was then applied to a 0.5-ml DOWEX-IX8 column equilibrated in 30% acetic acid, and the phosphorylated peptide was eluted with 30% acetic acid and stored at −80 °C. On the day of the PTP assay, the phosphorylated peptide was lyophilized using a Speedvac concentrator and resuspended in PTP assay buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1 mM mg bovine serum albumin, 10 mM dithiothreitol, 5 mM EDTA). Monophosphoseryl (Tyr(P)) peptides p1172 (SNLpYIDLDDLK, where pY represents phosphotyrosine), p1212 (LSTpYASINFQK), and bis-Tyr(P) peptide p1172-p1222 (LNPpYIDLLDLK, where x is 6-aminoacaproic acid), which correspond to the Shp2 binding sites on IRS-1, were described previously (19, 28). Phosphorylated substrates in assay buffer alone or with varying concentrations of activating mono- or bis-Tyr(P) peptides (or control) were preincubated at 30 °C for 5 min. Assays were initiated by adding 5 μM GST-Shp2-FL (25-μl final reaction volume) and terminated after 5 min by adding 775 μl of ice-cold charcoal solution. The resulting mixtures were centrifuged for 10 min at room temperature, and 400 μl of the supernatants were analyzed by scintillation counting. All assays were carried out in the linear range of the product-time curve (under these conditions, substrate hydrolysis was linear). The percentage of phosphorylated substrate was determined by thin-layer chromatography.

Isothermal Calorimetry—Measurements were conducted using the VITC unit (Micro-Cal) as described (29). Briefly, Tyr(P)-peptide p1172 was dissolved in 20 mM Hepes, pH 7.4, at 125 μM. GST fusion proteins were added at 25 μM. Measurements were performed at a cell temperature of 25 °C, using 21 total injections with an initial delay of 60 s and a reference power of 15 μcal/s. Constants were calculated with an iteration method using the Micro-Cal application program, assuming one set of binding sites as the model.

Kinetic Modeling—A detailed description of the equation modeling the activity of Shp2 as a function of activating peptide concentration is included in the supplemental methods. The maximum activity ($V_{max}$) of Shp2 against RCML was previously determined to be 41.4 pmol/min/30 μl (30), in good agreement with our observed maximum value of 41.02 pmol/min/30 μl; we took the former value as $V_{max}$ for our studies of Shp2 domain mutants. The $K_i$ calculated (Table 1) was determined by taking the ratio of the “open” fraction (activity in the absence of ligand) to the “closed” fraction (41.02 pmol/min/30 μl minus the activity in the absence of ligand). Global curve fitting was performed to mean values using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA), constraining all constants to be $>0$ and requiring $K_i$ to be shared.

Structural Interpretation and Molecular Modeling—Crystal structures of Shp2 were analyzed using computer graphics and molecular modeling in order to interpret the mutation data in the context of molecular structure. The structures examined were as follows: (i) Protein Data Bank structure 2SHP, comprising the two SH2 domains plus the PTP domain of Shp2 (this structure corresponds to the basal, inactive form of the enzyme in the absence of a Tyr(P) peptide ligand (20)); (ii) the crystal structure of both SH2 domains of Shp2, bound to the bis-Tyr(P) peptide used in our studies (kindly provided by Dr. [Downloaded from http://www.jbc.org/ by guest on July 24, 2018]
Expression and Purification of Recombinant Shp2 Proteins—We analyzed a panel of Shp2 mutations found in NS and/or neoplasia, affecting each of the domains within Shp2 (Fig. 1, a and b) (2–4, 7, 33). Some mutations are located in the N-SH2/PTP domain interface, including the “NS-only” mutation E76D, the “leukemia-only” mutations D61Y and E76K, and three mutations found in both NS and leukemia, D61G, T73I, and Q506P. (D61G had previously been observed only in NS, but a very recent study identified a D61G mutation in a case of sporadic leukemia (33).) Although we use the term “leukemia-associated” to refer to mutations associated with hematologic neoplasms, some of these mutations also occur in solid tumors (16). Other mutations map outside the N-SH2/PTP domain interface. Residue 308, the most common residue affected in NS patients (N308D and N308S), is buried within the PTP domain. T42A, an NS mutation, is located in the Tyr(P) peptide binding pocket of the N-SH2 domain. E139D, found in NS and leukemia, lies adjacent to R138, a conserved residue critical for Tyr(P)-peptide binding to the C-SH2 domain. The NS mutation D106A is located in the linker between the N- and C-terminal SH2 domains. Finally, the NS mutation L560F resides within the proline-rich domain in the C-terminal tail, which is not included in the crystal structure of Shp2 (20). The function of the proline-rich domain in Shp2 remains unclear.

We generated doubly tagged (N-terminal GST, C-terminal FLAG) wild type and mutant Shp2 proteins, expressed them in Escherichia coli, and purified them sequentially on glutathione-agarose and an anti-FLAG antibody column (Fig. 1 c). This strategy was chosen to obtain high yields of full-length protein, since the C terminus of Shp2 is highly protease-sensitive and susceptible to degradation in bacterial lysates. All of the recombinant proteins showed similar purity (ϕH1101197%: Fig. 1 c). Similar biochemical results were obtained with recombinant proteins from which the N-terminal GST tag was cleaved by thrombin digestion, followed by repurification using the C-terminal FLAG tag (see supplemental Fig. 1).

Activity of Shp2 Mutants—Using the standard artificial substrate 32P-RCML (Fig. 2, open bars), wild type GST/FL-Shp2 (WT) showed low basal activity of ϕH110111.2 pmol/min/pmol, comparable with the previously reported activity for full-length (tag-free) recombinant Shp2 (0.5 pmol/min/pmol) assayed under similar conditions (18). Also consistent with prior results, the addition of saturating amounts of a mono-Tyr(P) peptide corresponding to tyrosine 1172 of IRS1 (p1172), a known high affinity N-SH2 ligand (19), resulted in 4–5-fold activation of WT (Fig. 2, closed bars), comparable with that reported for untagged Shp2 (ϕ10-fold).
Previous reports suggested that all disease-associated Shp2 mutants have increased activity and that leukemia-associated Shp2 mutants are more active than NS-associated mutants. This has led to a model in which higher levels of Shp2 activation are required for leukemogenesis than for NS. Indeed, several mutants conformed to the predictions of this model. The most common mutants found in leukemia, D61Y and E76K, displayed the highest levels of basal activity against RCML (Fig. 2) and para-nitrophenyl phosphate (data not shown). Neither protein was activated further upon Tyr(P) peptide addition. Two mutants associated with both NS and leukemia (D61G, T73I) were activated more than WT but less than the leukemia-only mutants, both basally and upon Tyr(P) peptide addition. The NS-only mutant N308D was also activated basally and upon p1172 addition compared with WT, but to a lower level than the leukemia- and NS/leukemia-associated mutants. Therefore, all five of these mutants displayed increased basal and stimulated activation compared with WT, and the extent of basal and stimulated activity correlated with leukemogenicity.

Other mutants, however, did not conform to a simple “activity-centric” model (Fig. 2). The NS-associated mutant T42A displayed modest basal activation, but upon stimulation, its activity was higher than that of several NS/leukemia mutants (e.g. T73I). The NS/leukemia mutant E139D, in contrast, was robustly activated by Tyr(P) peptide, but its basal activity was lower than that of some NS-only mutants (e.g. N308D). Two NS-associated mutants (E76D and D106A) were hyperactivated compared with WT, but E76D only showed increased basal activation, whereas D106A was only hyperactivated upon Tyr(P) peptide addition. Finally, two mutants (N308S, associated with NS, and Q506P, associated with NS and leukemia) were not activated more than WT, either basally or after Tyr(P) peptide addition.

Biochemical and Kinetic Modeling Studies of SH2 Domain Mutants—To further understand the “outlier” mutants, we studied their activation properties in more detail. The EC_{50} values for activation by p1172 for T42A and E139D were lower (−10- and 2-fold, respectively) than for WT (Fig. 3a). D106A had an unaltered EC_{50} but exhibited increased responses to all doses of Tyr(P) peptide. Similar results were obtained for T42A and E139D stimulated with a bis-Tyr(P) peptide containing the sequences surrounding IRS-Tyr(P)_{1172} and IRS-Tyr(P)_{1222}, separated by a flexible linker. This peptide simultaneously binds to both SH2 domains of Shp2 and is a more potent activator of WT Shp2 than mono-Tyr(P) peptides (28). Notably, D106A was not more activated than WT by the bis-Tyr(P) peptide (Fig. 3a, right), in contrast to its enhanced activation by p1172 (Fig. 3a, left) or p1222 (data not shown). The NS mutant E76D, which displayed mild (~2.5-fold) basal activation compared with WT, showed the converse response to D106A; it was resistant to further activation by mono-Tyr(P) peptide (Fig. 3b, left panel) but was stimulated by the bis-Tyr(P) peptide (Fig. 3b, right panel).

The different extents to which these mutants were activated by Tyr(P) peptides could reflect different affinities of their SH2 domains for phosphopeptide. To address this possibility, we mathematically modeled the activity of Shp2 as a function of Tyr(P) peptide concentration (Fig. 4 and supplemental methods). We defined $K_e$ as the equilibrium constant between the inactive (closed) and active (open) forms of Shp2 in the absence of Tyr(P) peptide. $K_i$ and $K_i'$ are the affinity constants for Tyr(P) peptide binding to the Shp2 N-terminal SH2 domain in the open and closed forms, respectively; for simplicity, our model assumes that only binding to the N-terminal SH2 domain affects the relative amounts of the open and closed states. In addition to binding the SH2 domains, however, Tyr(P) peptides can compete with substrate at the Shp2 active site; we termed the equilibrium constant for this reaction $K_o$. Taking these parameters into account, we expressed Shp2 activity ($A$) as follows.

$$A = V_{max} \left( \frac{K_i + [L]}{K_i'} \left( \frac{K_i + [L]}{K_i'} + 1 \right) \right) \quad \text{(Eq. 1)}$$

Because binding of a bis-Tyr(P) peptide is more complicated (e.g. binding of one Tyr(P) moiety to the C-SH2 domain enhances binding of the other to the N-SH2 domain, and vice versa), we only fit the kinetic data for the SH2 domain mutants activated by p1172 to this model. Since the PTP domains of the SH2 mutants are identical to WT, we assumed that their $K_e$ for p1172 inhibition at the active site would be the same. E76D was excluded from analysis, since it could not be activated by p1172 (Figs. 2 and 35). For WT and the remaining SH2 domain mutants except D61Y, we obtained good fits ($R^2$ from 0.833 to 0.999; see supplemental Fig. 2 and Table I). We derived $K_e = 10.85$ mM, consistent with our failure to see significant inhibition of these forms of Shp2 even at peptide concentrations up to 0.5 mM. The derived $K_i$ values agreed well with those calculated from the turnover rate in the absence of ligand and the observed $V_{max}$ (see “Materials and Methods”). $K_o$ was 0.690 μM for WT Shp2 and ranged from 0.024 to 5 μM for the mutants,
suggesting substantial variation in the affinity of the open N-SH2 domain for Tyr(P) peptide. 

\[ K_c \text{ in the micromolar range for WT and all but one of the mutants, consistent with the relative inaccessibility of the N-SH2 domain in the closed form seen in the crystal structure of Shp2 (20).} \]

Based on the fit of the model to the data, the SH2 domain mutants fell into two classes: those that activate primarily by shifting the basal equilibrium constant, \( K_c \), toward the active state (D61G, D61Y, T73I, and E76K) and those that activate by increasing the affinity of Tyr(P) peptide for the open versus closed state (T42A, D106A, and E139D; \( K_c/K_o \) in Table I). Significantly, the former mutants are in the region of the N-SH2 domain that interacts with the catalytic domain (see below), whereas the latter are in regions that are likely to affect the affinity of the SH2 domains for Tyr(P) peptide or interactions between the N- and C-SH2 domains.

To directly test the effects of an SH2 domain mutation on Tyr(P) peptide binding, we purified GST fusions of the N-SH2 domains of WT and T42A and monitored their binding to the p1172 peptide by isothermal calorimetry (Fig. 5). Indeed, the T42A N-SH2 domain bound p1172 with 6.6-fold higher affinity than WT. This result was similar to the 3.5-fold increase in affinity predicted by our modeling data, although notably, the measured affinity for p1172 binding to each isolated N-SH2 domain was 10-fold higher than the affinity for the open state of the intact protein determined by the best fit of the model to the data (see “Discussion”).

**Biochemical Studies of PTP Domain Mutants** —Kinetic analyses performed on two PTP domain mutants (N308S and Q506P) and a C tail mutant (L560F) indicated that they were activated neither basally nor by a single (high) dose of mono-Tyr(P) peptide. Dose-response studies were performed on these mutants using mono-Tyr(P) (Fig. 6a) or bis-Tyr(P) peptides as SH2 domain ligands (Fig. 6b) and 32P-RCML as the substrate. As a control, the NS mutant N308D showed 3-fold basal activation (compared with Fig. 2) and enhanced activity (compared with WT) over a wide dose range of either peptide. In contrast, N308S and Q506P were neither basally activated nor activated more than WT with either Tyr(P) peptide. On the contrary, these two mutants showed apparent inhibition of catalytic activity at lower Tyr(P) peptide concentrations than WT (more easily seen in the bis-Tyr(P) dose-response curve). This apparent inhibition of catalytic activity presumably reflects the ability of any given Tyr(P) peptide to act both as a ligand for the SH2 domain(s) and to compete with the radiolabeled RCML substrate for the active site of the enzyme. Thus, these mutants may affect the substrate specificity of the catalytic site rather than the equilibrium between the open and closed states.
To experimentally test the substrate specificities of these mutants, we assayed the activities of N308S and Q506P against a peptide containing the inhibitory tyrosyl phosphorylation site 529 of c-Src (Fig. 6c). Consistent with a possible alteration in substrate specificity, we observed a reproducible and significant ($p < 0.01$) basal activation using this substrate, in contrast to the lack of significant basal activation of these mutants when assayed with RCML.

Structural Correlates of Altered Activities of Disease-associated Mutants—To further study the disease-associated mutants, we examined their positions within the Shp2 crystal structure (20) and performed molecular modeling studies. L560F could not be analyzed, because the C-tail of Shp2 was not included in the crystallization construct.

Asp61 participates in a hydrogen bond network with water molecules in the active site and is surrounded by several positively charged side chains in the PTP domain (Fig. 7a). Substitution of a noncharged amino acid for Asp61 probably disrupts the N-SH2/PTP domain interface proportionally to the size of the substituted residue, accounting for the enhanced basal activity ($K_e$) of D61G and the previously characterized D61A (22) and the further enhancement of $K_e$ in D61Y.

E61D participates in a hydrogen bond network with water molecules and is next to an arginyl residue (Fig. 7b). The full basal activation of E76K, like that previously seen with E76A (22), can therefore be explained by charge effects. E76D preserves charge/charge interactions in the N-SH2/PTP domain interface but may be basally activated, because the C-tail of Shp2 was not included in the crystallization construct.

Altered basal activities of disease-associated mutants may be transmitted to the Tyr(P) peptide-binding pocket, since they are near each other in the molecule (Fig. 7b). Thr42 is next to the binding pocket of the N-SH2 domain, and its side chain forms a hydrogen bond with the phosphate group of the bis-Tyr(P) peptide (Fig. 7d) (28). Although changing Thr42 to alanine should lead to the loss of this hydrogen bond, T42A actually exhibits increased affinity ($7$-fold) for mono-Tyr(P) peptide (Fig. 5), a $10$-fold lower $EC_{50}$ for peptide activation (Figs. 2 and 3), and lower predicted $K_o$ (Table I). Our calorimetric studies indicate that the increased affinity results from a greater decrease in (more negative) enthalpy overcoming a concomitant decrease in entropy (Fig. 5). Although structural studies are needed to provide a precise explanation, the increased contribution of the enthalpy term to binding affinity in T42A could reflect a release of strain in the Tyr(P)-bound structure. For example, the hydrogen bond between Thr42 and the Tyr(P) peptide in the WT protein may induce a conformational change in the N-SH2 domain, which is eliminated in the T42A mutant.

Asp106 is located in the linker between the two SH2 domains and forms a hydrogen bond with residue Asn10 in the N-SH2 domain (Fig. 7e). This hydrogen bond probably positions it properly over the PTP domain. Interestingly, in the N+C-SH2/Tyr(P) structure, the two SH2 domains are reoriented, causing Asp106 to interact with Arg8 (Fig. 7e). The increased basal activation of the D106A mutation may be due to disruption of the Asp106–Asn10 interaction and its slightly increased affinity for ligand in the open versus closed state ($K_e/K_o$; Table I) could reflect coupling between the two SH2 domains.

|        | N               | K               | $\Delta H$ | $\Delta S$ | $K_o$ |
|--------|-----------------|-----------------|------------|------------|-------|
| WT     | 1.005 ± 0.01001 | 1.997 ± 10³     | 2.353 ± 10⁷ | -2.201 ± 10⁷ | 322.7 | 40.43 | 5.1 ± 10⁷ M |
| T42A   | 1.061 ± 0.00396 | 1.297 ± 10³     | 1.836 ± 10⁷ | -2.767 ± 10⁷ | 185.3 | 55.69 | 7.7 ± 10⁷ M |

**Fig. 5.** Affinity of GST-N-SH2 WT (left) and T42A (right) for p1172 as measured by isothermal calorimetry. Binding constants (table) were calculated after curve fitting using the Micro-Cal application program according to the manufacturer's guidelines.
Glu\textsuperscript{139} lies in the C-SH2 domain and is solvent-exposed in the native and peptide-bound structures. Although Glu\textsuperscript{139} is adjacent to Arg\textsuperscript{138}, the critical residue for Tyr(P) binding, the Glu\textsuperscript{139} side chain points away from the Tyr(P)-binding pocket. Neither the full Shp2 structure nor the N-C-SH2 structure explains why the conservative E139D mutation is activated. As discussed above for T42A, this mutation seems to shift the activation of the enzyme, consistent with our data (Fig. 2) and prior in vivo studies (4, 23).

Serine is accommodated in position Asn\textsuperscript{308} in other PTPs (information available on the World Wide Web at science.novonorisk.com/ptp), such as PTP1B and RPTP\textsubscript{\alpha}, suggesting that N308S would have a subtle effect on Shp2 function. Indeed, the major effect of this mutation appears to be on substrate specificity (Fig. 6). Although it is difficult to explain this mutant's apparent switch in substrate preference based on structural data, it is interesting to note that PTP1B and RPTP\textsubscript{\alpha} both dephosphorylate the C-terminal Tyr(P) site of c-Src (34–37).

Gln\textsuperscript{506} is conserved in nearly all PTP family members (information available on the World Wide Web at science.novonorisk.com/ptp). The cognate residue in PTP1B (Gln\textsuperscript{529}) is required for proper positioning of a water molecule to hydrolyze the thiophosphate catalytic intermediate, and mutating Q262 in PTP1B severely impairs catalytic activity (38). In Shp2, in contrast, Q506P retains significant activity against RCML. This finding suggests that another residue positions this water molecule properly in Shp2. Inspection of the Tyr(P) peptide-bound structures of PTP1B (Protein Data Bank code 1G1H) shows that the side chain carbonyl oxygen of Q506 also forms a hydrogen bond to the main chain amide group of the substrate two positions downstream from the Tyr(P) (39). The sequence surrounding the Tyr(P) in RCML is DpYGILQI (i.e. isoleucine in the Tyr(P)\textsuperscript{12} position). In contrast, in the pSrc529 peptide (QpYpGpGEnL), this residue is a proline, which is unable to form a hydrogen bond to Gln\textsuperscript{506}. Prolyl substitution for Gln\textsuperscript{506} might remove the preference for a hydrogen bond partner at the Tyr(P)\textsuperscript{12} position and perhaps facilitate interaction with the pSrc peptide. To test this possibility in more detail, we modeled the interaction between the Q506P mutant and the pSrc peptide (Fig. 7h). Energy minimization was performed, keeping the PTP domain rigid except for residues 505–507 in the protein and the peptide. The result predicts a close hydrophobic interaction between the prolyl side chains of the protein and peptide, providing a potential explanation for the substrate specificity switch observed with this mutant.

**DISCUSSION**

Shp2 mutations are associated with NS and neoplastic disorders, but the biochemical basis for their effects has remained unclear. Previous studies suggested that all disease-associated Shp2 mutants have increased basal and stimulated enzymatic activity and that NS-associated mutants are less active than leukemia/cancer-associated ones (4). We find, however, that this “activity-centric” model cannot explain all mutant Shp2-associated pathology. Although most Shp2 mutants are basally activated, they differ substantially in their extent of basal activation, ability to be activated by SH2 domain ligands, and, possibly, in their substrate specificity. Furthermore, there is no absolute biochemical distinction between mutations found in NS, leukemia, or both.

Asn\textsuperscript{308} is buried in the interior of the PTP domain, away from the N-SH2/PTP domain interface. However, a side chain oxygen in Asn\textsuperscript{308} makes a hydrogen bond to a conserved arginyl residue (Arg\textsuperscript{501}) that may interact with and stabilize the phosphatase binding loop (Fig. 7f). In addition, the closest charged residue to Asn\textsuperscript{308} is Glu\textsuperscript{76}, a critical interface residue (see above). Although Asn\textsuperscript{308} and Glu\textsuperscript{76} are separated by 10.7 Å, an ionic interaction between these residues is possible, since buried charges are less susceptible to solvent effects. Using molecular modeling, we predict that the surface of the mutant PTP domain interface is slightly less positively charged (Fig. 7g). This could lead to repulsion of Glu\textsuperscript{76} and partial activation of the enzyme, consistent with our data (Fig. 2) and prior in vivo studies (4, 23).

Many disease-associated SH2 domain mutations in Shp2 enhance its basal activation and/or SH2 domain affinity. Mutations that disrupt the autoinhibitory NSH2/PTP interaction (D61G, D61Y, E76K) are basally activated, consistent with previous findings (4, 16, 22, 40). In contrast, some SH2 domain mutants (T42A, D106A, and E139D) have only slightly increased basal activity, yet are more potently stimulated by Tyr(P) peptides than WT. Our mathematical model suggested that these mutations increase SH2-Tyr(P) peptide affinity, which could lead to increased signaling at lower Tyr(P) levels and/or more potent competition with other SH2/PTB proteins for Tyr(P) sites. We confirmed this increase in affinity for T42A
by isothermal calorimetry, although the modeled value of the “open” enzyme’s affinity constant ($K_o$) did not agree precisely with that obtained experimentally. Conceivably, the N-SH2 domain in the open state in the context of full-length Shp2 has a lower affinity than it has as an isolated domain. Regardless, our model fit the mono-Tyr(P) activation data for these mu-
tants and accurately predicted that the N-SH2 domain of T42A has a higher affinity for Tyr(P) ligand compared with WT. Notably, while this manuscript was in revision, another mutant (L43F) was reported that maps adjacent to the N-SH2 binding pocket and is associated with congenital heart disease but not frank NS (41). We predict that, like T42A, this mutant probably has increased affinity for Tyr(P) peptides that bind the N-SH2 domains. Such an increase in N-SH2 affinity may have the same biological effect as more classic activated mutants that disrupt the inhibitory interaction between the N-SH2 and PTP domains, namely increased amounts of active Shp2 at lower levels of cell stimulation. Furthermore, SOCS proteins, which promote target protein turnover in several signaling systems, often bind to the same Tyr(P) sites as Shp2 (42). Thus, as a secondary consequence of increased “openness” and/or affinity of the N-SH2 domain for Tyr(P) peptides, these disease-associated Shp2 mutants may enhance signaling through both their own catalytic actions and by decreasing SOCS recruitment to these sites.

In our mathematical model, we assumed that Shp2 mutants differ from WT in $K_c$ (basal activation), $K_e$ (“open” SH2 domain affinity for Tyr(P) ligand), and $K_s$ (“closed” SH2 domain affinity for Tyr(P) ligand). This model accurately described the behaviors of many of the SH2 domain mutants in response to mono-Tyr(P) peptide. In contrast, this relatively simple model, which does not account for alterations in $V_{max}$ or substrate specificity, could not fully describe the behavior of PTP domain mutants. Attempts to model these mutants with a more complex equation were unsuccessful (data not shown), most likely because the number of PTP mutants analyzed was insufficient to determine the equation. Insights into their behavior may be obtained from structural studies and/or future investigations of additional mutants.

Some Shp2 mutants affect the affinity of the SH2 domains only for particular ligands. E76D and D106A, which are only slightly activated basally, undergo enhanced activation selectively in response to bis- or mono-Tyr(P) peptide, respectively. These data raise the interesting possibility that these mutants may have altered responses in vivo to singly versus doubly phosphorylated Shp2 binding sites (e.g. those found on the platelet-derived growth factor receptor (43, 44) versus those found on scaffolding adapters (45, 46)); such differences could, of course, translate into different effects on the NS phenotype.

We also found that some PTP domain mutations (N308S and Q506P) may confer altered substrate specificity, which could result in more effective dephosphorylation of selected target proteins. If these are substrate specificity mutants, they may be viewed as the PTP analogs of mutations in Ret found in multiple endocrine adenomatosis syndromes (47) or those in e-KIt found in mastocytosis (48). Further characterization of these mutants may help identify the physiologically relevant substrates of Shp2 in NS and cancer, which are presently unknown.

A single mutant, L560F, which maps to the proline-rich region in the C-tail of Shp2, showed no detectable biochemical differences in any of our assays. Previous studies have shown that deletion of the C-tail does not affect Shp2 activity in vitro (20). Furthermore, only a single patient with this mutation has been reported (49), so L560F could represent a polymorphism rather that a disease-associated mutation. This mutation may, however, cause disease by affecting binding of an as yet unknown Src homology 3 domain-containing protein.

It remains possible that intracellular events also may modulate the activity differences in Shp2 mutants that we observed in vitro. Some mutants may be more (or less) stable, so that their total activity is more (or less) than might be predicted from our kinetic studies. WT and mutant Shp2 proteins also might be subject to different post-translational modifications that regulate their activity. Further studies will be required to test these possibilities. Nevertheless, some of the mutants studied in this work have been characterized using immune complex phosphatase assays by other investigators (4, 40). Although the precise extent of activation differs among these reports, as well as with our results, the qualitative ranking of basal activities is remarkably similar. For example, a recent study found that the basal activities of D61Y, T73I, N308D, and E76D were 105, 60, 32, and 26% as high as that of E76A in immune complex assays (40), a ranking identical to that seen in our study (Fig. 2). We have had similar experiences with immune complex and in vitro phosphatase assays performed on other Shp2 mutants. Therefore, it is likely that the relative rankings of PTP activities in this report reflect their relative catalytic activities in vivo.

Although increased phosphatase activity is a nearly uniform feature of disease-associated Shp2 mutants, it may be only a component of pathogenesis in vivo. The PTP activity of leukemia-associated Shp2 mutants is essential for them to transform primary bone marrow cells to factor independence (50, 51), but our data show that transforming ability is not directly proportional to PTP activity; e.g. D61G is less transforming than other NS/leukemia mutants (T73I, Q506P, and E139D) (50), despite its higher basal and activated phosphatase activity. Although we do not favor such a model, it even remains possible for NS that the increased catalytic activity of Shp2 mutants could be an epiphenomenon, since it has not been demonstrated that catalytic activity is required for pathogenesis. Mutations in the N-SH2, C-SH2, or PTP domain could lead to the exposure of a common surface that interacts with an as yet unidentified protein. The enhanced ability of Shp2 mutants to compete with SOCS proteins (see above) could also be critical for pathogenesis. Therefore, PTP activation might not be required for all of the signaling disorders evoked by Shp2 mutants.

In conclusion, our studies show that pathogenic Shp2 mutations may cause increases in PTP activity, alterations in substrate specificity, and/or enhanced targeting to upstream proteins and argue that, in contrast to prior suggestions, the extent of activation alone cannot predict the potential leukemogenicity of an Shp2 mutant. These biochemical properties probably cooperate to increase the activity of Shp2 against a specific substrate or set of substrates at specific locations in the cell. Although there is no strict correlation between any one of these parameters and pathogenesis, it is likely that the biochemical differences that we have defined here play a role in determining the spectrum of disease caused by Shp2 mutants.

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