SHP-1 is a protein-tyrosine phosphatase with two Src homology 2 (SH2) domains. These SH2 domains determine which proteins SHP-1 associates with, but they also autoregulate the activity of the catalytic domain. In this report, we find that the murine SHP-1 transcript is processed to yield a series of alternatively spliced in-frame transcripts, the majority of which exclude exons encoding one or the other SH2 domain. We have examined the corresponding protein isoforms in several ways. First, our measurements of Vmax and Kmax under different conditions indicate that the SH2 variants have elevated activity because of lessened autoregulation. Second, to ascertain whether regulation by the SH2 domains reflects intra- or intermolecular effects, we analyzed the state of SHP-1 by high performance liquid chromatography and sucrose density gradient centrifugation. Our results showed that SHP-1 is a monomer and, thus, is regulated in an intramolecular manner. Third, our analyses detected shape differences between SHP-1 and the active splice variant protein deleted of the amino-terminal SH2 domain; i.e. SHP-1 was globular and resistant to proteolytic digestion, while the splice variant protein was “rod-shaped” and more susceptible to proteolytic digestion.

Protein-tyrosine phosphatases (PTPs) have major effects on the activity of numerous proteins that function in cell physiology and ontogeny. One such PTP, SHP-1 (previously called PTP1C, HCP, and SHPTP1) is predominantly expressed in hematopoietic cells and has gained much attention with the finding that SHP-1, HCP, and SHPTP1) is predominantly expressed in hematopoietic cells and has gained much attention with the activity of numerous proteins that function in cell physiology and ontogeny. One such PTP, SHP-1 (previously called PTP1C, HCP, and SHPTP1) is predominantly expressed in hematopoietic cells and has gained much attention. Affected animals have a puzzling phenotype, with characteristics of both immunodeficiency and autoimmunity. Affected animals die prematurely, usually of pneumonitis (3).

SHP-1 (4–7), SHP-2 (8–11), and csw in Drosophila melanogaster (12) form a class of protein-tyrosine phosphatases that have two tandem SH2 domains amino-terminal to the phosphatase domain. SH2 domains, which are present in many signaling molecules, bind phosphorylated tyrosine residue motifs in a sequence-specific manner (13) and hence function to bring two signaling molecules together to help propagate a signaling cascade. However, in the case of SHP-1 and SHP-2, their SH2 domains have a second function, namely to negatively regulate the activity of the catalytic domain (14–16). This inhibitory effect is relieved when the amino-terminal SH2 domain binds to phosphorylated peptides (17, 18). Biphosphoproteptides that bind to both SH2 domains of SHP-2 activate SHP-2 at lower concentrations than do single phosphopeptides, arguing that both SH2 domains participate in this inhibitory effect (19). This notion is also supported by the crystal structure for SHP-2 that shows an intramolecular interaction between both SH2 domains and the phosphatase domain (20). Despite the sequence similarity between SHP-1 and SHP-2 (~50% amino acid identity; Ref. 8), other comparisons have suggested that the SH2-mediated inhibition of SHP-1 and SHP-2 might be different. Thus, SHP-1 molecules that lack the carboxyl-terminal SH2 domain (SH2C) are still subjected to negative regulation by the amino-terminal SH2 domain (SH2N; Refs. 18 and 21). However, the polypeptide segment corresponding to SH2C is necessary in SHP-2 as a spacer to allow the SH2N to block the catalytic cleft (20). To accommodate the stereochemical restrictions, it is possible that autoregulation of SHP-1 might be due to intermolecular interactions between two or more SHP-1 molecules, as has been suggested before (21).

Two forms of SHP-1 transcripts have been detected in humans (22), denoted as (I)SHP-1 and (II)SHP-1. The cDNA sequences of I and II are identical except in the untranslated region and in the first few coding nucleotides (MLSRG are the first 5 amino acids of (I)SHP-1, and MVR are the first 3 amino acids of (II)SHP-1). Because the published mouse SHP-1 amino-terminal coding sequences (MVR) are identical (5, 6), probably corresponding to the human (II)SHP-1 isoform, it is unclear whether mouse has more than one species of SHP-1 protein. Here, we report findings that indicate that the mouse also produces a second isoform, similar to the human (I)SHP-1. In addition, we also found that these transcripts are each spliced in multiple ways, thus generating in-frame RNAs. In some cases, these SHP-1 RNAs lack exons that encode either of the SH2 domains. As the SH2 domains of SHP-1 possess autoregulatory functions (see above), proteins encoded by these...
assays were carried out essentially as described by Pei et al. (17). Each fusion protein was assayed at a concentration of ~150 ng in 50 μl of either buffer A (50 mM NaAc, pH 5.0, 2 mM EDTA, 2 mM dithiothreitol, and different concentrations of NaCl) or buffer B (50 mM Pipes, pH 6.0 or 7.0, 2 mM EDTA, 2 mM dithiothreitol, and 10% glycerol) at 30 °C for 2 h. After incubation, each reaction was stopped by adding 450 μl of 4% TCA and 45 μl of 10% sodium pyrophosphate.

RESULTS

**Characterization of Marine SHP-1 Isoforms**—As noted above, the two published sequences for mouse SHP-1 correspond more closely to the human type II isoform than to the type I isoform. It has also been reported that human (I)SHP-1 is exclusively expressed in epithelial cells, while (II)SHP-1 is only found in hematopoietic cells (22). To assess whether mice also produce a type I isoform of SHP-1, we sequenced 5′-RACE products from SHP-1 transcripts from a mouse adrenal epithelioid tumor cell line, Y-1(14). This strategy, described under *Experimental Procedures*,” we obtained two products, fragment X (~360 bp) and fragment Y (~250 bp) (Fig. 1). Sequencing of fragment X (Fig. 1A) showed that it encoded the amino-terminal amino acids (i.e. MVR) similar to that for human type II SHP-1. Fragment Y (Fig. 1B) contained sequences that encoded the amino-terminal amino acids (i.e. MLSRG) similar to that of human type I SHP-1 (4). These results indicate that...
mouse also produces two distinct SHP-1 isoforms differing at their 5'-ends.

**Analysis of the Murine Chromosomal SHP-1 Gene**—In humans, the two SHP-1 isoforms are generated from the same gene via the use of alternative 5' exons (22). To assess whether each transcript isoform of mouse SHP-1 is also produced from two alternatively used 5' exons, we characterized the entire mouse SHP-1 locus. Assuming that the human and murine genetic structure was similar, we designed primers to amplify the complete mouse SHP-1 gene. Six genomic DNA fragments (Fig. 2A) encompassing the complete SHP-1 gene were amplified by PCR from normal mouse DNA. Fig. 2A shows the locations of these PCR fragments and the primers used to obtain the intron-exon boundary sequences that are shown in Fig. 2B. Genomic DNA sequences were compared with the SHP-1 cDNA sequences in order to deduce the intron-exon boundaries. We obtained complete sequences for all introns except for the indicated four cases in which the introns were larger than 2 kb (GenBank™ numbers U65951–U65955). Similar to the human chromosomal SHP-1 gene, mouse SHP-1 has 17 exons (Fig. 2A). (I)exon 1, which contains 5'-untranslated sequences and nucleotides encoding the first 5 amino acids (i.e. MLSRG) of (I)SHP-1, is located −5.8 kb upstream of (II)exon 1, which contains 5'-untranslated sequences and nucleotides encoding the first 3 amino acids (i.e. MVR) of (II)SHP-1. Thus, both mouse and human (I)SHP-1 and (II)SHP-1 transcripts are generated by alternative 5' exon usage.

**Identification of Murine SHP-1 Splice Variants in Bone Marrow Macrophages**—(I)SHP-1 transcript variants lacking either one (exon 2) or both exons (exons 2 and 3) encoding the SH2N domain exist in human cells (22). To determine whether we could identify such transcripts in murine cells, we assessed the SHP-1 transcripts in murine bone marrow macrophages by Northern blots (Fig. 3A). These blots, using the phosphatase domain of SHP-1 as a probe, revealed the presence of multiple bands larger and smaller than the expected full-length transcript (Fig. 3A). To examine the sequence of these bands, we used RT-PCR to amplify the different RNA molecules. Expression of (I)SHP-1 transcripts was assessed using the primers (I)SHP-1–90-5' and SHP-1–1859-3'. We found not only the expected 1944-bp (I)SHP-1 mj product, but also a larger product (~2000 bp) and two smaller products (~1800 and ~1600 bp; Fig. 3B). The sequencing analysis of these products is summarized in Fig. 3C. As depicted in Fig. 3, the different RNA isoforms arise by alternative splicing. We denote these different isoforms with specific postscripts. Thus, the major forms indicated in Fig. 3C are denoted as mj. The spliced variants are denoted as S1–S6. Two SHP-1 structures are present within the ~2000-bp product; (I)SHP-1 S1 (2105 bp) retains the 171-bp intronic sequence between exons 15 and 16, and
Both SH2 Domains Regulate SHP-1 Intramolecularly

Fig. 2. Organization of the mouse SHP-1 chromosomal locus. A, schematic diagram of the SHP-1 gene. The six PCR genomic fragments used to characterize the gene are indicated below the map. Arrows denote the primers (A–V) used to sequence across the exon-intron boundaries. The four larger introns marked with a double slash on the map were not completely sequenced. B, sequences of exon-intron junctions. Exon sequences are in uppercase letters, and intron sequences are in lowercase letters.
(I)SHP-1 S2 (2051-bp) retains the 117-bp intronic sequence between exons 4 and 5 (Fig. 3C). Two SHP-1 structures are present within the 1800-bp product; (I)SHP-1 S3 (1811 bp) excludes sequences from exon 2, and (I)SHP-1 S4 (1820 bp) excludes a 114-bp segment from exon 4 (Fig. 3C). The 1600-bp product (I)SHP-1 S6 (1626 bp) excludes sequences from exons 2 and 3 (Fig. 3C). This structure is similar to the previously reported human SHP-1 variant (22). All of these transcript variants of SHP-1 retain the normal reading frame, and as a consequence, they are capable of encoding active phosphatases. Thus, most of these splice variants of SHP-1 would express proteins with altered amino-terminal or carboxyl-terminal SH2 domains. However, we found that all of these alternatively spliced transcripts of SHP-1 were present at low levels and that the corresponding protein products were not detected (see “Discussion”).

Expression and Purification of Murine SHP-1 in a Bacterial System—The SH2 domains of human SHP-1 inhibit the phosphatase domain by decreasing its affinity for its substrate (17). The naturally occurring mouse SHP-1 transcript variants encode SHP-1 proteins with deletions in their SH2 domains. These splice variant SHP-1 proteins can be used to gain information on the mechanism of autoinhibition. By comparing their kinetic properties relative to SHP-1 mj, we can assess whether they have elevated activities as predicted and thus map the motifs responsible for the inhibitory effects.

The SHP-1 cDNAs that were used in this study all encode proteins that have deletions affecting one or the other SH2 domain (Fig. 4A). In this study of autoregulation, we have compared these SHP-1 splice variant proteins with two other proteins: SHP-1 mj, which contains the complete SH2N and SH2C domains, and an engineered mutant protein of SHP-1, which consisted only of the phosphatase domain (PTPase domain; Fig. 4A). These cDNAs were cloned into the BamHI site of the pRSETb vector and transfected into the bacterial strain BL21(DE3) for protein expression. Cloning into the BamHI site produces fusion proteins that contain an N-terminal polyhistidine region that allows for purification by binding to nickel.

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columns (see Fig. 5A for schematic of the fusion peptide). To allow for removal of the fusion peptide, an enterokinase cleavage site was placed immediately amino-terminal to the initiator methionine of SHP-1 and its isoforms. Bacterial lysates were prepared, and the fusion proteins were partially purified and analyzed by staining SDS-PAGE gels with Coomassie Blue (Fig. 4B). As (I)SHP-1 S4 was expressed at low levels and thus contained many bacterial proteins even after purification, Western blots using a carboxyl-terminal specific antibody were performed in this study, data are only presented for one or the other. Kinetic analyses using the fusion proteins. As both (I)- and (II)SHP-1 mj had comparable results in all of the experiments (see below for more discussion on this point).

To test whether the presence of the oligo-His fusion peptide affected the kinetic behavior of the proteins in question, we expressed in bacteria both (I)- and (II)SHP-1 mj and selected SHP-1 splice variant proteins without the oligo-His fusion peptide (for simplicity, hereinafter referred to as “native”) and compared their kinetic parameters to those of their fusion protein counterparts. This analysis was applied to (I)- and (II)SHP-1 mj, S3, and S6. Fig. 6A depicts the bacterial expression of the (I)SHP-1 mj, S3, and S6, both as native protein and as fusion proteins. We then measured the \( K_m \) of unpurified native and unpurified fusion proteins and compared these values to those obtained with purified fusion proteins, as shown in Fig. 6B. In all cases, the enzyme activity curves were similar for unpurified native and unpurified fusion proteins, while bacterial lysates alone could not hydrolyze pNPP at any concentration (Fig. 6B). Furthermore, the \( K_m \) values for unpurified fusion protein were not significantly different than the values for the purified fusion proteins (Table I). We thus conclude that the fusion peptide had little effects on the kinetic properties of the isoforms and that we could therefore carry out enzyme kinetic analyses using the fusion proteins. As both (I)- and (II)SHP-1 mj had comparable results in all of the experiments performed in this study, data are only presented for one or the other.

Both SH2 Domains Are Required for Maximal Inhibition of the Phosphatase Domain—In these studies, we analyzed purified splice variant proteins together with an engineered mutant that consisted only of the phosphatase domain (PTPase). Since the effects on the \( K_m \) of deleting either one of the two SH2 domains were not known, the PTPase protein served to define the kinetic properties of the catalytic domain in its uninhibited state. Kinetic analyses at physiological conditions (i.e., pH 7.0, 150 mM NaCl) revealed that SHP-1 mj had very little activity, and thus it was not possible to calculate a \( K_m \) value from the data (Fig. 6C). However, the \( K_m \) for each of the other isoforms was readily measured. In some cases, the values approached that of the PTPase (Fig. 6C, Table II). Specifically, deletion of a

Fig. 5. Enterokinase digestion of (I)SHP-1 mj and S6. A, schematic diagram depicts location of the T7 epitope and the enterokinase cleavage site in both (I)SHP-1 mj and S6 used to remove the fusion peptide. B, Western blot of portions of enterokinase digestion products of 2 μg of (I)SHP-1 mj and S6 using polyclonal anti-SHP-1 antibody or anti-T7 antibody. Lanes 4 and 10–13 represent enterokinase digestions in the presence of 60 μM EpoR pY429 peptide.

| Time (h) | 0 | 6 | 12 |
|---------|---|---|---|
| Activity | 0 | 60 | 120 |

Fig. 4. Bacterial expression of SHP-1 isoforms. A, schematic diagram of SHP-1 splice variants used in this study. B, SDS-PAGE analysis of purified and partially purified SHP-1 and isoforms. Gel was stained with Coomassie Blue. Lanes 1, BL21 DE3 lysate; lanes 2, (II)SHP-1 mj; lanes 3, (II)SHP-1 S3; lanes 4, (II)SHP-1 S4; lanes 5, (II)SHP-1 S5; lanes 6, (I)SHP-1 mj; lanes 7, (I)SHP-1 S6; lanes 8, PTPase domain. The 45-kDa band present is a bacterial protein that consistently co-purified with the fusion proteins. C, Western blot of SHP-1 and isoforms using an anti-SHP-1 antibody. PTPase domain (lanes 8) was loaded in the following relative amounts, 3, 1, and \( \frac{1}{3} \), to show the linearity of the signal.
Both SH2 Domains Regulate SHP-1 Intramolecularly

Fig. 6. Kinetic analyses of SHP-1 mj and isoforms. A, expression of SHP-1 isoforms as "native" or fusion proteins. Lanes 1, 3, and 5 of this Coomassie Blue-stained SDS-PAGE represent the fusion proteins for (I)SHP-1 mj, S3, and S6, while lanes 2, 4, and 6 represent the native proteins for the same three proteins, respectively. B, initial velocities at varying substrate (pNPP) concentrations at pH 7.0, 150 mM NaCl. A 0.15 μM concentration of each protein was assayed for 1 min at different substrate concentrations and stopped with 0.2 N sodium hydroxide, and absorbance at 410 nm was determined as a measure of product generated. C, initial velocities of partially purified SHP-1 and isoforms at varying pNPP substrate concentrations at pH 7.0, 150 mM NaCl. A 0.15 μM concentration of each protein was assayed for 1 min. Values for $K_m$ and $V_{max}$ are presented in Table II. The solid lines represent the best fit of the data to the Michaelis-Menton equation, with the values of $V_{max}$ and $K_m$ in each case being presented in Table II. Because of the low level of activity for (II)SHP-1 mj, a satisfactory fit of the data with respect to the magnitude of the errors associated with $V_{max}$ and $K_m$ was not obtained.

TABLE I

The $K_m$ (millimolar concentrations of pNPP) of selected SHP-1 isoforms either "native" or as fusion proteins at the indicated pH. Bacterial lysates alone were found not to hydrolyze pNPP at any concentration.

| Purified protein | Unpurified fusion protein | Unpurified protein |
|------------------|---------------------------|-------------------|
| (I)SHP-1 mj, pH 5.0 | 39.3 ± 5.8 | 46.3 ± 3.3 | 40.4 ± 1.8 |
| (I)SHP-1 mj, pH 7.0 | >100 | >100 | >100 |
| (I)SHP-1 S3, pH 7.0 | >100 | >100 | >100 |
| (I)SHP-1 S6, pH 7.0 | >100 | >100 | >100 |
| (I)SHP-1 mj, pH 5.0 | 8.5 ± 0.6 | 13.5 ± 1.1 | 16.6 ± 2.5 |
| (I)SHP-1 S3, pH 7.0 | 5.5 ± 0.9 | 8.9 ± 1.3 | 11.7 ± 1.3 |
| (II)SHP-1 mj, pH 7.0 | 5.8 | 6.4 | 6.1 |
| (II)SHP-1 mj, pH 5.0 | 8.9 | 13.5 | 16.6 |

*These values were adopted from Table II for comparison.

NaCl concentration at 50 mM for this assay and at 150 mM for all others.

portion or the entire SH2N domain of SHP-1 (i.e. S3 and S6, respectively) resulted in the complete activation of the catalytic domain. (Fig. 6C, Table II); i.e. the $K_m$ values of S3 and S6 are similar to that of the PTPase, which was ~6 mM pNPP (Fig. 6C, Table II), similar to the value of 12.6 mM obtained previously for the catalytic domain of human SHP-1 (17). Internal deletions of the SH2C domain (i.e. S4 and S5) resulted in partial activation of the catalytic domain, as evidenced by its $K_m$ value of ~20 mM pNPP, which is about 3-fold higher than the $K_m$ of the PTPase.

Although the $K_m$ of S4 and S5 (both missing portions of the SH2C domain) ranged from ~10 to 27 mM when assayed at pH 7.0 and 50–150 mM NaCl, their $K_m$ were >100 mM when assayed at pH 7.0 and 250 mM NaCl (Table II). By contrast, the $K_m$ for the PTPase was at 6–8 mM under all assay conditions. This suggests that at high NaCl concentrations and in the absence of an intact SH2C domain, the interaction between the SH2N domain and the catalytic domain was stabilized. However, at pH 6.0 and 250 mM NaCl, the $K_m$ of S4 and S5 were ~17–44 mM, while the $K_m$ for (I)SHP-1 mj remained at >100 mM (Table II). This suggests that a more stable interaction exists between the SH2 domains and the catalytic domain of (I)SHP-1 mj compared with that of S4 or S5. These data suggest that the SH2N domain is principally responsible for the inhibitory effects but requires an intact SH2C domain to exert its full effects.

Activation of Murine SHP-1 mj—Human SHP-1 is inactive under physiological conditions, but this inhibitory effect is alleviated at pH 5.0 (24), in 80% glycerol (25), or in the presence of tyrosine-phosphorylated peptides that bind to the SH2N (17, 18). To test whether murine SHP-1 can be activated by similar means, we performed enzyme kinetic assays under these various conditions. We first assayed SHP-1 mj and splice variant proteins in 80% glycerol, the concentration that was previously shown to have maximum stimulatory effects for human SHP-1 (25). Although there was variability in the results due to the difficulty in carrying out reactions at this high glycerol concentration, all SHP-1 isoforms were found to be active in 80% glycerol at pH 7.0 (Table II).

Acidification of the reaction conditions to pH 5.0 activated (I)SHP-1 mj with a corresponding decrease in $K_m$ to ~20 mM pNPP (Table II). It is possible that at this low pH the electro-
static repulsive effects of amino acid side chains that have gained positive charges uncouple the SH2 domain(s) from the inhibitory site. In support of this premise, increasing the NaCl concentration to 250 mM reversed the activating effects of an acidified environment by increasing the $K_m$ (Table II), although it is possible that NaCl might directly affect the catalytic domain. Some SHP-1 splice variant proteins were inhibited at pH 5.0. The $K_m$ for S6 and the PTPase were inexplicably above 100 mM pNPP (Table II). It thus appears that the manner by which glycerol and an acidic environment activates SHP-1 and its isoforms is different.

Inhibition of (I)SHP-1 mj Is Intramolecular—Our data so far indicated that inhibition requires two intact SH2 domains but did not reveal whether the inhibitory SH2 domains act intra- or intermolecularly. To examine this issue, we subjected (I)SHP-1 mj to HPLC size exclusion analysis to assess whether the enzyme was a monomer or dimer. (I)SHP-1 mj was applied onto a Superdex 200 size exclusion column that was equilibrated to pH 7.0 (50 mM Pipes pH 7.0, 250 mM NaCl, 2 mM EDTA). Using the standard curve, (I)SHP-1 mj (calculated molecular mass of 72 kDa) was determined to migrate on the size exclusion column with an apparent molecular mass of 72 kDa. (Fig. 7B). No portion of SHP-1 mj was detected as a dimer (Fig. 7A). This suggests that (I)SHP-1 mj is a globular monomer and excludes the possibility that it is a stable dimer. Interestingly, (I)SHP-1 mj (calculated molecular mass of 59 kDa) migrated through the column with an apparent molecular mass of 92 kDa (Fig. 7B).

Proteins with extended structures elute earlier on size exclusion columns (i.e. higher apparent molecular mass) than do globular proteins of equal molecular mass. The anomalous elution volume for (I)SHP-1 mj on the HPLC column suggested that it existed either as a dimer or had an extended rather than globular conformation. To determine whether (I)SHP-1 mj was a dimer or had an extended structure, sucrose density gradient sedimentation analyses of (I)SHP-1 mj and S6 were carried out. This assay can resolve this issue because a protein with an extended conformation sediments slower than a globular protein with an equivalent molecular mass, while a protein of larger mass sediments faster; i.e. if (I)SHP-1 mj had an extended conformation, it would sediment slower than a 59-kDa globular protein, while if it was a dimer, it would sediment faster. In fact, as shown in Fig. 7C, S6 sedimented with an apparent molecular mass of about 45 kDa, indicating that S6 was most likely a monomer with an extended rather than globular conformation. Again, (I)SHP-1 mj sedimented as a globular monomer, confirming the results obtained by size exclusion chromatography and suggesting that inhibition arises predominantly through intramolecular interactions (Fig. 7C).

We were concerned that autoregulation might involve only a transient intermolecular interaction that was so short lived that it did not affect the exclusion and sedimentation properties. To test this possibility, the activity of (I)SHP-1 mj was assessed over a ∼100-fold range of enzyme concentration (17–1400 nM). We reasoned that if the inhibition was due to an intermolecular interaction, there should be an inverse concentration dependence to the specific activity observed. However, as can be seen in Fig. 7D, the specific activity of (I)SHP-1 mj was invariant over the concentration range tested. In addition, Fig. 7D also shows that the specific activity of (I)SHP-1 S6 did not vary significantly over the same range tested, indicating that it does not require dimerization to be catalytically active. The concentration independence of enzyme specific activity together with the hydrodynamic data indicating that (I)SHP-1 mj is a monomer at 17 nanomolar to 1.4 micromolar concentrations both argue that autoregulation of (I)SHP-1 mj is intramolecular.

Can the Activation Status of the Enzyme Be Attributed to the Structural Difference between (I)SHP-1 mj and (I)SHP-1 S6?—As presented above, the difference in enterokinase cleavage between (I)SHP-1 mj and S6 suggested a structural difference between the two enzymes. Structural differences between (I)SHP-1 mj and S6 were in fact detected by size exclusion chromatography and by sucrose density gradient analyses. These structural differences might be attributed to the activation status of (I)SHP-1 mj. To determine whether activation of (I)SHP-1 mj rendered the enterokinase recognition site more accessible, (I)SHP-1 mj was incubated with 60 μM of the phosphorylated erythropoietin receptor peptide pY429 (EpoR pY429). At this concentration, this peptide activates (I)SHP-1 mj maximally by binding to its SH2 domains (18). Nevertheless, enterokinase could not cleave at the enterokinase recognition site of (I)SHP-1 mj, but it still cleaved the same site on

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**Table II**

| pH 5.0 | pH 6.0 | pH 7.0 |
|---------|---------|---------|
| 50 mM NaCl | 15.2 ± 1.2 | 15.2 ± 1.2 |
| 150 mM NaCl (pH 7.0) | 15.2 ± 1.2 | 15.2 ± 1.2 |
| 250 mM NaCl | 23.6 ± 1.4 | 23.6 ± 1.4 |

| pH 5.0 | pH 6.0 | pH 7.0 |
|---------|---------|---------|
| 50 mM NaCl | 15.2 ± 1.2 | 15.2 ± 1.2 |
| 150 mM NaCl (pH 7.0) | 15.2 ± 1.2 | 15.2 ± 1.2 |
| 250 mM NaCl | 23.6 ± 1.4 | 23.6 ± 1.4 |

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a Enzymatic activity too low to obtain accurate $K_m$ and $V_{max}$ values.

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3 A. Martin, H. W. Tsui, F. W. L. Tsui, unpublished observations.
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Second, all variations involve the use of alternative splice junctions. For the generation of (I) and (II)SHP-1 S4, a cryptic splice site was used within exon 4. This alternative splice site sequence (CTTtgtgct) is similar to a common version of the consensus 5' splice site sequence (CCGtgtgct) (26). However, as further discussed below, these alternative transcripts are present at low levels, and as a consequence, we believe that they are not physiologically relevant.

Our finding that mice, like humans, produce both type I and type II forms of SHP-1 argues that this conserved feature serves an important function. It is not evident whether the different forms have arisen because of a need to regulate SHP-1 transcription using distinct promoters or to produce two SHP-1 isoforms with different functions or properties. However, considering that we detected, at most, minor differences between (I)SHP-1 mj and (II)SHP-1 mj proteins and that we found most of the same splice variants in both I and II forms, we favor the former view.

It is unclear at present whether the naturally occurring mRNA isoforms are translated into stable proteins at physiologically significant levels. The motheaten mutation, a single base pair deletion in exon 3 of the SHP-1 gene, creates a novel splice site that is subsequently utilized to splice to exon 4 (1, 2). However, this splicing is out of frame, and thus SHP-1 mj is not produced in motheaten mice (2). We found that the SHP-1 gene in motheaten macrophages is also produced as multiple spliced variant transcripts that resemble those identified in normal macrophages, except for the altered splicing caused by the motheaten mutation. Interestingly, we identified the (I)SHP-1 S6 transcript in motheaten macrophages, and since this transcript does not contain exon 3 and thus does not harbor the motheaten mutation, it might produce a SHP-1 variant protein. However, we failed to detect this protein in motheaten macrophages, suggesting that it is most likely not present at physiological levels in normal macrophages. Furthermore, the finding that the transcript variants in normal macrophages represent at most minor species (i.e. S3, S4, S5, and S6 are collectively at ~5% the level of (II)SHP-1 mj) suggests that the corresponding protein products are also not expressed at physiologically relevant levels. This is not surprising, because we found in this study that these splice variants encode deregulated phosphatases. That the catalytic activities of SHP-1 and SHP-2 are negatively regulated suggests that a constitutively active SHP-1 or SHP-2 is toxic. Thus, these active splice variant proteins might be toxic to cells if present at high levels.

Mechanism of Autoregulation—The structural similarity between SHP-1 and SHP-2 (~50% amino acid identity; Ref. 8) suggests that autoregulation of these two phosphatases is accomplished by similar mechanisms. The finding that human SHP-1 chromatographs as a monomer suggests a role for intramolecular interactions between the SH2 and the catalytic domains (24). Indeed, the crystal structure for SHP-2 (20) shows that the SH2 domains fold back onto the catalytic domain. In the SHP-2 structure, there are extensive interactions between the SH2N and the catalytic domain. Asp61, located within the SH2N domain of SHP-2 (Asp59 for SHP-1), blocks the catalytic pocket and hydrogen bonds to the catalytic cysteine in the phosphatase domain (20). However, other data suggest that SHP-1 and SHP-2 might be autoregulated in different ways. First, the SH2 domains and catalytic domain of SHP-2, when expressed as separate polypeptide chains, can associate and inhibit the catalytic domain in vitro (16), a feature that was not reproduced with SHP-1 (17). Second, agents that activate SHP-1, such as glycerol and other 1,2-diols, inhibit the activity of SHP-2 (25). Finally, complete deletion of the SH2C domain from human SHP-1 did not impair autoreg-

Fig. 7. Structural analyses of SHP-1 mj and S6. A, HPLC analysis. Elution profiles for (I)SHP-1 mj (solid line) and S6 (dashed line). Elution volumes at peak absorption values (shown at the top of each peak) were used to construct the plot shown in B. Peaks were confirmed to contain (I)SHP-1 mj and S6 by Western blotting using the carboxy-terminal specific anti-SHP-1 antibody (data not shown). B, HPLC elution positions for (I)SHP-1 mj and S6 relative to standard curve. Data are presented as elution volume (V_e) divided by the void volume (V_v) of the Superdex column measured using blue dextran. The molecular masses of (I)SHP-1 mj and S6 employed for calculating the y coordinate represent the theoretical value of the monomer species in each case. Data are representative of three experiments. C, sucrose density gradient sedimentation analysis. Data are presented as peak elution fraction measured by both protein determination and phosphatase activity assays. Data plotted as molecular weight to the 5/2 power (m). The molecular masses of (I)SHP-1 mj and S6 employed for calculating the y coordinate represent the theoretical value of the monomer species in each case. Data are representative of three experiments. D, activities over a concentration range. At a substrate concentration of 35 mM pNPP, (I)SHP-1 mj and S6 were assayed in 50 mM Pipes, pH 7.0, 250 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, and 0.1 mg/ml BSA for up to 5 min. (I)SHP-1 mj and S6 were assayed at approximate concentrations of 17, 50, 150, 450, and 1400 nM. Data points are averages of three experiments. Error bars are omitted for simplicity (range is less than 30% of the average).
Both SH2 Domains Regulate SHP-1 Intramolecularly

This is surprising because, based on the crystal structure for SHP-2, the SH2N domain would not be expected to reach the catalytic pocket without the SH2C domain acting as a spacer (20). Pregel et al. (21) suggest that inhibition of the human SH2C deletion mutant, as well as normal SHP-1, might be mediated by the intermolecular association of two molecules in a head to tail manner.

Our analyses of the full-length protein showed that SHP-1 mj is regulated intramolecularly; i.e. we showed using size exclusion chromatography and sucrose density gradient sedimentation analyses that murine SHP-1 mj is a globular monomer and that the specific activity of (I)SHP-1 mj was invariant over an ~100-fold concentration range. These results indicated that SHP-1 does not form stable dimers, and consequently, this concentration independence of specific enzyme activity indicates that SHP-1 mj is not inhibited by the SH2 domains from another SHP-1 molecule, thus ruling out transient intermolecular interactions. Our finding that partial deletions of the SH2C of murine SHP-1 (i.e. S4 and S5) activate the catalytic domain is also consistent with SHP-1 mj being inhibited intramolecularly. As argued above, such deletions would be expected to impede the SH2N from reaching and blocking the substrate binding pocket, based on the structure of SHP-2. It is not clear why our data differ from previous work (18, 21). As there is a high degree of amino acid identity between human and mouse SHP-1 sequences (~95%; Ref. 6), it appears unlikely that species specificity is the reason for the discrepancy. A possible reason for the difference is that our splice variants (i.e. S4 and S5) only deleted portions of the SH2C, which contrasts with the mutants from the previous report that delete the entire SH2C (18, 21). Perhaps the portions of the SH2C domain in our splice variants prevented the proposed intermolecular interactions (21). Nevertheless, we conclude that SHP-1 mj is autoregulated through intramolecular interactions between the SH2 domains and the catalytic domain, just as with SHP-2.

Evidence for Conformational Change Associated with Activation—Many laboratories have proposed that an inactive SHP-1 exists in a closed state and undergoes a conformational change upon activation (open state). This is exemplified by the number of models presented in publications illustrating how SHP-1 “opens up” when it engages a substrate with its SH2 domains (e.g. Ref. 17). The open state for SHP-1 has been only hypothetical, since there are no data to suggest that such a state exists for an active SHP-1. Our data, which correlate the structural differences between (I)SHP-1 mj and S6 to the catalytic state of the phosphatase, support models in which SHP-1 mj “opens up” when activated. Both our HPLC size exclusion and sucrose density gradient sedimentation analyses detected hydrodynamic differences between SHP-1 mj and S6. These assays were consistent in showing that inactive SHP-1 mj was a globular protein (closed state) and that active S6 had an extended conformation (open state). The conformational differences between SHP-1 mj and S6 were also detected with proteolytic sensitivity assays (Fig. 5). These results indicate that our HPLC gel filtration and sucrose density gradient sedimentation assays were sensitive enough to detect such structural differences. Unfortunately, activating SHP-1 mj with either pH 5.0 buffers or activating concentrations of EpoRPy420 induced aggregation,3 so we were not able to test directly if SHP-1 mj undergoes a conformational change upon activation.

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