Determination of the molecular reach of the protein tyrosine phosphatase SHP-1

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ABSTRACT Immune receptors signal by recruiting (or tethering) enzymes to their cytoplasmic tails to catalyze reactions on substrates within reach. This is the case for the phosphatase SHP-1, which, upon tethering to inhibitory receptors, dephosphorylates diverse substrates to control T cell activation. Precisely how tethering regulates SHP-1 activity is incompletely understood. Here, we measure binding, catalysis, and molecular reach for tethered SHP-1 reactions. We determine the molecular reach of SHP-1 to be 13.0 nm, which is longer than the estimate from the allosterically active structure (5.3 nm), suggesting that SHP-1 can achieve a longer reach by exploring multiple active conformations. Using modeling, we show that when uniformly distributed, receptor-SHP-1 complexes can only reach 15% of substrates, but this increases to 90% when they are coclustered. When within reach, we show that membrane recruitment increases the activity of SHP-1 by a 1000-fold increase in local concentration. The work highlights how molecular reach regulates the activity of membrane-recruited SHP-1 with insights applicable to other membrane-tethered reactions.

SIGNIFICANCE Immune receptors transduce signals by recruiting (or tethering) cytoplasmic enzymes to their tails at the membrane. When tethered, these enzymes catalyze reactions on other substrates to propagate signaling. Precisely how membrane tethering regulates enzyme activity is incompletely understood. Unlike other tethered reactions, in which the enzyme tethers to the substrate, the substrate in this case is a different receptor tail. Therefore, the ability of the receptor-tethered enzyme to reach a substrate can be critical in controlling reaction rates. In this work, we determine the molecular reach for the enzyme SHP-1 and use it to quantify the impact of molecular reach on receptor signaling.

INTRODUCTION

Immune receptor signal transduction proceeds by the recruitment of cytoplasmic enzymes to their unstructured cytoplasmic tails before they catalyze reactions on other membrane substrates (1–3). Well-studied examples include inhibitory checkpoint receptors, such as programmed cell death protein 1 (PD-1) on T cells, that can contain immunoreceptor tyrosine-based inhibition (ITIM) and switch (ITSM) motifs (2). Ligand binding induces phosphorylation of these motifs, which can then recruit the tyrosine phosphatases SHP-1 and SHP-2 by their SH2 domains. When tethered to inhibitory receptors, these promiscuous phosphatases are thought to dephosphorylate diverse membrane substrates, including the T cell receptor, the costimulation receptor CD28, the membrane adaptor LAT, and even autoinhibition of inhibitory receptors in trans (Fig. 1A; (4–8)). Precisely how membrane recruitment regulates and directs the activity of SHP-1 is incompletely understood.

Biochemical and structural studies have clearly demonstrated that engagement of the SH2 domains of SHP-1 and its family member SHP-2 can induce a conformational change from a closed low-activity state into an open high-activity state (9–18). When quantified, this binding-induced allosteric activation can increase catalytic rates by ~80-fold (9), and therefore, this is a mechanism by which membrane recruitment can regulate enzyme activity and has motivated the development of therapeutic allosteric inhibitors (15).

Membrane recruitment can also tether SHP-1 in a small volume, increasing the local concentration of SHP-1 experienced by substrates (3). Tethering is prevalent in cellular signaling (19), and experimental and mathematical work
has shown that it can dramatically increase local concentrations and hence reaction rates (16,20,21); it can also overcome enzyme specificity (22). How tethering impacts the local concentration of SHP-1 is presently unknown.

In contrast to previously studied tethered reactions (20,21,23), in which the enzyme tethers directly to the substrate, the situation is more complicated for immune receptors. The local concentration of SHP-1 when this is the case. The molecular reach of PD-1 ($L_{PD-1}$), SHP-1 ($L_{SHP-1}$), and the substrate ($L_{substrate}$). It determines whether the substrate is within reach (within gray area) and the local concentration of SHP-1 is determined by the molecular reach of SHP-1 based on sequence (maximal stretch), crystal structure, and experimental measurement in this work. To see this figure in color, go online.

Molecular reach of SHP-1

FIGURE 1 Molecular reach in immune receptor signal transduction. (A) Schematic of a tethered dephosphorylation reaction mediated by the tyrosine phosphatase SHP-1 (red) recruited to an inhibitory receptor (pink), such as PD-1, acting to dephosphorylate a membrane substrate (orange). The molecular reach of the reaction, $L$ (gray area), is determined by the molecular reach of PD-1 ($L_{PD-1}$), SHP-1 ($L_{SHP-1}$), and the substrate ($L_{substrate}$). It determines whether the substrate is within reach (within gray area) and the local concentration of SHP-1 when this is the case. (B) Estimates of SHP-1 molecular reach ($L_{SHP-1}$) based on sequence (maximal stretch), crystal structure, and experimental measurement in this work. To see this figure in color, go online.

METHODS

SHP-1 molecular reach estimates from structure

Using the structure of SHP-1 in the open conformation (Protein Data Bank, PDB: 3PS5) and sequence data from UniProt (P29350), we can estimate a range of reach values for SHP-1. Direct measurement from the PDB structure of the N-SH2 binding site to the catalytic site gives a reach estimate of 13.0 nm. For our maximal reach estimate, we subdivide SHP-1 into three structured domains (N-SH2, C-SH2, and PTP) and two linker domains. For the structured domains, distances were measured from the structure in PDB: 3PS5: between binding pocket and linker for N-SH2, between two linkers for C-SH2, and from the linker to the active site for PTP. We then count the number of residues in the two intervening disordered linker domains and compute contour length assuming these are fully extended. Adding these five numbers together (N-SH2, linker, C-SH2, linker, PTP)
yields a value of 20.4 nm. All measurements of structured domains were calculated using the measurement tool in PyMol.

**Peptides and SHP-1**

All phosphopeptides were custom synthesized by Peptide Protein Research and were N-terminally biotinylated. Peptide sequences, including peptides conjoined with polyethylene glycol (PEG), are listed in Table 1. Human SHP-1 with an N-terminal 6×His tag was produced in *Escherichia coli* BL21-CodonPlus (DE3)-RIPL strain (Agilent Technologies, Santa Clara, CA) and purified on Ni²⁺-NTA agarose (Invitrogen, Carlsbad, CA) (16). Aliquots were stored at −80°C. On the day of experiment, SHP-1 was further purified by size-exclusion chromatography on an AKTA fast protein liquid chromatography system equipped with a Superdex S200 10/300 GL column (both from GE Healthcare Life Sciences; Marlborough, MA) equilibrated with 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 supplemented with 1 mM dithiothreitol. SHP-1 concentration was determined from absorbance at 280 nm measured on a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

**SPR**

Experiments were performed on a Biacore T200 instrument (GE Healthcare Life Sciences) at 37°C and a flow rate of 10 μL/min. Running buffer was the same as for size-exclusion chromatography. Streptavidin was coupled to a CM5 sensor chip (Cytiva, Marlborough, MA) and purified on Ni²⁺-NTA agarose (Invitrogen, Carlsbad, CA) (16). The molar ratio of peptide/streptavidin was kept below 0.25 to avoid generating streptavidin complexes with more than one peptide. Usually, FC1 and FC3 were used as references. Before SHP-1 injection, the chip surface was conditioned with 10 injections of the running buffer, and SHP-1 was then injected over all FCs; the duration of injections was the same for conditioning and SHP-1 injection (45 s).

**Solution assay for allosteric activation of SHP-1**

The reaction mixture contained (final concentrations) 80 mM HEPES (pH 7.4), 1 mM dithiothreitol, 60 μM PEG0-PD-1 peptide, 5% dimethyl sulfoxide (DMSO, vehicle), 10 mM p-nitrophenyl phosphate, and 0.1 mM Na₂VO₃. Absorbance at 405 nm was measured on the Nanodrop ND-2000. In the control, the quenching solution was added before SHP-1, and the mixture was kept either on ice or at 37°C for the duration of the time course. The efficiency of quenching was confirmed by the absence of a difference in absorbance between samples kept on ice or at 37°C.

**MPDPDE model and parameter fitting**

We have previously derived a multicenter particle density partial differential equation (MPDPDE) model that accurately captures the stochastic and spatial features of tethered reactions in SPR (16). The nondimensional MPDPDE system is as follows:

\[
\frac{\partial n_A}{\partial t} = -\left(p_1 + p_3\right)n_A + p_2n_B
\]

\[
-4\pi\left(\frac{3}{2\pi}\right)^{3/2}p_3n_A n_B \int_0^\infty dr' \left[(r')^2 e^{3(r')^2/2} Y(r')\right],
\]

\[
\frac{\partial n_B}{\partial t} = p_1n_A - p_2n_B,
\]

\[
\frac{\partial Y}{\partial t} = \frac{p_1n_A}{n_B}(X_A - Y) + \frac{p_2n_B}{n_A}(X_B - Y)
\]

\[
-\left(\frac{3}{2\pi}\right)^{3/2} p_A e^{-3(r')^2/2} Y,
\]

\[
-2\pi \left(\frac{3}{2\pi}\right)^{3/2} p_3 n_B Y \int_0^\infty dr' \int_{|r-r'|}^{r+r'} dq \left[qr' e^{3(r')^2/2} Y(r')\times (X_B(q) - 1)\right],
\]

\[
\frac{\partial X_A}{\partial t} = \frac{2p_2n_B}{n_A}(Y - X_A),
\]

\[
-4\pi\left(\frac{3}{2\pi}\right)^{3/2} p_3 n_B X_A \int_0^\infty dr' \int_{|r-r'|}^{r+r'} dq \left[qr' e^{3(r')^2/2} Y(r')\times (Y(q) - 1)\right].
\]

**TABLE 1 Peptides used in this study**

| Name     | Sequence                                                                 |
|----------|---------------------------------------------------------------------------|
| PEG28-PD-1 | biotin-(PEG)₂₈-SVPEQTYE*ATIVFPFG                                       |
| PEG12-PD-1 | biotin-(PEG)₁₂-SVPEQTYE*ATIVFPFG                                       |
| PEG6-PD-1  | biotin-(PEG)₆-SVPEQTYE*ATIVFPFG                                        |
| PEG3-PD-1  | biotin-(PEG)₃-SVPEQTYE*ATIVFPFG                                        |
| PEG0-PD-1  | biotin-SVPEQTYE*ATIVFPFG                                                 |
| PD-1       | biotin-SRAARTIGARRTGQPPLKEDPS/AVPFSVDYGELDFQ WREKTP                     |
|           | EPPVPVSPQTYE*ATIVFPFG                                                   |
| SLAM       | biotin-QLRRRGTHTNYQVTVEKK SLTYYAVQKPGQLKLD SFPQ QDCTTYYVAATEPVPESQVET  |
|           | NSITVY*ASVTILPES                                                        |

Phosphotyrosines are denoted as Y*.
and
\[
\frac{\partial X_B}{\partial t} = \frac{2p_1 n_A}{n_B} (Y - X_B),
\]
with initial conditions \( n_A(t = 0) = 1, n_B(t = 0) = 0, X_A(t = 0, r) = 1, X_B(t = 0, r) = 1, \) and \( Y(t = 0, r) = 1. \) In these equations, \( A \) refers to the free phosphorylated peptide, and \( B \) refers to the SHP-1-bound phosphorylated peptide. Specifically, \( n_A \) and \( n_B \) represent the concentration of free phosphorylated peptide and SHP-1-bound phosphorylated peptide, respectively, and \( X \) and \( Y \) describe the auto- and pair correlations, respectively. The five fitting parameters \( p_1, p_2, p_3, p_4, \) and \( p_5 \) are related to the five biophysical and biochemical constants as follows: \( p_1 = k_{on} \) [SHP-1], \( p_2 = k_{off} \) [Peptide], \( p_3 = k_{cat}(\text{tethered}) \times \text{[Peptide]}, p_4 = k_{cat}(\text{tethered}) \sigma^*, \) and \( p_5 = k_{cat}(\text{solution}) \times \text{[SHP-1]} \). The complete derivation can be found in our previous work (16).

These data exhibited nonspecific binding of the enzyme to the surface that differed in magnitude between the control and experimental FCs and, as a result, produced a different baseline before and after the SHP-1 injection. We therefore modified the original model to include nonspecific binding with rate \( n_{ns} \) that changed linearly with time (see Fig. S2) between the start \( (p_{\text{start}}) \) and end \( (p_{\text{end}}) \) of the SHP-1 injection. Therefore, the equation that we fitted directly to our SPR traces, which report the amount of buffer resumes.

For the initial guess of \( k_{on} \), we first fit an exponential curve to the SPR time series data in the dissociation phase after SHP-1 injection ceases (e.g., after \( t = 45 \) s in Fig. 2 A). We find the parameters generated by simulated annealing are in close agreement with parameters found from MATLAB’s (The MathWorks Natick, MA) least-squares curve fitting (lsqcurvefit) function (data not shown). However, the sum of square error for the parameters found using simulated annealing is consistently smaller. We perform simulated annealing three times on each data set, using the fit with the lowest sum of square error for our analysis. All model evaluation and fitting are implemented in MATLAB 2017b.

To test for under-constrained parameter fitting, we perform a Markov chain Monte Carlo (26,27). Specifically, we use the Metropolis-Hastings algorithm (27,28) with flat, unbounded priors for \( p_1, p_2, p_3, p_4, p_5, \) and \( p_{\text{start}} \). We bounded \( p_{\text{start}} \) to be less than 0.3 s. The Metropolis algorithm proposes configurations using a perturbation size that is adaptive, increasing or decreasing until the acceptance rate is 0.44 (26). We repeat parameter proposals until the sequence of samples has reached a stationary distribution, which we define when the third quarter and fourth quarter of the sequence have the same distribution according to the Kolmogorov-Smirnov statistics. The resulting posteriors are shown in Fig. S3. All of the parameters exhibit compact posterior distributions in which most of the probability mass is concentrated in a single peak. Some weak correlations are evident, but these are away from the peaks. This suggests that the parameters can be independently determined.

Estimation of molecular reach

The molecular reach of the reaction, \( L \), in our SPR assays is influenced by the reach for the tether, \( L_{\text{tether}} \) and the reach of the enzyme, \( L_{\text{SHP-1}} \). For a worm-like chain model, the probability density of a site on the molecule at location \( x \) is

\[
P(x; L) = \frac{3}{2\pi L^2}^{3/2} \exp\left(-\frac{x^2}{2L^2}\right),
\]

where \( L \) is a property of the molecule. For a worm-like chain model, \( L = \sqrt{2L_c}\), where \( L_c \) is the contour length and \( l_p \) is the persistence length. However, we note that Eq. 1 arises in more general molecular models, so we use it to describe the behavior of the enzyme, without the interpretation of \( L \) in terms of a contour length and persistence length. In (16), we show that this leads to a local concentration kernel

\[
\sigma(r) = \frac{3}{2\pi L^2}^{3/2} \exp\left(-\frac{3r^2}{2L^2}\right),
\]

where \( r \) is the distance between the anchors of the two tethers and

\[
L = \sqrt{L_{\text{tether}}^2 + L_{\text{SHP-1}}^2}.
\]

For disordered domains and PEG linkers, we interpret \( L \) in terms of the worm-like chain model (20,29), so the reach can be estimated from the contour length and the persistence length of the domain. For the constructed PEG-PD-1 peptides, the contour length (from the surface anchor to binding site of SHP-1) is the number of PEG linkers \( N_{\text{PEG}} \) times the length of a single PEG, \( l_{\text{PEG}} \approx 0.4 \) nm (30). From this, we derive an approximation for the reach of SHP-1,

\[
L^2 = 4 \times N_{\text{PEG}} \times l_{\text{PEG}} \times l_p + L_{\text{SHP-1}}^2,
\]

predicting that the reach is given by the intercept of the line \( L^2 \) vs. \( N_{\text{PEG}} \).

Uncertainty quantification for derived parameters

For each PEG length, \( L^2 \) is calculated by averaging the fitted parameter \( \sigma^* \) for all replicates and transforming the average to a single \( L^2 \)-value for the peptide. Error propagation is used to convert the standard deviation of \( \sigma^* \) to an error for \( L^2 \).
Best-fit lines with associated R-values and p-values for PEG28-PD1 parameters versus phosphatase and peptide concentrations, shown in Figs. 2 and 3, were determined using MATLAB’s robust fitlm function.

We use MATLAB’s anova1 and multcompare functions to conduct multiple comparison t-tests on paired PEG-peptide parameters to establish significant differences. Pairs that are significantly different at the 0.05, 0.01, and 0.001 level are shown.

Implications of reach for reactions at the cell membrane

For a given receptor density \( \rho_0 \), the effective concentration experienced by a substrate is

\[
C_{\text{eff}} = \iint \sigma(r) \rho_0 \, dA
\]
1 injection, for example, visible in Fig. 2, B and C. Using alkaline phosphatase, we found that this difference was not a result of phosphate mass being lost from the surface (Fig. S1) but rather by nonspecific binding of the enzyme (Fig. S2). We therefore extended the MPDPDE model to capture nonspecific binding by introducing three additional parameters \( p_{\text{start}}, p_{\text{stop}}, p_{\text{nonsb}} \), and in addition, we included the dissociation phase in the fit; see Methods for details.

With these changes, we found that the extended eight-parameter MPDPDE model \( (k_{\text{on}}, k_{\text{off}}, k_{\text{cat(tethered)}}, \sigma^*, k_{\text{cat(solution)}}, p_{\text{start}}, p_{\text{stop}}, p_{\text{nonsb}}) \) closely fits the 37°C SPR data (e.g., Fig. 2, B and C). We perform Markov chain Monte Carlo analysis to assess whether the parameters can be uniquely identified and found this to be the case (Fig. S3).

We next determined whether the fitted parameters were independent of the SHP-1 and PEG28-PD-1 concentrations. We repeated the experiments at various concentrations and found that the fitted parameters associated with catalysis \( (k_{\text{cat(tethered)}}, k_{\text{cat(solution)}}, \text{and } \sigma^*) \) were independent of concentrations (Fig. 2 D, correlations are not significant). However, binding parameters \( (k_{\text{on}}, k_{\text{off}}, \text{and } K_D = k_{\text{off}}/k_{\text{on}}) \) exhibited a correlation with SHP-1 concentration, with a significant correlation for \( k_{\text{on}} \) and \( K_D \) after correcting for multiple hypotheses (indicated by red asterisks in Fig. 2 D). This correlation may arise because higher concentrations of SHP-1 could lead to steric crowding effects on the surface, whereby volume exclusion reduces the ability for more SHP-1 molecules to bind to the surface reducing apparent binding. We concluded that the catalytic parameters, including reach, can be determined using this fitting procedure.

Isolating the molecular reach of SHP-1 by varying the tether length

The molecular reach of the reaction, \( L = (\sigma^*)^{-1/3} \), involves two components: the reach of the PEG-peptide tether and the reach of the enzyme. As the reach contributed by the tether is progressively decreased (e.g., by shorter tethers), eventually the molecular reach of the reaction will be wholly determined by the reach of the enzyme. Indeed, assuming that the reach of the tethers and enzyme can be effectively modeled by worm-like chains, an equation can be derived to relate \( L \) with the contour length of the tether (Eq. 4; see Methods). This model predicts that the squared molecular reach of the reaction should be linearly related to the length of the tether (Eq. 5), with the reach of the enzyme being the vertical intercept (i.e., when the tether length is nil).

Therefore, we performed the SPR-based assay using a different number of PEG repeats \( (N_{\text{PEG}} = 0, 3, 6, 12, 28) \) coupled to the same short PD-1 ITSM peptide (Fig. 3 A). As before, the extended MPDPDE model was able to fit the data and produced binding and catalysis parameters that were similar for different length PEG linkers with the exception of \( \sigma^* \), which progressively increased as the

\[
\rho_0 - \frac{L_{\text{run}}}{r} = \left(\frac{3}{2\pi}\right)^{3/2} \rho_0 L_{\text{run}}. 
\]
number of PEG linkers was reduced (Fig. 3 B). This is expected because with shorter PEG linkers, the local volume that SHP-1 is confined to decreases, thereby increasing local concentration.

As expected, the squared molecular reach of the reaction (determined by converting the averaged $\sigma^*$ to $L$) increased with the number of PEG linkers (Fig. 3 C). Using regression on all data except PEG0, we determined the vertical
intercept, and hence the molecular reach of SHP-1, to be \( L_{\text{SHP-1}} = 13.0 \pm 0.8 \) nm. This value is between estimates obtained using crystal structure and maximal stretch (Fig. 1 B).

When directly coupling the PD-1 peptide without any PEG repeats (PEG0), we found a molecular reach of 10.9 ± 0.3 nm. Although this value is also within theoretical estimates for the reach of SHP-1 and similar to the value obtained by the intercept method above, we reasoned that it may be less accurate because this very short peptide can introduce steric hindrance to binding and catalysis (e.g., by more readily adopting conformations in which the binding site is near the surface), which is reflected in the larger value of \( K_D \) and smaller value of \( k_{\text{cat(solution)}} \) that this peptide produces compared with peptides with PEG linkers.

PD-1 contributes less than SHP-1 to the molecular reach of the reaction

Given that the molecular reach of the reaction is determined by both the enzyme and tether, we next sought to determine the molecular reach of the receptor tail. We injected SHP-1 over immobilized peptide corresponding to the cytoplasmic tail of PD-1 from the membrane to the ITSM. This N-terminally biotinylated peptide contained 64 aa, with the phosphorylated tyrosine in the ITSM being 55 aa from the membrane (position 248 in the native sequence). The extended MPDPDE model was fitted to the SPR traces (Fig. 4 A) and provided estimates of the biophysical parameters (Fig. 4 B).

Using the value of \( \sigma^* \), we calculated the combined molecular reach of the reaction for PD-1-bound SHP-1 acting on PD-1 to be 16 nm. Given that we already obtained an estimate for the reach of SHP-1, we were able to back calculate the reach of PD-1 (see Eq. 4) to be 6.55 nm (Fig. 4 C). Thus, we find that PD-1 contributes less to the overall molecular reach of the reactions compared with the SHP-1 reach contribution of 13.0 nm.

We note that the worm-like chain model would predict a 3.0 nm reach for the PD-1 peptide we have used, assuming a persistence length of 0.4 nm that applies to random aa chains (20,25). Therefore, the experimentally measured reach of PD-1 appears to be twice that predicted by the worm-like chain model, suggesting a preference for extended conformations of this peptide.

The binding affinity between SHP-1 and singly phosphorylated PD-1 was determined to be 11 ± 2 \( \mu \)M. Using a different assay, Hui et al. (5) reported an affinity of 4.28 \( \mu \)M. The ~2-fold higher affinity they report is likely a result of using a doubly phosphorylated PD-1 peptide.

\[
L_{\text{SHP-1}} = \left( \frac{L^2 - L_{\text{PEG}}^2}{2} \right)^{1/2}, \text{ where } L \text{ is the molecular reach of the reaction calculated from } \sigma^* \text{ in (B) and } L_{\text{SHP-1}} = 13.0 \text{ nm. To see this figure in color, go online.}
\]
The ratio of $k_{\text{cat(tethered)}}/k_{\text{cat(solution)}}$ provides an estimate for the strength of allosteric activation of SHP-1 upon SH2-domain binding to PD-1. We find a modest twofold increase in activity from this effect (Fig. 4 B). Because larger fold increases have been reported previously (9,11,16), we further explored this finding. First, we used a standard solution assay whereby SHP-1 acted on a low-molecular-weight synthetic substrate and confirmed that catalytic activity increased only twofold upon addition of a phosphorylated PD-1 peptide (Fig. S4). Second, we previously reported a larger allosteric activation for murine SHP-1 binding to the inhibitory receptor LAIR-1, but at 10°C, and therefore performed experiments at this lower temperature, finding again only a modest increase in activity (Fig. S5). We conclude that human SHP-1 exhibits only modest allosteric activation upon binding to singly phosphorylated PD-1.

As a positive control to ensure our SPR-based assay is sensitive to reach, we repeated the experiments using the longer cytoplasmic tail of SLAM, a surface receptor that is also known to recruit SHP-1 (32). The N-terminally biotinylated peptide contained 77 aa, with the phosphorylated tyrosine in the ITSM being 69 aa from the membrane (position 327 in the native sequence). Performing the analysis as for PD-1, we find that the molecular reach contributed by SLAM is 20 nm (Fig. 4). This is markedly more than the reach of SHP-1 and comprises 72% of the predicted contour length for the SLAM peptide ($l_c \sim 69 \times 0.4 \text{ nm} = 27.6 \text{ nm}$). This suggests that SLAM has a larger persistence length than would be expected for random aa’s and/or is otherwise biased toward extended conformations.

Interestingly, we observed a larger 6.2-fold allosteric activation for SHP-1 interacting with SLAM (Fig. 4 B), and this is highlighted when plotting the ratio of $k_{\text{cat(tethered)}}/k_{\text{cat(solution)}}$ across all experimental conditions (Fig. S6). However, this allosteric activation for SLAM was a result of a lower $k_{\text{cat(solution)}}$, not a higher $k_{\text{cat(tethered)}}$, compared with PD-1. We also observe a much smaller on rate for SHP-1 binding to SLAM compared with PD-1. A possible explanation for both observations is that the SLAM peptide may have fewer configurations in which the phosphorytosine is available for interaction with SHP-1 when in solution.

Lastly, we noted that temperature had a large impact on these tethered reactions. We observed ~2-fold slower binding kinetics, ~10-fold slower catalytic rates, and an ~4.5-fold larger value of $\sigma^\ast$ (960 vs. 210 $\mu$M) at 10°C compared to 37°C using PEG28-PD-1 (Table 2). This underlined the importance of the extended SPR assay in overcoming the technical issues associated with making measurements at physiological temperatures.

### Control of surface receptor signaling by the molecular reach of SHP-1

We next used a mathematical model to explore how molecular reach regulates the activity of SHP-1 upon recruitment to an inhibitory receptor confined to the two-dimensional membrane (Fig. 5 A). The difference in receptor distribution between our experiments and the membrane is that in our experiments, the receptor is randomly distributed in three dimensions. By using a mathematical model in the previous section that accounted for this three-dimensional geometry, we are able to produce geometry-independent parameters that can now be used to predict the impact of reach for any receptor distribution, including the two-dimensional membrane distribution. Using PD-1 as a prototype, we calculated the combined reach of receptor-SHP-1 complexes as 14.6 nm ($\sqrt{L_{PD-1}^2 + L_{SHP-1}^2}$). Using this number, we first consider the effective concentration of SHP-1 that a substrate would experience when receptors are randomly distributed on the membrane. At typical physiological densities of inhibitory receptor, this effective concentration is ~1000 $\mu$M (Fig. 5, B and C), which is ~1000-fold larger than the ~1 $\mu$M concentration of SHP-1 in the cytosol, assuming it is uniformly distributed (16,31).

In these tethered reactions, even though the effective concentration can be large, the coverage can in principle be low because a random or uniform distribution of surface receptors can allow some substrates to be out of reach (Fig. 5 A). We therefore calculated the fraction of substrates that can be accessed by receptor-SHP-1 complexes for different values of the molecular reach of the reaction and receptor density (Fig. 5, E and F). If receptors are uniformly distributed on the cell surface, we estimate that they are only able to

### Table 2: Average biophysical parameter values for each peptide

| Substrate | N | $k_{\text{on}}$ ($\mu$M$^{-1}$ s$^{-1}$) | $k_{\text{cat}}$ (s$^{-1}$) | $K_\theta$ (M) | L (nm) | $\sigma^\ast$ ($\mu$M) | $k_{\text{cat(tethered)}}$ ($\mu$M$^{-1}$ s$^{-1}$) | $k_{\text{cat(solution)}}$ ($\mu$M$^{-1}$ s$^{-1}$) |
|-----------|---|-----------------|-----------------|-------------|------|-----------------|-----------------|-----------------|
| PEG0      | 3 | 0.19 ± 0.02     | 1.9 ± 0.1       | 10.4 ± 0.2  | 10.9 ± 0.3 | 1300 ± 100      | 0.041 ± 0.008   | 0.020 ± 0.001   |
| PEG3      | 5 | 0.22 ± 0.03     | 1.6 ± 0.2       | 8.0 ± 1.0   | 13.4 ± 0.6 | 690 ± 90        | 0.047 ± 0.005   | 0.040 ± 0.009   |
| PEG6      | 6 | 0.31 ± 0.02     | 1.5 ± 0.1       | 4.9 ± 0.3   | 16.0 ± 1.0 | 410 ± 77        | 0.033 ± 0.004   | 0.027 ± 0.004   |
| PEG12     | 5 | 0.25 ± 0.02     | 1.7 ± 0.2       | 7.2 ± 0.9   | 16.0 ± 1.6 | 400 ± 100       | 0.034 ± 0.005   | 0.030 ± 0.007   |
| PEG28     | 14| 0.34 ± 0.03     | 1.8 ± 0.2       | 6.1 ± 0.8   | 19.7 ± 1.3 | 210 ± 40        | 0.042 ± 0.006   | 0.031 ± 0.007   |
| PEG28 (10°C) | 8 | 0.28 ± 0.03     | 0.8 ± 0.06      | 2.9 ± 0.2   | 12.0 ± 1.1 | 960 ± 260       | 0.0036 ± 0.0006 | 0.0047 ± 0.0007 |
| PD1       | 3 | 0.21 ± 0.03     | 2.4 ± 0.3       | 11.0 ± 2.0  | 16.0 ± 1.4 | 400 ± 100       | 0.040 ± 0.007   | 0.023 ± 0.002   |
| SLAM      | 3 | 0.02 ± 0.01     | 1.7 ± 0.2       | 130 ± 4.0   | 31.2 ± 1.2 | 55 ± 6          | 0.036 ± 0.003   | 0.0058 ± 0.001   |

All experiments conducted at temperature 37°C except where noted. Uncertainty is computed as the SE of the mean among the N different experiments (shown in second column).

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achieve a low \( \sim 15\% \) coverage of the substrates (Fig. 5, E and F). Coclustering of receptors and substrates would lead to a higher local density and could therefore be important to improve both the effective concentration and coverage. We find that a clustered density of 0.0034 nm\(^{-2}\) (\(\sim\)10-fold higher than a uniform estimate) is required to achieve a 90% coverage.

We next explored the contribution of the substrate reach to both concentration and coverage. Previously, we noted that the number of aa's between the membrane and activating or inhibiting tyrosine motifs differed with a median of 33 aa (or 13 nm) or 65 aa (or 26 nm), respectively (16). We therefore repeated the calculations by increasing the number of aa's between the membrane and activating or inhibiting tyrosine motifs differed with a median of 33 aa (or 13 nm) or 65 aa (or 26 nm), respectively (16).

**DISCUSSION**

We provide the first estimates of the molecular reach for an enzyme at physiological temperatures. The molecular reach has several implications for how membrane recruitment regulates and directs the activity of SHP-1.

The two-state allosteric activation model of SHP-1 (33,34) is based on crystal structures showing a closed auto-inhibitory conformation, in which the N-SH2 domain blocks the catalytic pocket (13), and an open conformation, in which the N-SH2 is rotated, exposing the catalytic pocket (14). Interestingly, the molecular reach of SHP-1 that we report when tethered in the higher activity state (13.0 nm) is longer than the reach obtained from the structure of the open conformation (5.3 nm). This suggests that SHP-1 utilizes flexible linkers to achieve a spectrum of open states with a longer reach.

The membrane activity of SHP-1 can be regulated not only by allosteric activation but by the molecular reach, which determines both concentration and coverage (3,24). We found that tethering increases the concentration of SHP-1 from \(\sim 1\mu M\) in solution (cytosol) to over \(\sim 1000\mu M\) when tethered (membrane), but importantly, clustering is necessary for the majority of substrates to experience this high local concentration. Interestingly, this 1000-fold increase is much larger than the twofold increase in the...
catalytic rate by allosteric activation when the SH2 domain of SHP-1 is engaged.

Although previous reports have demonstrated allosteric activation of SHP-1 using singly phosphorylated peptides engaging a single SH2 domain (9,11,16), recent reports have suggested that allosteric activation of SHP-2 requires simultaneous binding of both SH2 domains on the same (18) or across different PD-1 peptides (17). Although SHP-1 in our assay could in principle bind across two PD-1 peptides, the observed kinetics were characteristic of single SH2 domain binding and not high-affinity tandem SH2 binding, as, for example, observed for ZAP-70 and Syk in SPR (35,36). Moreover, using PD-1 peptides with both ITIM and ITSM phosphorylated produced SPR traces similar to those with only the ITSM phosphorylated (data not shown). Therefore, SHP-1 and SHP-2 may exhibit differences in their allosteric mechanisms.

There is evidence in T cells that SHP-1 and SHP-2 may function through different inhibitory receptors, with PD-1 more readily utilizing SHP-2 compared to SHP-1 (6,7,37). We found that SHP-1 bound to PD-1 with an affinity typical of SH2 domains but that binding was rapidly abolished by autoinhibition in trans, whereby SHP-1 dephosphorylated other nearby PD-1 molecules. Although this autoinhibition process was also observed for SHP-2, it took place on the minute timescale and therefore appears to be less efficient than for SHP-1 (5). This may suggest that the interaction of SHP-1 with PD-1 may be important to limit, rather than promote, the activity of PD-1.

Using mathematical modeling, we found that the molecular reach of SHP-1 tethered to inhibitory receptors means that it would only be able to reach 15% of substrates but that co-clustering at 10-fold higher density can increase coverage to 90%. Indeed, microscopy experiments have found that inhibitory receptors that can recruit SHP-1 co-cluster with their substrates (5,37,38), although the precise density is presently unknown. This result is based on the assumption that inhibitory receptors and their substrates have limited mobility within clusters. We have previously used simulations to show that increasing molecular reach can increase or decrease inhibitory receptor potency when diffusion is slow or fast, respectively (24). Although it is reasonable to expect that the diffusion coefficient of inhibitory receptors would be reduced when they bind their ligands and cluster, direct measurements have yet to be performed. Another mechanism that can potentially control molecular reach within cells is the dynamic and regulated association of the cytoplasmic tails of immune receptors with the membrane (39–42), which may allow receptor tails to adopt more extended conformations.

The cellular environment is crowded (43,44) and rheologically more complex (45,46) than the fluid environment of our assay. Crowding can effectively change the biophysical parameters that we have reported, including the molecular reach, in a manner that likely depends on the density, size, and shape of the crowding molecules (43,47,48). Although the fluid phase in our assay is dilute, we have noted that it is possible for high concentrations of SHP-1 to accumulate on the surface. This can potentially lead to crowding, explaining why the binding affinity appears to decrease as the SHP-1 concentration increases (Fig. 2 D). Studying tethered reactions in the presence of crowding agents in our assay will require overcoming two challenges: extension of the model and simulation to explicitly account for crowding and careful characterization of the relevant in vivo crowding parameters that are to be replicated. Ultimately, these in vitro experiments would benefit from direct in vivo measurements of reach, in which discrepancies between measurements can shed light on both passive and active mechanisms that may be acting in vivo.

The experimental assay and subsequent mathematical analysis we have used can readily be implemented in SPR. An important assumption of the mathematical analysis is that the peptides are randomly distributed. However, given that SPR is based on a flow chamber, it is conceivable that more peptide is deposited near the injection inlet. To reduce this bias, immobilization takes place using a fast flow rate so that a similar peptide concentration is experienced by the entire flow cell. In the future, a complementary method can be used whereby peptides are immobilized at defined distances using DNA origami platforms that themselves are immobilized in SPR. This has recently been used to study antibody-antigen interactions in SPR (49).

It is increasingly clear that cellular signaling relies on tethered reactions (3,19,50,51), and studies have shown how tethering can increase the rate of these intramolecular reactions (20,21). A feature of tethered reactions by immune receptors and many other membrane-confined reactions is that they are intermolecular. This work has highlighted that at typical receptor densities, the short molecular reach of the reaction means that other processes, such as co-clustering, are required for efficient signaling, and moreover, small nanometer changes in molecular reach can have large changes on receptor potency. This suggests the possibility of modulating receptor activity by molecular reach inhibitors that can target unstructured receptor tails or flexible linkers within enzymes, which can have advantages over the targeting of structured domains (52–54).

SUPPLEMENTARY MATERIAL
Supporting material can be found online at https://doi.org/10.1016/j.bpj.2021.03.019.

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