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Functional characterization and conformational analysis of the *Herpesvirus saimiri* Tip-C484 protein

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SUMMARY

Tyrosine kinase interacting protein (Tip) of *Herpesvirus saimiri* (HVS) activates the lymphoid-specific Src-family kinase Lck. The Tip:Lck interaction is essential for transformation and oncogenesis in HVS-infected cells. As there are no structural data for Tip, hydrogen exchange mass spectrometry was used to investigate the conformation of a nearly full-length form (residues 1-187) of Tip from HVS strain C484. Disorder predictions suggested that Tip would be mostly unstructured so great care was taken to ascertain whether recombinant Tip was functional. Circular dichroism and gel filtration analysis indicated an extended, unstructured protein. *In vitro* and *in vivo* binding and kinase assays confirmed that purified, recombinant Tip interacted with Lck, was capable of strongly activating Lck kinase activity and was multiply phosphorylated by Lck. Hydrogen exchange mass spectrometry of Tip then showed that the majority of backbone amide hydrogens became deuterated after only 10 seconds of labeling. Such a result suggested that Tip was almost totally unstructured in solution. Digestion of deuterium labeled Tip revealed some regions with minor protection from exchange. Overall, it was found that although recombinant Tip is still functional and capable of binding and activating its target Lck, it is largely unstructured.

Keywords

Lck; unstructured protein; hydrogen exchange; mass spectrometry; kinase activation

INTRODUCTION

Herpesvirus saimiri (HVS) is a rhadinovirus regularly found in South American squirrel monkeys (reviewed in ¹). HVS does not cause disease in its natural host but in New World primates like marmosets and owl monkeys, HVS strains A and C cause fatal acute

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lymphoproliferative diseases (reviewed in ²). HVS strain C is the most oncogenic and can transform human, rabbit and rhesus monkey lymphocytes ³⁻⁶.

In HVS strain C, the production of tyrosine kinase interacting protein (Tip) is responsible for oncogenic transformation ^{3,7-14}. There are at least two variants of Tip, differing by a 42 amino acid deletion in the N-terminal region. Tip-C484 was found in HVS strain C484 and contains 214 amino acids while Tip-C488 was found in HVS strain C488 and contains 256 amino acids. Tip-C484 is a 23.2 kDa protein shown to bind to and activate the lymphoid-specific Src-family tyrosine kinase Lck (reference 7, reviewed in ^{2,15}). Tip interacts with Lck through two motifs termed LBD1 (aka SH3B) and LDB2 (aka CSKH) ¹⁶⁻²⁰ (see Figure 1A). The region between LBD1 and LBD2 has also been shown to be important for Lck association, perhaps by providing a spacer. Given the importance of Tip in HVS-induced cellular transformation, a better understanding of the interaction between Tip and Lck and the structural details of the interaction is desirable.

While the physiological impact of Tip on HVS-infected cells has been studied, much less is known about the structural details of the Tip protein. Tip is anchored to the membrane by a short C-terminal membrane spanning region, leaving the remaining ~80% of the protein in the cytoplasm ⁹. Analysis of short Tip peptides spanning the LBD1 region, an SH3 binding motif matching the consensus sequence for class II polyproline helices, has been performed with both NMR and circular dichroism ^{17,21}. Although LBD2 shares some sequence homology with the Lck kinase domain and may interact with the C-terminal half of Lck ^{17,18}, NMR of a peptide spanning both LBD1 and LBD2 (Tip 140-191) demonstrated that polyproline helix in LBD1 is the only structure in this region ¹⁹. Outside of residues 140-191, there is no information for the conformation of the full-length Tip protein.

A major stumbling block in obtaining conformational data for Tip is that the protein may not be structured. Predictions of the disorder in the Tip protein indicate that the protein is likely to be mostly disordered (Figure 1A). As such, full-length Tip is not amenable for crystallography or for conventional NMR analysis. Overexpression and purification of significant quantities of material for conformational analysis are also difficult for many unstructured proteins. In addition, any overexpression and purification procedure must address whether or not the purified material is biologically active prior to conformational investigations. In spite of the difficulties, and given its significant role in T-cell transformation, a detailed knowledge of Tip conformation would be valuable.

In the current study, the full-length cytoplasmic portion of Tip (residues 1-187 from strain C484) was overexpressed in bacteria and purified. Recombinant full-length Tip protein was highly active in both Lck binding and phosphorylation assays *in vitro* and was capable of activating Lck in cells. Confident that the prepared protein represented a state with the correct biological function and activity, the conformation of full-length Tip was investigated by hydrogen exchange mass spectrometry (HX MS). Because only picomoles of material were required for HX MS analysis, it was possible to work with the small quantities of full-length Tip that could be prepared. Remarkably, HX MS results indicated that the protein had little or no structure in solution and that Tip should be added to the growing list of functional yet intrinsically unstructured proteins.

RESULTS and DISCUSSION

Overexpressed Tip

A vector encoding the cytoplasmic component of Tip from strain C484 (missing residues 188-214 from the whole protein) was overexpressed in *E. coli* [this protein is referred to as full-length Tip]. A large percentage of the overexpressed protein went to inclusion bodies and

adjusting the growth conditions to reduce the formation of inclusion bodies was not effective (data not shown). Tip was isolated from inclusion bodies with denaturant and renatured as described in the Materials and Methods. Renatured Tip was stable at room temperature and on ice for 15 hours and the protein could be stored for several weeks at -80 °C with no adverse effects to sample integrity or difference in the results. All the data presented below are for renatured protein that was isolated from inclusion bodies. In addition, a small amount of soluble protein was isolated from *E. coli* overexpression and analyzed. In multiple trials, there was no difference in any of the results for the soluble or renatured Tip.

A variety of experimental observations suggested that recombinant full-length Tip was unstructured (see also ¹⁸). A PONDR (Predictors Of Natural Disordered Regions) prediction ²² suggested that Tip would be mostly disordered (Figure 1A), although a few regions of potential order were predicted to exist around residues 80-100. Analytical gel filtration was performed to determine if the purified, renatured protein was monomeric. The results showed that full-length Tip, with a theoretical molecular weight of 23.2 kD, eluted at a retention time similar to BSA, a 66 kD protein (Figure 1B). Based on size alone, monomeric Tip was expected to elute significantly after BSA. As gel filtration reports on both size and shape, it is useful to consider the theoretical dimensions of Tip and BSA. The theoretical radius of gyration for a globular protein the size of Tip residues 1-187 is ~47 Å (calculated according to ²³) while that of BSA is ~85 Å. Because the gel filtration standards are globular proteins under the conditions used, the retention time of Tip either indicated that it was a trimer or that it had an extended conformation causing it to appear larger in gel filtration than a 23.2 kD globular protein. Based on the HX MS results that are presented and discussed below, it was concluded that Tip was not trimeric in solution but that it was monomeric and non-globular. Note that only one peak for Tip was observed in gel filtration and that there was no evidence for a mixed population involving other potential oligomeric forms. Circular dichroism of the recombinant, renatured full-length Tip was consistent with an absence of secondary structure (Figure 1C). Taken together, the data in Figure 1 show that Tip does not exhibit attributes of a conventionally folded, globular protein.

Recombinant Tip was functional

Because the full-length Tip studied with gel filtration and circular dichroism was renatured from inclusion bodies, we were concerned that perhaps it appeared unstructured in our assays because it was not able to refold correctly. To confirm that recombinant Tip was functional, a variety of functional assays were performed.

Tip is known to bind to the SH3 domain of the Src-family kinase member Lck ⁷. A glutathione-S transferase (GST) pull-down assay was performed to test whether recombinant, renatured Tip could bind to Lck SH3. When purified Tip was incubated with a GST-LckSH3 fusion protein immobilized on GSH beads, an additional band corresponding to the Tip protein appeared in the pull-down assay (Figure 2, lane 5, far right). Controls were run concurrently and indicated that Tip was not directly interacting with the GSH beads or GST (lanes 3, 4). Therefore, in spite of the apparent lack of structure suggested by the data in Figure 1, renatured Tip was still capable of binding to Lck SH3.

Interaction with Tip leads to Lck kinase activation and Tip phosphorylation *in vitro* ^{7,17,18,24}. The activity of recombinant, renatured Tip was tested by monitoring the phosphorylation state of Tip before and after incubation with Lck. In these experiments, a downregulated form of recombinant Lck termed LckYEEI was used. Similar to what was done to facilitate Hck crystallography ²⁵, this form of Lck has the first ~ 80 N-terminal amino acids removed and multiple mutations in the C-terminal tail that change the wild-type tail sequence to the sequence YEEI. HckYEEI tail mutants are crystallographically indistinguishable from the wild-type proteins but are downregulated due to the higher affinity of the C-terminal tail for the SH2

domain. LckYEEI is highly downregulated but can still be selectively activated by Tip *in vitro* and *in vivo*²⁶. When recombinant, renatured Tip was incubated with LckYEEI, Tip was able to activate Lck and itself became doubly phosphorylated (Figure 3). Consistent with previous observations^{7,16-18,24,27}, Tip appeared as multiple bands in SDS-PAGE, all of which have larger apparent molecular weights than the true mass of Tip, 23.2 kD. In the gel in Figure 2 there were three bands while the gel in Figure 3 there were two bands. When combined with the gel filtration results from above, the observation of multiple bands is suggestive that Tip is not a traditional, globular protein or, as speculated previously, it is differentially phosphorylated and therefore runs uncharacteristically on gels. We show below, particularly with mass spectrometry, that the later is likely not the case. In Figure 3, both bands were shifted to higher apparent molecular weight upon incubation with LckYEEI (Figure 3A) indicating that Tip was capable of activating LckYEEI such that LckYEEI could then phosphorylate Tip. The gel indicates that LckYEEI was also phosphorylated in the process (see also below), but this is not necessarily a requirement for Tip:Lck interaction²⁴. Electrospray mass spectra of Tip (same material as in Figure 3A, lane 1) indicated that although Tip appeared as multiple bands in SDS-PAGE, there was in fact only one species with the correct molecular weight (Figure 3B). In the full m/z spectrum (data not shown) there was no evidence for other species. Mass spectra of Tip after interaction with LckYEEI indicated that Tip became doubly phosphorylated. Peaks corresponding to +1 and +2 phosphorylations (+80 for each phosphate) were readily apparent in the mass spectra. The sites of phosphorylation will be reported separately (manuscript in preparation). As further confirmation of Tip-induced Lck activation, LckYEEI activity towards a peptide substrate was assayed *in vitro* in the absence or presence of purified Tip at different molar ratios (Figure 3C). LckYEEI was strongly activated by Tip even at a 1:1 molar ratio. In contrast to Tip, a non-activating control protein (HIV-1 Nef) did not affect LckYEEI activity even when present in a 20-fold molar excess. These results show that renatured Tip was functional and active towards LckYEEI *in vitro*.

To test whether the cytoplasmic portion of Tip (residues 1-187) was sufficient to activate Lck in cells, we turned to a yeast expression system. Yeast cells provide a useful model system for the study of Src family kinase regulation because they do not express orthologs of c-Src or other mammalian tyrosine kinases²⁸. However, ectopic expression of c-Src and other Src family members causes growth arrest in yeast that correlates with kinase activity, providing a simple assay for kinase activation in cells^{26,28,29}. Tip and Lck were expressed either alone or together in yeast, followed by analysis of growth and protein-tyrosine phosphorylation. As shown in Figure 4, expression of Tip alone had no effect on yeast cell growth or protein-tyrosine phosphorylation. Expression of wild-type Lck alone produced mild growth inhibition, consistent with its low basal kinase activity in yeast. However, co-expression of the two proteins produced a dramatic inhibition of yeast cell growth that correlated with a strong increase in tyrosine phosphorylation of yeast cell proteins, consistent with Tip-induced Lck activation.

To determine whether Tip could also activate a tail-phosphorylated, down-regulated conformation of Lck, we co-expressed Tip with LckYEEI. As described above, the YEEI modification induces autophosphorylation of the tail and subsequent downregulation in a manner similar to co-expression with Csk²⁸. Figure 4 shows that LckYEEI did not induce growth suppression on its own, consistent with effective downregulation in yeast. However, coexpression of LckYEEI with Tip induced strong growth suppression and a parallel increase in protein-tyrosine phosphorylation similar to that observed with wild-type Lck. Co-expression of the HIV-1 Nef protein, which does not interact with Lck, failed to activate either Lck or LckYEEI. These results show that cytoplasmic Tip residues 1-187 are sufficient to activate Lck in cells, and support the use of this Tip region for conformational studies with hydrogen exchange and mass spectrometry.

Hydrogen exchange and protein conformation

The mechanisms of hydrogen exchange (HX) and its utility for investigating protein conformation in combination with mass spectrometry have been reviewed³⁰⁻³². Briefly, protons that are located at amide linkages (also referred to as the backbone amide hydrogens) in proteins undergo replacement with deuterons naturally. In folded proteins the rate of amide hydrogen exchange can be dramatically reduced over that in unfolded proteins as a result of restricted solvent access and intramolecular hydrogen bonding. It is not possible to differentiate between solvent access and hydrogen bonding by HX as the two factors occur concomitantly. Hence, elements of secondary structure cannot be identified. Protection from exchange, however, is readily apparent and is diagnostic for the presence of structure. Subtle changes to protein conformation can also be monitored by measuring hydrogen exchange rates in different states of the same protein (folded vs. unfolded, active vs. inactive, bound vs. unbound, etc.).

Intact Tip HX analysis

Deuterium incorporation into recombinant Tip was analyzed with electrospray mass spectrometry. Initial spectra without deuterium were taken to be certain that Tip was amenable to electrospray and to gauge spectral quality and limits of detection. The experimental molecular mass was 23240.25 (theoretical mass, 23241.77) and high quality spectra were possible with as little as 100 picomoles of material (data not shown). These results established that Tip was suitable for HX MS analysis.

A four hour HX time-course experiment was conducted for recombinant Tip (Figure 5). Only one population of Tip molecules existed as all the mass spectra of deuterium labeled Tip had binomial isotope patterns (explained in detail in Ref³³) (Figure 5A). The average mass at each incubation point was determined and plotted. The data were adjusted to account for the loss of deuterium during analysis due to back-exchange. An increase of 174 Da was observed for the first time point of 10 seconds (Figure 5B). The maximum possible mass increase due to isotope exchange at all backbone amide hydrogens was 191 Da (206 residues minus 14 proline residues minus the N-terminal NH, which exchanges rapidly³⁴). Since deuteration within 10 seconds at neutral pH occurs primarily for backbone amide hydrogens that are highly solvent exposed and not involved in intramolecular hydrogen bonds³⁵, these Tip deuterium levels suggested that a large portion of the protein was not hydrogen bonded and was highly solvent exposed. Furthermore, the deuterium level of Tip remained unchanged over the entire hydrogen exchange time course, with a difference of only ~3 Da between the 10 second and 4 hour time points.

Multiple purifications of 6xHis tagged, full-length Tip were performed and analyzed with HX MS to verify the deuterium levels. In addition to the Tip that had been renatured from inclusion bodies, minute quantities of soluble Tip were also purified without denaturation and analyzed by HX MS. In all cases, the deuterium levels were within 2-3 Daltons for each time point. This result implied that conformations of the soluble and renatured proteins were the same and the lack of structure was not a side-effect of protein renaturation from inclusion bodies.

Approximately ten percent of the backbone amide positions in Tip were protected from exchange in the intact protein HX MS analyses and did not become deuterated even after four hours of labeling. To explore the possible involvement of the affinity tag in the protected regions of Tip, the 6xHis tag was cleaved off and an HX MS experiment was performed on the cleavage product. After thrombin cleavage, the first 16 residues (15 backbone amide hydrogens) were removed. Of the 190 amino acids in the cleaved Tip protein, there were 175 backbone amide hydrogens that could be followed (less prolines and the N-terminus). After back-exchange correction, the thrombin-cleaved, renatured Tip protein incorporated 170 deuterium atoms after 10 seconds of labeling (95% labeled) (data not shown). The magnitude

of exchange into Tip without a 6xHis tag (~170 D) closely matched the exchange level into Tip with the 6xHis affinity tag (~174 D). It can be concluded that removal of the affinity tag had little consequence on the number of deuteriums that exchanges into Tip and that most or all of the protection from exchange that was observed in intact Tip could be attributed to the affinity tag. The HX MS peptide data below also suggest that there was protection from exchange in the N-terminal regions of Tip that contained the affinity tag.

We ruled out the possibility that Tip was trimeric (above) for multiple reasons. The possibility that the 6xHis tag caused Tip to oligomerize was ruled out because gel filtration results with and without the 6xHis tag were identical (data not shown). The lack of protection from exchange in Tip also argues against the formation of trimers in solution. If Tip formed trimers in solution, some regions would be expected to be protected from exchange. Certainly there would be evidence at the earliest exchange-in time points if protection occurred due to trimer formation. As the intact protein data and the peptide data below indicate, there was almost no protection and the much more likely explanation for the retention time of Tip in gel filtration was that it was not a globular protein.

Localizing HX to short fragments

Deuterium incorporation for full-length Tip indicated that of the 191 backbone amide hydrogens available for exchange, only ~20 were resistant to exchange. Exchange at the protein level did not provide information about where these changes might be. To establish which regions of Tip were partially protected, labeled Tip was digested with pepsin and analyzed by MS. Peptic peptides covering 95% of the Tip sequence were identified with MS/MS experiments (see Supplementary Table 1). The deuterium incorporation into each of the peptides was determined. Example results for four peptides are shown in Figure 6. Most peptides were heavily deuterated and even after 10 seconds of labeling, they resembled the deuteration level of the heavily deuterated control. Again, these results suggested that renatured Tip was largely unstructured as deuterium levels of this magnitude would only be observed for highly-solvent exposed residues not involved in hydrogen bonding.

Previous structural analysis of short Tip LBD1 and LBD2 peptides indicated that, aside from a short polyproline helix, no structural elements were present, even after the addition of high concentrations of TFE to induce folding²¹. It is difficult to detect protection in polyproline helices with HX MS. Such helices often involve only a few residues other than proline, which does not even contain a backbone amide hydrogen. Polyproline helices are not held together by backbone amide hydrogen bonds as is the case the α -helix. As a result, the formation of a polyproline helix does not reduce hydrogen exchange rates as drastically as formation of an α -helix and detection of the presence of a polyproline helix with HX MS is therefore difficult.

As shown in summary in Figure 7, most regions of Tip were heavily deuterated after only 1 minute in deuterium. The number of backbone amide hydrogens in each peptide (shown as a small number at the top right of each bar in Figure 7) must be taken into account when interpreting such results (see also the far right column of Supplementary Table 1). Peptides 109-126 and 127-144 both have 17 residues while peptide 63-81 has 18 residues. The number of backbone amide hydrogens in these three peptides is quite different however (14, 11, 17, respectively). The percent unprotected will vary therefore according to the number of backbone amide hydrogens available in each fragment. A difference of 0.5 daltons in the measurement of the average deuterium level, therefore, can account for a change as large as 5-7% in the value of the percentage unprotected when a peptide contains 10 potential sites of backbone deuteration. The error of the percentage value therefore is approximately $\pm 3-4\%$. The majority of Tip was very unprotected (>85% deuterated within 10 seconds) with minor protection of only a few residues in peptides 27-62 and 145-177. The most significant

protection from exchange was observed in peptides covering the N-terminal region, which included the affinity tag.

CONCLUSIONS

The results of this study of Tip have shown that the cytoplasmic portion of the Tip-C484 protein is largely if not totally unstructured in solution. Despite this conformation, as we have shown, Tip remains highly functional and can bind to and activate its target Lck.

The lack of structure in a functional protein is not unique to Tip. Intrinsically unstructured proteins and protein domains are receiving more attention in recent years³⁶. There are potential advantages for proteins that exist normally in an unfolded state including the potential for many diverse interactions with a variety of binding partners³⁶. For a folded protein to interact with more than one binding partner, it generally must possess more than one binding domain. If each binding domain has a unique fold, proteins with multiple binding domains can become quite large. Unstructured and disordered proteins, in contrast, may be able to alter their structures to create a variety of conformations for interaction thereby increasing the diversity of the protein and its binding pool. Tip is known to bind to a number of different proteins (Lck, p80, STAT factors) and a disordered configuration may support these diverse interactions.

A disordered protein like Tip may become ordered upon binding and interaction. For example, the kinase inducible-transcriptional-activation domain (KID) of the cyclic-AMP-response-element-binding protein (CREB) is entirely disordered but folds into two orthogonal helices upon interaction with CREB binding protein, CBP³⁷⁻³⁹. Stimulated folding was also discovered in large protein domains, such as the 116-residue N-terminal domain of DNA-fragmentation factor 45 (DFF45)⁴⁰ where DFF45 is completely unstructured in its free form but takes on a globular conformation when it binds to DFF40. It has been speculated and is possible that regions of Tip become structured upon binding to Lck or assembly of Tip:Lck complexes at the plasma membrane.

HX MS analyses may be able to reveal changes in Tip conformation in the presence of LckYEEI and help determine if and where Tip becomes structured when bound to Lck. Obtaining such data will, in the future, provide much insight into the mechanism of the biological function of Tip. While the analysis of the HX in Lck in the Tip:Lck complex has been completed (D.D. Weis & J. R. Engen, unpublished results), analysis of HX in the Tip protein requires the development of new methods for isolating Tip from Lck post-labeling but prior to mass analysis. For weakly associating complexes such as Tip and Lck, massive amounts of Lck must be added in order to produce a population of Tip molecules that are >75% bound during solution labeling. Removing Tip from the large quantities of Lck under HX quench conditions is a ongoing technical problem. Once solved, such HXMS methods will be readily transferrable to other systems and should prove instrumental in future understanding of intrinsically unstructured proteins.

MATERIALS AND METHODS

Recombinant Tip expression: Native protein

The cytoplasmic region of Tip (residues 1-187) was subcloned into pET-15b (Novagen) from the pFastBac HT vector described in²⁴. The sequence GSSHHHHHHSSGLVPRGSH was present before the N-terminal methionine of Tip-C484 (Genbank #M31964, see also⁹) and the protein terminated at isoleucine 187. The 6xHis-tagged Tip protein was over-expressed in Rosetta (DE3) pLysS *E. coli*. Starter cultures (100 mL) of transformed cells were grown at 37 °C overnight and diluted into 3 L the following morning. The cultures were grown to OD₆₀₀= 0.6 and expression was induced with 2 mM IPTG for four hours at room temperature.

All subsequent purification steps were performed at 4 °C. Cells were lysed by sonication in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM β -mercaptoethanol (β -ME) and 1 mM PMSF. The lysate was clarified by centrifugation (15,000 RPM for 20 minutes) and incubated with Qiagen Nickel-nitrilotriacetic acid (Ni-NTA) beads for 2 hours (1 mL bead suspension per L culture). After incubation, the beads were washed with 200 mL of 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 20 mM imidazole, 5 % glycerol, and 10 mM β -ME and then with another 200 mL of the same buffer containing 1 M NaCl. Protein was eluted from the Ni-NTA beads with 25 mM Tris-HCl, pH 8, 100 mM NaCl, and 50 mM EDTA. Approximately 55 μ g of protein was purified for every liter of culture. For both native and renatured Tip purification (see below), the final protein concentration was determined by the Bradford Assay⁴¹ and the purity and molecular mass of the protein were verified with SDS-PAGE and electrospray mass spectrometry.

Recombinant Tip expression: Renatured protein

The vector, cell growth and protein expression parameters were identical to the native protocol described above. Tip was purified from inclusion bodies based on a previously described protocol⁴² with slight modifications. Cells were lysed in the lysis buffer described above with the addition of 6M guanidine hydrochloride (GuHCl) and incubated for 20 minutes prior to centrifugation. The supernatant was incubated with Ni-NTA beads for 2 hours, the slurry was transferred to an empty glass chromatography column and washed with 200 mL wash buffer (20 mM Tris-HCl, pH 8.0, 8M urea, 150 mM NaCl, 20 mM imidazole, 5% glycerol, and 10 mM β -ME). The concentration of urea in the wash buffer was lowered step-wise over an 8-hour period to allow the protein to renature. Renaturation occurred at room temperature until the urea concentration reached 3M at which point it was transferred to 4 °C for the remainder of the protocol. Proteins were eluted from the Ni-NTA resin with the final buffer (elution and storage) containing 20 mM Tris-HCl, pH 8.0, 0M urea, 150 mM NaCl, 5% glycerol, 10 mM β -ME, and 50 mM EDTA. Amicon ultra-15 centrifugal filter devices were used to concentrate the protein to approximately 100 pmol/ μ L. Approximately 1.5 mg of renatured Tip were purified from every liter of bacterial culture. The renatured protein was aliquoted and stored at -80 °C. For Tip purification in which the 6xHis tag was cleaved off the renatured protein, thrombin cleavage of the tag was performed on the Ni-NTA beads after the urea concentration reached 0M. Eight units of thrombin/ μ L protein solution were used and the cleavage reaction was allowed to proceed overnight at room temperature. The supernatant containing the cleavage product was removed and cleavage confirmed by SDS-PAGE and electrospray mass spectrometry.

Disorder predictions

Calculations of naturally disordered regions were made with the PONDR program²² (www.pondr.com) using the default settings and the sequence for Tip-C484 from Genbank #M31964 (see also⁹).

Gel filtration

Gel filtration experiments were performed on an Amersham Pharmacia Biotech ÄKTA FPLC system using an Amersham Superose 12 HR 10/30 gel filtration column at a flowrate of 0.1 mL/min. Control proteins were purchased from Sigma. Separation was performed at 4 °C with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% glycerol, 10 mM β -ME, and 50 mM EDTA as the mobile phase. UV₂₈₀ monitored the elution of the proteins.

Circular dichroism

Circular dichroism spectroscopy was performed on an Aviv Model 202 CD Spectrometer with a 0.1 mm path length at 25 °C. Spectra were recorded at a Tip protein concentration of 0.6 mg/mL in 10 mM sodium phosphate buffer, pH 7.4, in the presence of 150 mM NaCl.

Tip binding to purified GST-LckSH3

GST-LckSH3 and GST expression vectors were as described⁴³. Transformed BL21 cells were grown at 37 °C overnight and expression was induced with 1 mM IPTG for four hours. Cells were lysed by sonication at 4 °C in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM β -ME and 1 mM PMSF. Sigma glutathione-agarose (GSH) beads were independently incubated with the clarified lysates of GST or GST-LckSH3 for thirty minutes at room temperature. The beads were washed three times with 25 mM Tris-HCl, pH 8.0, 100 mM NaCl to remove unbound proteins. For Tip binding to GST-LckSH3, an aliquot of GSH beads containing approximately 25 μ g of bound GST-LckSH3 (by Bradford assay) was incubated with approximately 150 μ g of purified, renatured Tip for thirty minutes at room temperature. Prior to loading onto an SDS-PAGE gel, the beads for all experiments were washed three times with 25 mM Tris-HCl, pH 8.0, 150 mM NaCl after incubations to remove unbound protein. The beads were then mixed with SDS-PAGE loading buffer, boiled and the supernatant run on a 10% SDS-PAGE gel.

In vitro Tip phosphorylation

The coding sequence of human Lck was modified by PCR to replace the wild-type C-terminal tail with the high-affinity SH2-binding sequence Tyr-Glu-Glu-Ile (YEEI). The coding sequence of the N-terminal unique domain was replaced with a hexa-histidine tag, and this modified Lck cDNA was subcloned into the baculovirus transfer vector pVL1393. The resulting plasmid was used to create a recombinant baculovirus for expression of LckYEEI in Sf9 insect cells as described elsewhere²⁶. LckYEEI was purified by a combination of ion exchange and affinity chromatography as described²⁵ for HckYEEI. Purified recombinant LckYEEI (1 μ M) and Tip (10 μ M) were combined in a 1:10 molar ratio (1 μ M LckYEEI and 10 μ M Tip) in 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 0.5 mM ATP (total reaction volume 20 μ L) and incubated for 30 min at 30 °C. The reaction was quenched by heating in SDS-PAGE loading buffer and the entire reaction was loaded onto a 10% gel. For MS analysis, LckYEEI and Tip were mixed in the same way as for the gel analysis except a 1:5 molar ratio (1.9 μ M LckYEEI and 9.7 μ M Tip) was used and the reaction was held at room temperature for between 1–4 hours. The reaction was quenched with the addition of 0.15 M H_3PO_4 to reduce the pH to 2.6. The samples were then flash frozen on liquid nitrogen and stored at -80 °C until analyzed. Tip phosphorylation was assayed by LC/ESI-MS (Shimadzu HPLC / Waters LCT Premier). Prior to mass analysis, the LckYEEI/Tip mixture was desalted and concentrated on a protein trap (Michrom Protein Microtrap) using 5% ACN/95% H_2O . LckYEEI and Tip were then separated online with a C-4 column (50 mm $\times$ 1 mm, Phenomenex Jupiter, 5 μ m particles, 300 Å pore size) using a 5 minute 5–60% ACN/ H_2O gradient. 20 pmoles of LckYEEI and 100 pmoles of Tip were injected on each run. All mobile phases contained 0.05% (v/v) TFA. Infused myoglobin was used as an internal mass calibrant after the protein eluted. Tip spectra were then transformed from the m/z domain into the mass domain using MassLynx software (Waters).

In vitro Lck activation assay

Tyrosine kinase assays were performed in 384-well plates using the FRET-based Z'-lyte kinase assay system and the Tyr 2 peptide substrate (Invitrogen). Reactions (10 μ L) were conducted in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% BRIJ-35). LckYEEI (15–30 ng/assay) was pre-incubated with various molar ratios of Tip for 5 min at room temperature. ATP (50 μ M final) and Tyr2 substrate (2 μ M final) were then added to the

reaction followed by a 1 h incubation. Development reagent, containing a protease that digests non-phosphorylated peptide, was then added to the reaction for an additional 60 min at room temperature, at which time the reaction was terminated with the proprietary stop reagent. Fluorescence was assessed at an excitation wavelength of 400 nm; coumarin fluorescence and the fluorescein FRET signal were monitored at 445 nm and 520 nm, respectively. The coumarin emission excites fluorescein by FRET in the phosphorylated (uncleaved) substrate peptide only. Reactions containing unphosphorylated peptide and kinase in the absence of ATP served as 0% phosphorylation control, while a stoichiometrically phosphorylated peptide was used as a 100% phosphorylation control. Raw fluorescence values were corrected for background and reaction endpoints were calculated as emission ratios of coumarin fluorescence divided by the fluorescein FRET signal. These ratios were then normalized to the ratio obtained with the 100% phosphorylation control. Each condition was assayed in quadruplicate, and results are presented as the mean $\pm$ S.D. The entire experiment was repeated twice with comparable results.

Yeast Lck activation assay

Coding sequences for Lck, LckYEEL, Tip, and HIV-1 Nef were amplified by PCR to introduce a yeast translation initiation sequence (AATA) immediately 5' to the ATG start codon. A Flag epitope tag was also added to the N-terminus of the Tip coding sequence. The Lck cDNA clones were subcloned downstream of the Gal1 promoter in the yeast expression vector pESC-Ura (Stratagene). The Tip and Nef cDNAs were subcloned downstream of the Gal10 promoter in pESC-Trp (Stratagene). *Saccharomyces cerevisiae* strain YPH 499 (Stratagene) was co-transformed with pESC-Ura and pESC-Trp plasmids containing the genes of interest via electroporation (BioRad Gene Pulser II). Transformed yeast colonies were selected on synthetic drop-out plates lacking uracil and tryptophan (SD/-U-T) with glucose as the sole carbon source to repress protein expression. Positive transformants were grown in liquid SD/-U-T medium plus glucose, normalized to OD₆₀₀ = 0.2 in water, and then spotted in four-fold dilutions onto SD/-U-T agar plates containing galactose to induce protein expression. Plates were incubated for three days at 30° C and imaged on a flatbed scanner. Yeast patches appear as dark spots against the translucent agar background. To verify protein expression, aliquots of the yeast cultures used for the spot assay were grown in SD/-U-T medium plus galactose for 18 h. Cells were pelleted, treated with 0.1 N NaOH for 5 min at room temperature, and normalized with SDS-PAGE sample buffer to 0.02 OD₆₀₀ units per μ L. Aliquots of each lysate (0.2 OD₆₀₀ units) were separated via SDS-PAGE, transferred to PVDF membranes, and probed for protein phosphotyrosine content with a combination of the anti-phosphotyrosine antibodies PY99 (Santa Cruz Biotechnology) and PY20 (Transduction Laboratories). Protein expression was verified by immunoblotting with antibodies to Lck (2102; Santa Cruz), the FLAG epitope for Tip (M2; Sigma) and Nef. Nef antibodies (EH1 monoclonal) were obtained from the NIH AIDS Research and Reference Reagent Program.

Deuterium exchange

Deuterium labeling was similar to previous descriptions^{44,45}. Approximately 3 nmol of protein (as determined by the Bradford Assay) were diluted 15-fold with labeling buffer (20 mM phosphate, 150 mM NaCl in D₂O, pH 7.2). The pH value was that given by the pH meter and was not corrected for the deuterium isotope effect⁴⁶. At selected times ranging from ten seconds to four hours after D₂O introduction, an aliquot of the original 3 nmol solution was transferred to a microtube containing an equal volume of quench buffer (100 mM phosphate buffer, pH 2.62, H₂O) and immediately set on dry ice. Samples were stored at -80 °C until mass analysis. Highly deuterated Tip was prepared by boiling renatured Tip in labeling buffer for 5 minutes, cooling Tip back to room temperature and quenching in the same manner as each labeling time-point sample was quenched.

Mass analysis: Intact protein

The deuterium content of intact Tip was measured with a Shimadzu SCL-10A VP HPLC coupled to a Waters QTOF2 with standard electrospray interface. Each frozen, deuterium-labeled sample (~400 pmoles) was rapidly thawed to 0 °C, injected onto a C-5 column (50 mm × 1.00 mm Phenomenex Jupiter, 5μ particles) and eluted directly into the mass spectrometer with a 6 minute gradient of 2-98 % ACN:H₂O at 50 μL/min. Both mobile phases contained 0.05% trifluoroacetic acid. Mass increases reported only on peptide amide linkage deuteration because side-chain deuterium was washed away during the HPLC step³⁰. The deuterium content of the intact protein was determined by transforming the raw m/z mass spectrum for each time-point to the molecular weight scale and subtracting the average mass of an undeuterated protein from the average mass at each time-point. The deuterium level at each time-point was determined 2 or 3 times from independently prepared samples from separate protein purifications. The error of mass determination for intact proteins in the calibrated QTOF2 mass spectrometer averaged +/- 0.02 m/z). To minimize deuterium back-exchange, the injector, column, and all associated tubing were kept at 0 °C with an ice bath⁴⁷. The average amount of back-exchange for the instrumental setup was approximately 17% for intact Tip. Back-exchange was corrected for by analyzing undeuterated and highly deuterated protein on the same day as experimental samples and applying the published formula⁴⁷ to adjust for back-exchange.

Mass analysis: Peptic peptides

Deuterium exchange was repeated as described above to prepare proteins for digestion. Each frozen sample (~400 pmoles) was rapidly thawed at 0 °C and injected onto a 50 mm X 200 mm column stainless steel column packed with pepsin immobilized on POROS-20AL beads (PerSeptive Biosystems)⁴⁸. The resulting peptides were trapped on a C-18 trap column. The total digestion time was one minute. The trapped peptides were eluted from the C-18 trap onto a Magic C-18 column (Michrom BioResources, Inc.) and eluted directly into the mass spectrometer with a 9 minute gradient of 2-98% acetonitrile-water. The injector, column and tubing were all cooled with an ice bath as described above. The pepsin column was located above the ice bath at an approximate temperature of 18 °C. Samples were analyzed using the same mass spectrometer as for intact proteins. The isotope distributions corresponding to the +2, +3 or +4 ion on the peptic peptides were centroided using MagTran software (available from Zhongqi Zhang, Amgen, Thousand Oaks, CA). As for intact proteins, controls were analyzed to correct for back exchange, which averaged 22%. Back exchange was corrected for in each peptide by analyzing digests of undeuterated and highly deuterated protein on the same day as experimental samples and applying the formula referenced above. The identity of each peptic peptide was determined with both ESI-MS/MS on the same mass spectrometer and MALDI MS/MS on an Applied Biosystems 4700 proteomics analyzer. Each parent ion was assigned to the sequence of Tip after manual interpretation of the MS/MS fragmentation pattern.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Conformational properties of Tip. (A). Prediction of the degree of disorder in the Tip protein as calculated from its primary structure using the program PONDR²². A score of >0.5 indicates a high probability of disorder. A schematic of the Tip construct used (residues 1-187 of Tip-C484) is shown above the graph. The location of Lck binding domains LBD1 and LBD2, as well as the transmembrane and extracellular portions (removed in this work), are indicated. (B). Analytical gel filtration results for recombinant, renatured, purified Tip (dotted line) compared with standard proteins. The standards and Tip were analyzed on the same day, under identical conditions. (C). Circular dichroism spectrum of the same protein as shown in (B).

The spectrum was recorded with a 0.1 mm path length and thermostatically controlled temperature of 25 °C at a final Tip concentration of 0.6 mg/mL.

Figure 2.

Tip:Lck SH3 binding assay. GST-LckSH3 was purified as described in the Materials and Methods and loaded alone (lane 1). For co-incubations, proteins were mixed as indicated (approximate 1:6 ratio for LckSH3:Tip) and incubated for 30 minutes prior to washing. Approximately 5 μ g of protein were loaded per lane onto a 10% SDS-PAGE gel, resolved and stained with Coomassie. The mass of Lck SH3 is 7929 Da. Tip normally appears as multiple bands in SDS-PAGE (see explanation in text).

Figure 3.

Phosphorylation of Tip by Lck. **(A)**. Coomassie-stained SDS-PAGE of Tip and LckYEEI coinubation. The proteins were allowed to interact for 30 minutes at a 1:10 Lck:Tip ratio (lane 2) in the presence of ATP and magnesium. Tip normally appears as multiple bands in SDS-PAGE (see explanation in text). **(B)**. Electrospray mass spectra of Tip phosphorylation by LckYEEI (as in lanes 1,2 from part A). The vertical dashed lines show the theoretical masses for native Tip and for singly (+1P) and doubly (+2P) phosphorylated Tip. A small peak at +78 Da is due to the formation of a Tip/ β -mercaptoethanol adduct. The LckYEEI:Tip molar ratio was 1:5 with the Mg^{2+} and ATP concentrations provided in the Materials and Methods. **(C)**. Recombinant LckYEEI was purified from Sf9 insect cells and assayed for kinase activity with

a peptide substrate either alone or in the presence of purified recombinant Tip or Nef in the molar ratios shown. Details of the FRET-based tyrosine kinase assay can be found under Materials and Methods. Each condition was repeated in quadruplicate, and the extent of phosphorylation is expressed as mean percent phosphorylation relative to a control phosphopeptide $\pm$ S.D. The overall experiment was repeated twice with comparable results.

Figure 4.

Tip is functional in yeast. **(A)**. *S. cerevisiae* cultures were transformed with combinations of galactose-inducible expression plasmids for Tip (T), HIV-1 Nef (N), wild-type Lck, Lck with a high-affinity SH2-binding tail sequence (LckYEEI), or no insert (-) as indicated. Four-fold dilutions of each culture were spotted on galactose plates to assess growth suppression. Plates were scanned and yeast patches appear as dark circles²⁶. **(B)**. Immunoblots from yeast cultures shown in part A. Lck activity was assessed on anti-phosphotyrosine immunoblots (pTyr) while control blots verify expression of Lck, Nef and Tip. In this system, Tip strongly activates both Lck and LckYEEI, suggesting that tail release from SH2 is not required for activation. Tip runs as multiple bands in the presence of Lck due to phosphorylation.

Figure 5.

Deuterium incorporation into intact Tip. **(A)**. Mass spectra of the +26 charge state of intact recombinant Tip. The time in D₂O is indicated on the right. A dotted line is provided at m/z 900 for optical guidance. **(B)**. Deuterium levels for intact, recombinant, renatured Tip. Each data point was determined from the mass scale transformation of the raw m/z spectra (see Materials and Methods) and is an average of triplicate measurements. Variation between measurements was within 2 Da so error bars are not shown. The maximum number of exchangeable backbone amide hydrogens in Tip is 191 (indicated by the dotted line). These results have been adjusted for back exchange (see Materials and Methods).

Figure 6.

Mass spectra of selected Tip peptic peptides during deuteration. (A) residues (-19)-6, (B) residues 145-177, (C) residues 89-110, (D) residues 109-126. The time spent in D₂O is shown only in panel (D) but was the same for panels (A-C). The charge state of each peptide is indicated. The peptide in panel (A) contains sequence prior to the first official amino acid of Tip and is therefore numbered negatively. The highly deuterated (TD) protein was prepared as described in the Materials and Methods. A complete list of peptic peptides and deuteration is shown in Supplementary Table 1.

Figure 7.

Summary of deuterium exchange in Tip peptic peptides. Each bar represents a peptic peptide. Residues are labeled beginning with -19 to account for affinity tag spanning the first 19 residues of recombinant Tip. The percent unprotected after 1 minute of deuterium exchange was calculated by dividing the number of residues that exchanged after one minute (after back-exchange correction) by the total number of possible exchangeable backbone amide hydrogens in each peptide. A schematic of Tip, aligned with the protection data, is shown at the top of the diagram. The number of exchangeable backbone amide hydrogens in each peptide is shown in the upper right of each bar. Some bars were obtained by subtraction of overlapping peptides. A full list of deuterium levels in all peptic peptides can be found in Supplementary Table 1.