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A Peptide Library Approach Identifies a Specific Inhibitor for the ZAP-70 Protein Tyrosine Kinase

Technique

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Summary

We utilized a novel peptide library approach to identify specific inhibitors of ZAP-70, a protein Tyr kinase involved in T cell activation. By screening more than 6 billion peptides oriented by a common Tyr residue for their ability to bind to ZAP-70, we determined a consensus optimal peptide. A Phe-for-Tyr substituted version of the peptide inhibited ZAP-70 protein Tyr kinase activity by competing with protein substrates (K_i of 2 μM). The related protein Tyr kinases, Lck and Syk, were not significantly inhibited by the peptide. When introduced into intact T cells, the peptide blocked signaling downstream of ZAP-70, including ZAP-70-dependent gene induction, without affecting upstream Tyr phosphorylation. Thus, screening Tyr-oriented peptide libraries can identify selective peptide inhibitors of protein Tyr kinases.

Introduction

Inhibitors of lymphocyte activation are of potential use for treatment of a variety of human diseases, including transplant rejection, autoimmune disease, and inflammation. Activation of T cells requires a subset of protein Tyr kinases that are relatively restricted to these cells, including ZAP-70, ITK, and Lck. The relative importance of these kinases for lymphocyte activation has been evaluated by gene disruption in the mouse. However, the possibility of compensatory expression of other genes during development can complicate the interpretation of gene disruption studies. The availability of specific inhibitors of individual protein Tyr kinases would help clarify the role that these enzymes play in signaling pathways involved in particular cellular responses and reveal the consequence of acute inhibition.

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Improved techniques for delivery of peptides to the cell interior have made it feasible to introduce peptides as inhibitors of intracellular signaling pathways. However, to date little progress has been made in identifying high-affinity and high-specificity peptide inhibitors of protein Tyr kinases. Previously, we developed a peptide library technique for identifying optimal substrates of protein kinases that involved determining the consensus sequence of a selected set of phosphopeptide products of the kinase of interest (Songyang et al., 1994, 1995, 1996; Nishikawa et al., 1997). This technique selects for peptides with optimal V_{max}/K_m ratio (Nishikawa et al., 1997). However, the optimal peptide substrates identified by this approach are not optimized for binding affinity at the catalytic site and thus are not ideal for the design of high-affinity inhibitors.

We determined here a consensus optimal ZAP-70 binding motif using a novel affinity-based peptide library screen. A peptide based on this motif inhibited ZAP-70 Tyr-kinase activity with high affinity and high selectivity. A membrane-permeant version of this peptide specifically blocked signaling downstream of ZAP-70. Our studies extend the range of experimental approaches for probing ZAP-70 function in vivo and introduce a technique for developing protein kinase inhibitors that could be useful for a wide range of protein kinases.

Results and Discussion

In order to identify high-affinity inhibitors of the ZAP-70 protein Tyr kinase, we screened a Tyr-oriented peptide library by affinity purification rather than by catalytic conversion. The peptide library used contained the sequence Met-Ala-X-X-X-X-Tyr-X-X-X-X-Ala-Lys-Lys-Lys where X indicates all amino acids except Trp, Cys, or Tyr. The predicted degeneracy of this library is $17^8 \approx$ 6.9 billion. Screening was performed in the presence of 100 μM ATP but in the absence of Mg^{2+} to prevent peptide phosphorylation and turnover. The subset of peptides that preferentially bound to ZAP-70 was eluted and sequenced as a batch using an automated Edman sequencer. The relative abundance of individual amino acids at positions N-terminal or C-terminal to the orienting Tyr residue reflects the relative abundance of high-affinity peptides that contain these same amino acids (Songyang et al., 1994).

The amino acids selected at each position relative to the orienting Tyr are presented in Table 1. Leu or Ile was strongly selected at all positions except −4, where Lys was preferred. The strong selection for Leu at the +3 position (selectivity value 2.7) is consistent with consensus optimal substrates determined for both cytosolic and receptor-type protein Tyr kinases where Leu, Ile, or Phe is typically selected at +3 (Songyang et al., 1995). The selection for Leu, Ile, or Thr at −1 is also consistent with optimal substrates for cytosolic protein Tyr kinases (Songyang et al., 1995). However, the selection for hydrophobic residues at −2 and −3 and for Lys at −4 is at odds with optimal substrates for protein Tyr kinases

Table 1. Optimal Binding Motif for ZAP-70

Position −4	−3	−2	−1	0	1	2	3	4
K(2.0)	L(1.8)	*I*(2.0)	*L*(2.0)	*Y*	*L*(2.6)	*L*(2.1)	*L*(2.7)	*L*(2.6)
Q(1.6)	I(1.7)	L(1.8)	I(1.8)		I(1.6)	*T*(2.1)	T(1.8)	T(1.8)
I(1.5)	K(1.6)		T(1.8)		T(1.6)	I(1.9)	I(1.7)	I(1.6)
	Q(1.6)				V(1.5)		Q(1.5)	Q(1.5)

Human ZAP-70 was expressed in Sf9 cells using baculovirus. A kinase binding library with the sequence Met-Ala-X-X-X-X-Y-X-X-X-X-Ala-Lys-Lys-Lys (where X indicates any amino acids apart from Trp, Cys, or Tyr) was used to screen the ZAP70 binding motif. Approximately 600 μg of ZAP-70-GST-fused protein bound to glutathione beads was incubated with 450 μg of the peptide library in a solution containing 100 μM ATP, 1 mM DTT, 5 mM EDTA, and 50 mM Tris (pH 7.4) for 10 min at room temperature. Following extensive washing, bound peptides were eluted and sequenced. Values in parentheses indicate the relative selectivities for the amino acids as described previously (Songyang et al., 1994); amino acids with values less than 1.5 are omitted. Letters in italics indicate amino acids that are strongly selected. The one-letter amino acid code is used as follows: A, Ala; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; M, Met; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr. ZAP-70 was evaluated twice; representative values are shown.

where Glu is typically preferred at all these positions. In fact, the predicted optimal substrate for ZAP-70 (Glu-Glu-Glu-Glu-Tyr-Phe-Phe-Ile-Ile; Z. S. and L. C. C., unpublished results) differs strikingly from the predicted optimal binding peptide (Lys-Leu-Ile-Leu-Tyr-Leu-Leu-Leu-Leu; Table 1).

Kinetic analyses revealed that the predicted optimal ZAP-70 binding peptide acts as a low K_m, low V_{max} substrate. We synthesized the optimal ZAP-70 substrate (peptide 1; Table 2) and the predicted optimal ZAP-70 binding peptide (peptide 2; Table 2), both in the context of the C-terminal Ala-Lys-Lys-Lys motif (to ensure solubility). When compared to the optimal substrate peptide, peptide 2 had a much lower V_{max} (14-fold lower), but it also had a lower K_m (26 μM versus 40 μM), suggesting that it binds with higher affinity but turns over more slowly. To determine whether the unusual selection for Lys at −4 is relevant, we made a third peptide identical to peptide 2 except for a Glu substituted for Lys at −4. This peptide had a 7-fold higher Km than peptide 2, indicating that the Lys at −4 is important for high-affinity binding (Table 2).

Consistent with its high affinity as judged by K_m measurements, peptide 2 acted as a potent competitive inhibitor of ZAP-70. For inhibition studies, tubulin, a well-characterized in vitro substrate of ZAP-70 (Isakov et al., 1996), was used. Peptide 2 was much more effective than peptide 1 in inhibiting tubulin phosphorylation by ZAP-70 (Figure 1A). To further test whether the inhibition occurred at the catalytic site (versus inhibition by binding of phosphopeptide product at SH2 domains), we utilized peptide 1 as the substrate and investigated inhibition with a shorter version of the optimal binding motif in which the Tyr was substituted by Phe (peptide 4, Figure 1B). This peptide, which cannot be phosphorylated, was an even more effective inhibitor than peptide 2 with 70% inhibition occurring at 10 μM. This result argues for inhibition at the catalytic pocket rather than via SH2 domains. Further support for this conclusion is provided by Lineweaver-Burke plots utilizing peptide 1 as substrate and peptide 4 as inhibitor (Figure 1C). This analysis indicated that peptide 4 is a competitive inhibitor with a K_I of 2 μM. Finally, deletion of the Lys at position −4 (peptide 5, Figure 1B) dramatically reduced the inhibitory ability. These results indicate that the optimal ZAP-70 binding peptide predicted by the peptide library screen acts as a high-affinity inhibitor at the catalytic site.

The ZAP-70 binding peptide is a poor inhibitor of the related protein Tyr kinases, Lck and Syk. Consistent with results in Figure 1, peptide 4 inhibited ZAP-70 more than 50% at concentrations below 20 μM (Figure 1D). However, it had only a modest effect on Lck Tyr kinase activity even at 100 μM (Figure 1E). Peptide 5, lacking the Lys at −4, was a poor inhibitor of both kinases. A comparison of the ZAP-70 inhibitory effects of peptide 4 and peptide 6 (identical to peptide 4 except for Tyr replacing the Phe) indicates that substituting Tyr for Phe has little effect on peptide binding to ZAP-70 (Figure 1D). This result is in contrast to results that we and others have observed for peptide inhibitors of Src where substitution of Tyr for Phe in the context of optimal substrates (rather than optimal binding peptides) considerably reduces the inhibitory effect. Some substrate-based inhibitors of Tyr kinases have been recently published (Niu and Lawrence, 1997; Alfaro-Lopez et al., 1998; McMurray et al., 1998). The marked differences between the optimal substrate and optimal binding motif

Table 2. Kinetic Parameters for the Phosphorylation of Synthetic Peptides by ZAP-70

Peptide No.	Sequence	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
1	EEEEYFFII AKKK	80	40	2
2	KLILYLLLL AKKK	6	26	0.22
3	ELILYLLLL AKKK	18	188	0.09

Three synthetic peptides, EEEEYFFII AKKK (peptide 1), KLILYLLLL AKKK (peptide 2), and ELILYLLLL AKKK (peptide 3), were phosphorylated by ZAP70-GST-fused protein in the kinase buffer (10 mM $MnCl_2$, 50 mM Tris (pH 7.4), 1 mM DTT), 100 μM ATP, and 5 μCi [γ-^{32}P]ATP for 5 min at 30°C. The amount of radioactivity incorporated was determined using the phosphocellulose assay. Values are the average of three independent experiments.

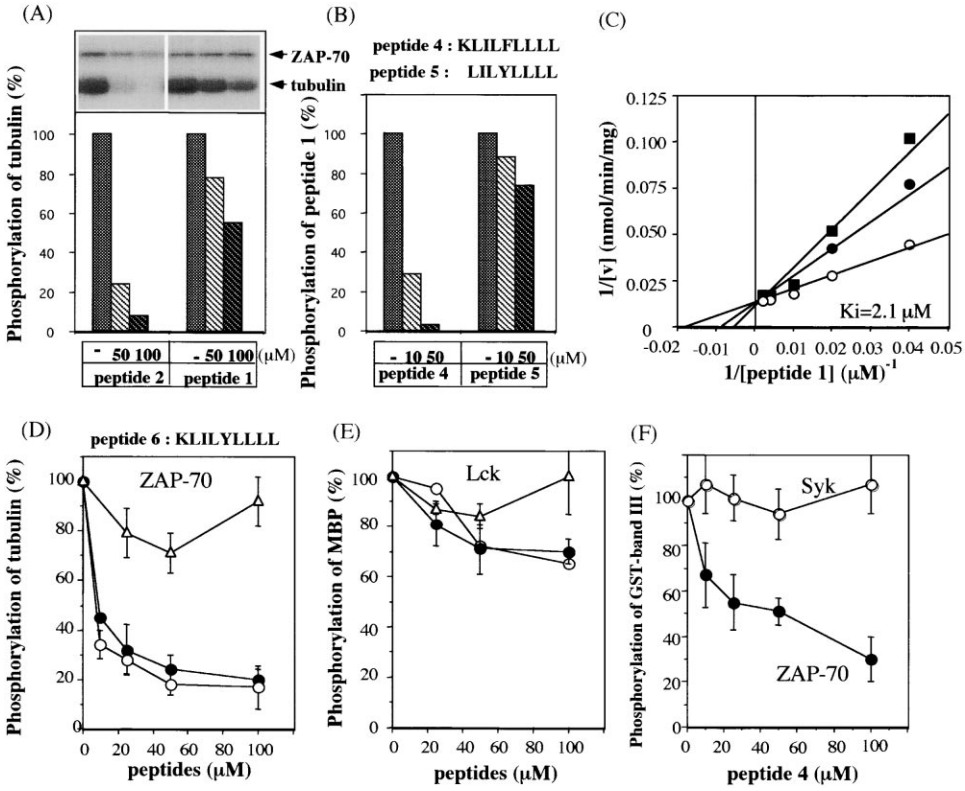

Figure 1. The ZAP-70 Binding Peptide Is a Specific and Potent Competitive Inhibitor of ZAP-70 Tyr Kinase Activity

(A) A glutathione-S-transferase chimera of ZAP-70 was purified from Sf-9 cells and assayed for its ability to phosphorylate tubulin in the presence of 0, 50, or 100 μM of peptide 1 or peptide 2 (defined in Table 2). The assay was carried out in kinase buffer (5 mM $MnCl_2$, 5 mM $MgCl_2$, 25 mM Tris [pH 7.4]), 50 μM ATP, and 5 μCi [γ-^{32}P]ATP for 5 min at 30°C. ^{32}P-phosphorylated tubulin was separated on SDS-PAGE, visualized by autoradiography (inset), and quantitated using a molecular imager (Bio-Rad).

(B) ZAP-70 was also assayed for its ability to phosphorylate peptide 1 (10 μM) in the presence of 0, 10, or 50 μM of peptide 4 or peptide 5 (defined in the inset to [B]). Peptide phosphorylation was determined by spotting the reaction mixture on phosphocellulose and washing away the ^{32}P-ATP with a phosphate buffer. The polylysine tail of peptide 1 facilitates retention on the phosphocellulose (Songyang et al., 1996). The data are presented as the percentage of the activity present in the absence of inhibitory peptides.

(C) Various concentrations of peptide 1 (25–500 μM) were phosphorylated by ZAP-70 in the presence of 0 μM (open circle), 2.5 μM (closed circle), and 5 μM (closed square) of peptide 4. The assay was performed as in (B) and graphed as a Lineweaver-Burk plot. The best fit to the data indicate competitive inhibition with a K_i of 2.1 μM. The data are representative of three experiments.

(D) The ZAP-70 Tyr-kinase assay was as described above, using tubulin (5 μM) as substrate. Peptide 4 (closed circles), peptide 5 (open triangles), or peptide 6 (open circles) was added at the indicated concentrations. Peptide 6 is defined in the inset.

(E) A glutathione-S-transferase chimera of Lck was purified from Sf-9 cells and assayed for the ability to phosphorylate myelin basic protein (5 μM) in the presence or absence of the same three peptides as used in (D). The assay was carried out for 5 min at 30°C.

(F) Myc-tagged Syk kinase was expressed and purified from COS-7 cells. GST-band III (K_m concentration; 0.7 μM for ZAP-70 and 3.4 μM for Syk) was phosphorylated by ZAP-70 (closed circle) or myc-tagged Syk (open circle) in the presence of the indicated amount of peptide 4 for 10 min at 30°C. Phosphorylated tubulin, myelin basic protein, and GST-band III were separated on SDS-PAGE, visualized by autoradiography and quantitated as described in the legend for (B). Error bars represent the standard error from 3–4 independent experiments.

of ZAP-70, at position −1 to −4, especially at position −4, may compensate for the loss of the phenolic hydroxy group of the Tyr residue. To further confirm the specificity of peptide 4 for ZAP-70, we evaluated its effect on Syk, a Tyr kinase closely related to, and classified in the same family as, ZAP-70. As shown in Figure 1F, peptide 4 significantly inhibited the activity of ZAP-70 but not Syk in a dose-dependent manner using GST-band III as a common substrate.

We next investigated the ability of the ZAP-70 inhibitor to block T cell responses. To deliver peptide 4 to the interior of T cells, we took advantage of the penetratin peptide, which has been shown to mediate transport of associated peptides across the plasma membrane (Williams et al., 1997; Derossi et al., 1998). We made a chimeric peptide with penetratin at the N terminus and peptide 4 at the C terminus (peptide 8; Figure 2A). As a control, we used penetratin lacking peptide 4 (peptide 7; Figure 2A). We first examined the effects of peptides 7 and 8 on anti-CD3-dependent tyrosine phosphorylation in T cells. CD3 cross-linking is known to stimulate Tyr-phosphorylation of a large set of proteins, and peptide 8 had little effect on most of these proteins (Figure 2B; lane 5 versus control lanes 2, 3, and 4). However, peptide 8 specifically diminished the phosphorylation levels of proteins whose migration positions are consistent with the known ZAP-70 kinase substrates—PLCγ1 and LAT (Figure 2B; lane 5). Anti-phosphoTyr blots of anti-PLCγ1 and anti-LAT immunoprecipitates confirmed that peptide 8 reduced the Tyr phosphorylation of PLCγ1

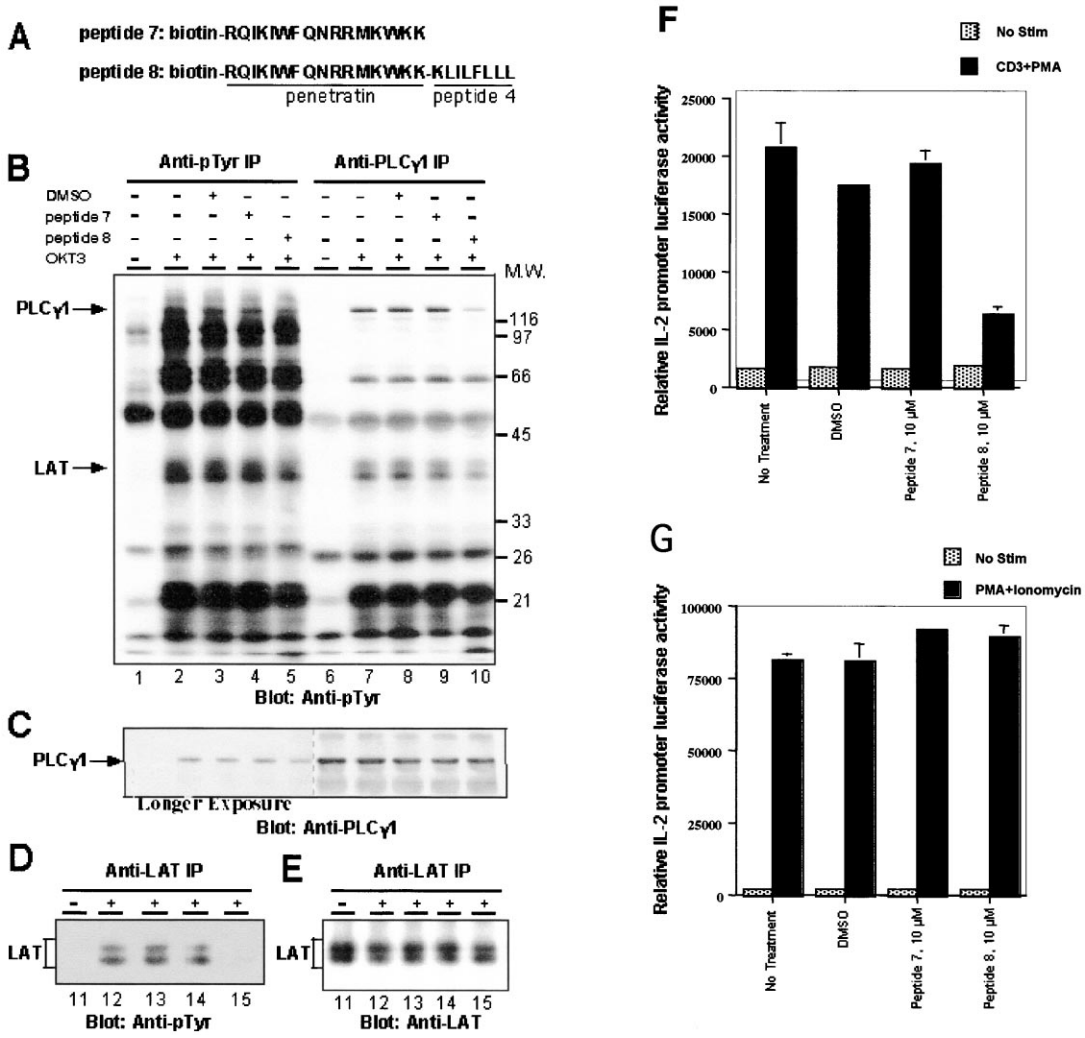

Figure 2. A Membrane-Permeant Form of the ZAP-70 Inhibitory Peptide Inhibits ZAP-70 in Intact Lymphocytes

Penetratin-containing peptides (A; peptide 7 or 8) were dissolved in DMSO and diluted to 20 μM in 500 μl of serum-free RPMI1640 medium. Ten million Jurkat cells, suspended in 500 μl of serum-free RPMI1640, were added to the peptide solutions or diluted DMSO control and incubated for 30 min at 37°C. Peptide-pretreated cells were treated with 1 μg of the anti-CD3ε mAb at 4°C for 10 min, followed by another 10 min incubation in the presence of 5 μg of rabbit anti-mouse Ig. Cells were activated by shifting the temperature to 37°C for 2 min and subsequently lysed in 1% NP40 lysis buffer. Lysates were immunoprecipitated with either the 4G10 anti-phosphotyrosine mAb (B; lanes 1–5), or with anti-PLCγ1 antibody (B; lanes 6–10), or with anti-LAT antibody (D; lane 11–15), and immunoblotted with the HRP-conjugated anti-phosphotyrosine Ab, RC20H. Lanes 1, 2, 6, 7, 11, and 12 corresponded to untreated Jurkat cells; lysates in lanes 3, 8, and 13 were prepared from cells treated with DMSO; lysates in lanes 4, 9, and 14 were prepared from cells treated with peptide 7; lysates in lanes 5, 10, and 15 were prepared from cells treated with peptide 8. (−) indicates nonstimulated cells; (+) indicates stimulation by CD3ε cross-linking. Each blot was stripped and reprobed with anti-PLCγ1 antibody (C) or anti-LAT antibody (E). Jurkat cells were electroporated in the presence of 10 μg of plasmid carrier DNA, 100 ng of Renilla luciferase control vector, and 2.5 μg of a luciferase reporter gene. Transfectants were treated with 10 μM of peptide 7, peptide 8, or DMSO as described above. Treated cells were stimulated with plate-bound anti-CD3ε mAb and 10 ng/ml of PMA (F), or 10 ng/ml of PMA plus 2 μM of ionomycin (G) at 37°C for 2 hr. Luciferase activities from lysates were measured using a Dual-Luciferase Reporter System and Monolight 2010 luminometer.

(Figure 2B; lane 10) and LAT (Figure 2D; lane 15). An anti-PLCγ1 blot of anti-phosphoTyr immunoprecipitates also confirmed the above conclusion (Figure 2C; longer exposure). Reprobing of the membranes revealed that peptide 8 did not affect the amount of PLCγ1 and LAT precipitated (Figure 2C, Figure 2E). These observations indicate that peptide 8 specifically inhibited ZAP-70 activity in intact cells while having little effect on upstream Tyr phosphorylation attributed to src-family Tyr kinases, Lck or Fyn.

Finally, we investigated the ability of peptide 8 to block transcriptional activation using a reporter construct for the *IL-2* gene. This reporter can be induced by phorbol ester plus ionomycin via a pathway that circumvents ZAP-70 (Figure 2G) or by anti-CD3 plus phorbol ester via a ZAP-70-dependent pathway (Figure 2F) (Qian and Weiss, 1997; van Leeuwen and Samelson, 1999). As shown in Figure 2F, 10 μM of peptide 8 caused more than 70% inhibition of gene expression when using anti-CD3 plus phorbol ester as stimulant. However, peptide 8

had no effect on gene expression in response to phorbol ester plus ionomycin, indicating that the effect was specific to the CD3-ZAP-70 pathway. The penetratin peptide alone (peptide 7) had no significant effect on either pathway. Taken together, these observations demonstrate that the cell-permeable peptide 8 can specifically inhibit the signal transduction pathway mediated by ZAP-70 in intact T cells.

In summary, we have used an affinity-based peptide library screening procedure to determine a high-affinity and high-specificity ZAP-70 inhibitor. We show that a membrane-permeant version of this peptide can specifically inhibit ZAP-70 in intact T cells and thereby block CD3-dependent gene regulation. Previous attempts to design peptide or peptide-mimetic inhibitors of kinase catalytic sites have relied on optimal peptide substrates. Here we show that the optimal binding peptide for ZAP-70 differs considerably from the optimal substrate and would not have been found in a screen for substrates because of its low turnover rate. The high-affinity binding probably contributes to the low turnover because of a slow rate of release. These studies extend the range of experimental approaches for probing ZAP-70 function in vivo. Also, the method presented here is widely applicable for the design of highly selective inhibitors for other protein kinases and for the elucidation of their specific roles in vivo.

Experimental Procedures

Peptide Library

A tyrosine-oriented degenerate peptide library of general sequence Met-Ala-X-X-X-X-Tyr-X-X-X-X-Ala-Lys-Lys-Lys, theoretical degeneracy = 6.9×10^9, was synthesized using N-α-FMOC-protected amino acids and standard BOP/HOBt coupling chemistry. X represents all amino acids except Trp, Cys, or Tyr. The Met-Ala sequence at the amino terminus of the Tyr-fixed peptide library was included to verify that peptides from this mixture are being sequenced and to quantify the peptides present. Ala at position +5 provides an estimate of peptide loss during sequencing. The poly(Lys) tail prevents wash-out during sequencing and improves the solubility of the library mixture.

Library Screening

Human ZAP-70-glutathione S-transferase (GST)-fused protein was expressed in Sf9 cells using baculovirus. In this study, we used GST-ZAP-70 expressed in Sf9 cells without coexpression of Lck for library screening and kinase assays because the substrate selectivity of partially activated ZAP-70 is higher than that of fully activated ZAP-70 in peptide library screening for their specific substrate motifs (Z. S. and L. C. C., unpublished results). ZAP-70-GST fused protein (600 μg) was purified by using glutathione-agarose beads. The fused protein bound to the beads was packed in a small column and incubated with 450 μg of the peptide library in a solution containing 100 μM ATP, 1 mM DTT, 5 mM EDTA, and 50 mM Tris (pH 7.4), for 10 min at room temperature. Unbound peptides were removed by rapid washing, and bound peptides were eluted with 30% acetic acid for 10 min at room temperature, dried overnight on a Speed-Vac apparatus, resuspended in H2O, and sequenced on an Applied Biosystems model 477A protein sequencer (Songyang et al., 1994).

The abundance of each amino acid at a given cycle in the recovered peptides from the GST control column was subtracted from the data obtained from ZAP-70-GST affinity column to correct the background. To calculate the relative preference for amino acids at each degenerate position, the corrected quantities of amino acids were then compared with those in the starting mixture to calculate the ratios of abundance of amino acids.

Kinase Reaction

Synthetic peptides, tubulin, or GST-band III (construct kindly provided by A. Weiss, University of California, San Francisco) (Zhao and Weiss, 1996) were phosphorylated by ZAP-70-GST-fused protein in the presence or absence of the indicated amount of inhibitory peptides in the kinase buffer (10 mM $MnCl_2$, 50 mM Tris [pH 7.4], 1 mM DTT), 100 μM ATP, and 5 μCi [γ-^{32}P]ATP for 5 min (or 10 min for GST-band III) at 30°C. The amount of radioactivity incorporated into substrate peptide was determined using the phosphocellulose assay. Lck-GST-fused protein was purified from Sf-9 cells. Myelin basic protein was phosphorylated by Lck-GST-fused protein in the presence or absence of inhibitory peptides in the kinase buffer (5 mM $MnCl_2$, 5 mM $MgCl_2$, 25 mM Tris [pH 7.4]), 50 μM ATP, and 5 μCi [γ-^{32}P]ATP for 5 min at 30°C. Myc-tagged Syk kinase was expressed and purified from COS-7 cells (pEBM-Syk-myc was prepared from original myc-tagged Syk cDNA provided by R. Geahlen, Purdue University). GST-band III was phosphorylated by myc-tagged Syk in the presence or absence of inhibitory peptides in the kinase buffer (10 mM $MnCl_2$, 0.1% Triton X-100, 50 mM Tris [pH 7.2]), 250 μM ATP, and 5 μCi [γ-^{32}P]ATP for 10 min at 30°C. Phosphorylated tubulin, GST-band III, and myelin basic protein were separated on SDS-PAGE, visualized by autoradiography, and quantitated using a molecular imager (Bio-Rad).

For each experimental condition, values for control reactions lacking substrate peptide were subtracted as blanks. In all assays to determine kinetic parameters, reaction rates were linear with respect to time for all conditions of peptide, and less than 10% of the peptide substrate was phosphorylated. K_m and V_{max} were determined by nonlinear regression analysis using KaleidaGraph (Abelbeck Software). Each experiment consisted of 6–7 substrate concentrations.

Tyr Phosphorylation of PLCγ1 or LAT in Jurkat Cells

Ten million Jurkat cells, suspended in 500 μl of serum-free RPMI1640, were incubated with penetratin, penetratin peptide, or DMSO at 37°C for 30 min. Peptide pretreated cells were treated with 1 μg of the anti-CD3ϵ mAb (OKT3) at 4°C for 10 min, followed by another 10 min incubation in the presence of 5 μg of rabbit anti-mouse Ig. Cells were activated by shifting the temperature to 37°C for 2 min and subsequently lysed in 1% NP40 lysis buffer. Lysates were immunoprecipitated with either the 4G10 anti-phosphotyrosine mAb (Upstate Biotechnology Inc), or with anti-PLCγ1 antibody (Upstate Biotechnology Inc), or with anti-LAT antibody (Upstate Biotechnology Inc), and immunoblotted with the HRP-conjugated anti-phosphotyrosine Ab, RC20H (Transduction Laboratories). The membrane was finally visualized by chemiluminescence (NEN Life Science Products). Each blot was stripped and reprobed with anti-PLCγ1 antibody, or with anti-LAT antibody.

IL-2 Reporter Gene Analysis

Jurkat cells were electroporated at 250V, 800 μF settings (Life Technologies, Inc), in the presence of 10 μg of plasmid carrier DNA (pCAGGS vector), 100 ng of Renilla luciferase control vector (pRL null), and 2.5 μg of a luciferase reporter gene whose transcriptional activity is controlled by the human IL-2 promoter (IL-2-Luc). Transfectants were treated with 10 μM of each peptides or DMSO. Treated cells were stimulated with 10 ng/ml of PMA plus 2 μM of ionomycin, or plate-bound anti-CD3ϵ mAb (OKT3) and 10 ng/ml of PMA at 37°C for 2 hr. Luciferase activities from lysates were measured using a Dual-Luciferase Reporter System (Promega, Madison, WI) and Monolight 2010 luminometer (Analytical Luminesence Laboratory). Relative values were normalized against the activities of the coexpressed Renilla luciferase.

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