duced evidence to suggest that, in birds, distinct allelic forms of Lps influence survival during Gram-negative infection. It is possible that mutations of human Tlr4 also affect susceptibility to Gram-negative infection, or its clinical outcome.

References and Notes
27. BLAST searches were performed against the nonredundant (NR) GenBank database, the TIGR database of ESTs, and the dbEST database of ESTs. Searches against NR and TIGR databases were performed at both the nucleotide (blastn) and amino acid (blastx) levels. dbEST searches were carried out at the nucleotide only level.
29. A. Poltorak et al., data not shown.
31. Supplementary Web material for Fig. 2 is available at www.sciencemag.org/content/feature/data/985613.shl.
32. To monitor the efficiency of reverse transcription and PCR, we used primers specific for the transferrin receptor (TfR) as a positive control when attempting to detect the low-abundance Tlr4 mRNA in macrophage or fetal RNA samples by RT-PCR.
34. RAW264.7 cells, obtained from the American Type Culture Collection, are immortalized LPS-responsive cells, frequently used in studies of LPS signal transduction and TNF gene regulation. RAW 264.7 cells, like primary macrophages, become refractory to LPS for a variable interval of time after a primary stimulus with LPS. C. A. Janeway Jr., J. Immunol. 126, 973 (1986).
38. E. Eldon et al., Development 120, 885 (1994).

Exploiting the Basis of Proline Recognition by SH3 and WW Domains: Design of N-Substituted Inhibitors
Jack T. Nguyen, Christoph W. Turck, Fred E. Cohen, Ronald N. Zuckermann, Wendell A. Lim*

Src homology 3 (SH3) and WW protein interaction domains bind specific proline-rich sequences. However, instead of recognizing critical prolines on the basis of side chain shape or rigidity, these domains broadly accepted amide N-substituted residues. Proline is apparently specifically selected in vivo, despite low complementarity, because it is the only endogenous N-substituted amino acid. This discriminatory mechanism explains how these domains achieve specific but low-affinity recognition, a property that is necessary for transient signaling interactions. The mechanism can be exploited: screening a series of ligands in which key prolines were replaced by nonnatural N-substituted residues yielded a ligand that selectively bound the Grb2 SH3 domain with 100 times greater affinity.

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Table 1. Reduction in binding affinity (21) caused by alanine (A) or sarcosine (Ac) substitutions within proline-rich ligands of Sem5 SH3 domain and Yap WW domain (22). "Required" prolines are underlined.

<table>
<thead>
<tr>
<th>Site</th>
<th>Peptide</th>
<th>Kin mutant/kin wild type</th>
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<tr>
<td>Wild type</td>
<td>SH3 ligands</td>
<td>X = A*</td>
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<tr>
<td>P0</td>
<td>PPPVPPR</td>
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<td>P2</td>
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<td>P5</td>
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<td>P6</td>
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<td>p2</td>
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<tr>
<td>p3</td>
<td>GTPPPYTVG</td>
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Randomized ШН° (22) substitutions within proline-rich ligands of Sem5 SH3 domain and Yap WW domain (22). "Required" prolines are underlined.

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Randomized ШН° (22) substitutions within proline-rich ligands of Sem5 SH3 domain and Yap WW domain (22). "Required" prolines are underlined.
shape but simply because they are the only naturally available N-substituted residue. This unusual recognition code has been used to guide design of SH3 inhibitors with improved affinity and selectivity.

We used a chemical minimization scheme to identify essential ligand recognition elements for two domains, the COOH-terminal SH3 domain from the *Caenorhabditis elegans* adapter protein Sem5, and the WW domain from the human signaling protein Yap. The Sem5 SH3 domain recognizes the core PXXP sequence, flanked by a specific arginine residue (1). WW domains recognize the consensus motif PPXY (Y = tyrosine) (2). We scanned through the proline-rich core of each ligand and made the following substitutions:

![Fig. 1. Backbone substitution requirements for SH3 and WW domain recognition.](image)

(A) Structural mapping of alanine and sarcosine scanning results (Table 1). Peptide/domain complex interfaces (8, 9) shown schematically. Ligands adopt a PPII conformation, depicted schematically as a triangular prism. Residue positions (spheres) are color-coded by class: white—does not require either Cα- or N-substitution (alanine and sarcosine tolerant); green—requires Cα-substitution (alanine tolerant, sarcosine intolerant); orange—requires N-substitution (sarcosine tolerant, alanine intolerant).

(B) Minimally sufficient recognition unit for SH3 and WW domain binding grooves. Schematic view of a single binding groove cross-section, looking down the PPII helical axis (viewed from left side of Fig. 1A). Minimally required atoms defined in this study, a sequential pair of Cα- and N-substituted residues, are solid black. The van der Waals binding surface that these atoms present is shaded. (C) Distinct mechanisms of proline recognition. Proline can be recognized by a lock and key mechanism, utilizing the full chemical potential of the side chain. In contrast, SH3 and WW domains recognize key prolines based on N-substitution. This mechanism utilizes relatively little of the binding potential of ligand or protein (hatched surface) but is still highly discriminatory for proline among natural amino acids.

![Fig. 2. Replacing required ligand prolines with peptoids can increase affinity and selectivity for SH3 domains.](image)

(A) Effects of peptoid substitutions at proline-requiring sites of SH3 ligand (23). Wild-type background is YEVPPPVPERRR (24). Required proline sites (P₂ and P₋₁) are shown shaded in the chemical structure. Binding was measured to the Sem5 COOH-terminal SH3 domain, the mouse Crk NH₂-terminal SH3 domain, the human Grb2 NH₂-terminal SH3 domain, and the mouse Src SH3 domain (21). Changes in free energy of binding upon mutation (ΔΔG) relative to proline are color coded (orange—favorable; blue—unfavorable). Dissociation constants for the wild-type 12-mer peptide are as follows: Sem5, *Kₐ* = 48 μM; Crk, *Kₐ* = 6 μM; Grb2, *Kₛ* = 5 μM; Src, *Kₛ* = 25 μM. (B) Peptide 45 selectively binds Grb2 SH3 with 10²-fold improved affinity. Binding curve of peptide 45 [N-(S)-phenylethyl peptoid at P₋₁] to Grb2 NH₂-terminal SH3 domain (filled circles), Src SH3 domain (white circles), and Crk SH3 domain (triangles), as measured by fluorescence perturbation. Data were fit (solid lines) as described (21). Data for the Sem5 SH3 domain are not shown, as this domain binds with 50-fold lower affinity than the other domains tested. For reference, isotherm of wild-type peptide binding to Grb2 is shown by a dashed line (overlaps with Crk binding curve). (C) Inhibition of Grb2 SH3 binding by peptoid 45. Binding of biotinylated Grb2 SH3 domain to Sos peptide/GST fusion protein in the presence of peptide 45 or wild-type peptide (25). The *Kᵢ* of inhibitor is estimated to be 1/10th of the observed IC₅₀.
Both substitutions destroy the proline ring, but each leaves a single methyl group bonded to a different main chain atom.

Proline recognition by the Sem5 SH3 domain and the Yap WW domain was almost exclusively based on amide N-substitution (Table 1)—other unique properties of proline, such as its unusual side chain shape and conformational rigidity, were dispensable (6). Critical prolines of the SH3 core PXXP motif (sites P$_2$ and P$_{-1}$) are sites where alanine or other amino acid replacements are not tolerated (1, 4). However, these sites tolerated sarcosine replacement. Thus nearly complete deletion of the proline side chain was acceptable as long as N-substitution was maintained. An identical tolerance pattern was seen at site P$_{-2}$ of the WW domain ligand. The scanning results also revealed a second backbone requirement: a C$_\text{α}$-substituted residue must precede the required N-substituted residue. At Site P$_1$, in the SH3 ligand and site P$_{-3}$ in the WW ligand, alanine and other C$_\text{α}$-substituted residues were acceptable, but sarcosine was not (7).

Examination of the crystal structure of the Sem5 SH3 domain complex (8) and the NMR (nuclear magnetic resonance) structure of the Yap WW domain complex (9) reveals the basis for these requirements. In both complexes, the ligand binds in a polyproline II (PPII) helical conformation, a left-handed helix with three residues per turn. Turns on one face of the helix pack into a series of grooves on the domain surface (Fig. 1A). Each groove accommodates two peptide residues. The minimal and sufficient requirement at each binding groove is a pair of sequential C$_\text{α}$- and N-substituted residues (10). The C$_\text{α}$/N-substituted pair may be required because, in this arrangement, substituent groups are separated by only a single backbone carbon atom, forming a relatively continuous ridge that can pack efficiently into the domain grooves (Fig. 1B).

SH3 and WW domains appear to read their signature sequences by a mechanism fundamentally different from the “lock and key” mechanism (12) of canonical sequence-specific recognition proteins (Fig. 1C). Such interactions utilize an array of surface pockets optimized to fit the shape and size of anchor side chains displayed along the ligand peptide backbone (13). In such cases, even small perturbations in side chain properties can be deleterious. Proline is recognized in this fashion at many protein interfaces. In contrast, the proline-requiring pockets of SH3 and WW domains actually recognize a unique backbone property: N-substitution. Specificity is achieved, not by favoring binding to proline but by disfavoring binding to any other natural amino acid, all of which lack N-substitution.

This backbone discrimination mechanism reveals a strategy for inhibitor design: maintain the required hybrid C$_\text{α}$- and N-substituted scaffold, but vary side chain identity along this scaffold to optimize complementarity. We tested this strategy by synthesizing a series of SH3 ligands in which each of the two “required” PXXP prolines was replaced by a diverse set of 11 nonnatural N-substituted glycine, or “peptoid,” residues (Fig. 2A). Such groups could exploit the untapped chemical potential of this interface. We tested binding of these ligands to four SH3 domains—Sem5, Crk, Grb2, and Src—all of which share a preference for ligands with the consensus sequence PXXPXR (14). More than half the 22 N-substituted ligands bound as well or better than natural proline-containing peptides. In contrast, no other natural amino acids are tolerated at these sites (4).

Peptide 45 (Fig. 2B) bound the Grb2 SH3 domain with an affinity ($K_d = 40 \text{ nM}$) > 100 times that of the wild-type peptide (15). This substitution of an N(4)-phenylethyl group at site P$_{-1}$ results in a favorable increase in binding energy of $\Delta G = -2.8$ kcal/mol, a 40% increase in total interaction energy. Peptide 41, which has an N(4-hydroxy)phenyl substitution, bound the Sem5 SH3 domain with 25-fold improved affinity. Four other specific domain-ligand pairs showed 5- to 10-fold improved affinity.

Recognition of these peptoid side chains is stereospecific, as is typical for interactions with high complementarity. For example, the Grb2 SH3 domain bound peptide 45 with 10$^3$-fold greater affinity than the related R-stereoisomer (peptide 44). In addition, peptide 45 acted as a potent competitive inhibitor (Fig. 2C), blocking binding of the Grb2 SH3 domain to a Sos peptide fusion protein with an $IC_{50}$ about 1/50th that of the wild-type proline peptide.

The peptoid ligands have improved domain selectivity, overcoming a second major problem posed by SH3 domains as drug targets—members of the family are highly cross-reactive (16). This enlarged range of N-substituted residues can be used to exploit subtle differences between individual SH3 domains. For example, peptide 45 binds potently to Grb2 but shows only modest to negligible improvement in binding to the other domains, resulting in about 10$^2$-fold selectivity for Grb2 (Fig. 2B).

We crystallized and solved the structures of three SH3-peptoid ligand complexes (17). These structures (Fig. 3) confirmed that the peptoid side chains bind at the proline-requiring sites and suggest how peptoids increase affinity and domain discrimination. The peptoid side chains insert into these sites more deeply than proline, packing slightly differently. Thus, specific side chains can make better fitting and more extensive contacts with the domain, including contacts with regions on the SH3 sur-
face that show higher sequence or structural variation. The chemistry of peptoid synthesis allows for exploration of greater side chain diversity than examined here and could yield optimized ligands for other SH3 domains.

The recognition strategy of SH3 and WW domains allows for high specificity binding that need not be of high affinity. In vivo, binding to proline peptides is highly selective, despite suboptimal shape complementarity, because there are no other natural sequences that can satisfy the minimal ligand backbone requirements. The resultant weak but specific interactions are ideal for intracellular signaling domains. These modules must recognize ligands with high enough selectivity to maintain proper information flow but with low enough affinity to allow for sensitive and dynamic modulation in response to changing signals. In contrast the high-affinity and high-specificity interactions that result from typical lock and key recognition are ill-suited for such a function. The ability to recognize proline in this way may explain why proline-rich motifs are so commonly used in regulatory interactions.

References and Notes


2. The SH3 ligand must recognize ligands with high enough groove binding sites, because glycine, which lacks any side chain, is not tolerated. (J.T.N., unpublished data.)


6. Proline has several distinct properties that could be used for recognition, including its unusually shaped pyrrolidine side chain and the conformational constraints that result from its cyclic structure. However, our data do not support a dominant energetic role for proline’s conformational constraints. Breakage of the proline ring always incurs a detectable energetic cost in binding and may explain the preference for a high density of ligand prolines. However, from substitutions at apical sites, which make no contact with the domain, we estimate that the conformational cost of breaking the proline ring by either alanine or sarcosine replacement is 0.4 to 0.9 kcal/mol (affinity of one-half to one-fifth of proline). This is far smaller than the energetic affinity of 7 to 10 kcal/mol (protein of containing required C-...N-substituted groups. Thus the conformational constraints are not a critical element in how these domains recognize prolines. The unique ability of proline to adopt the cis peptide bond conformation also does not appear to play a role in SH3 or WW recognition because bound ligands adopt an all-gauche conformation.

7. Site P1 of the SH3 ligand is included in this set because it can tolerate valine (wild-type peptide), proline, and alanine but not sarcosine (23). Site P3 in the Sem5 SH3 ligand does not conform to this pattern of required backbone substitution, perhaps because it is at a proline residue that is not part of the ligand. This site does not appear to have a dominant role in recognition and can tolerate even glycine substituents (J.T.N., unpublished data).


10. Minimally, methyl substitution at the appropriate position appears to be required at both of these groove binding sites, because glycine, which lacks any side chain, is not tolerated. (J.T.N., unpublished data.)

11. Canonical SH3 binding sequences contain two pairs of XP dipeptides (18). The only way to achieve the required C-...N-substituted structure with natural amino acids is with an XP dipeptide. SH3 domains bind proline-rich peptides in two possible NH2- to COOH-terminal orientations (18, 18). When binding orientation is switched, the sites along the SH3 surface that require proline are switched. As shown in Fig. 1B, when binding orientation is reversed, maintenance of groove interactions would necessitate a reversal of sites requiring an N-substituted unit (8). It is because the C-...N-substituted unit is twofold rotationally symmetric that it can pack into SH3 grooves.

12. We use the term lock and key to mean recognition of two molecules with high complementarity of shape. Complementary structures may be formed or induced by the conformational constraints. Breakage of the proline ring always incurs a detectable energetic cost in binding and may explain the preference for a high density of ligand prolines. However, from substitutions at apical sites, which make no contact with the domain, we estimate that the conformational cost of breaking the proline ring by either alanine or sarcosine replacement is 0.4 to 0.9 kcal/mol (affinity of one-half to one-fifth of proline). This is far smaller than the energetic affinity of 7 to 10 kcal/mol (protein of containing required C-...N-substituted groups. Thus the conformational constraints are not a critical element in how these domains recognize prolines. The unique ability of proline to adopt the cis peptide bond conformation also does not appear to play a role in SH3 or WW recognition because bound ligands adopt an all-gauche conformation.


14. The COOH-terminal Sem5 SH3 domain was purified as the NH2-terminal peptide portion of the intact human immunodeficiency virus Nef protein [J. T. N., unpublished data].


16. The Sem5 SH3 domain requires the binding of the WW domain, which has a higher affinity for the WW ligand than the 7-mer used in Table 1. This peptide from mSos has a nonproline anchor residue that locks the WW ligand into only one conformation; this is at variance with the WW ligand.

17. Site P0 of the SH3 ligand is included in this set because it can tolerate valine (wild-type peptide), proline, and alanine but not sarcosine (23). Site P3 in the Sem5 SH3 ligand does not conform to this pattern of required backbone substitution, perhaps because it is at a proline residue that is not part of the ligand. This site does not appear to have a dominant role in recognition and can tolerate even glycine substituents (J.T.N., unpublished data).


21. Dissociation constants were determined by fluorescence perturbation and fit by nonlinear least-squares analysis with the program Sigmagraft or ProFit [W. A. Lim, R. O. Fox, M. Richards, Proc. Natl. Acad. Sci. U.S.A. 93, 7 (1996)]. Throughout this paper, dissociation constants are expressed as dissociation constants for the wild-type peptide, which was used at 0.05 to 1 μM for each binding experiment, yielding conditions for most ligands in which Kd >> P. For high-affinity ligands (Kd < 0.5 μM), where Kd = [P], data were fit to the following equation:

\[ F = K_d + P + P \times \frac{1}{K_d} + 2 \times P + P \]

where \( F \) = fluorescence bound and \( [P] \) is total peptide ligand concentration. Data are the averages of two or three measurements. Errors for all measurements were between 5% and 15%.

22. These represent ligands of minimal length containing a required nonproline anchor residue for the Sem5 SH3 domain, Y for the YAP WW domain at one terminus. The Sem5 SH3 domain requires the binding motif PXPPPXR, and removal of the R results in a decrease in affinity of another 20-fold (W. A. Lim, unpublished data). Removal of the Y in the WW ligand also results in loss of detectable binding (9). Thus these nonproline anchor residues lock the ligand into only one possible binding register, making compensatory binding arrangements unlikely. Dissociation constants for the wild-type peptides (Kd wild-type) were as follows: Sem5 SH3 domain, Kd = 190 μM; WW ligand, Kd = 40 μM. Peptides were synthesized on an Applied Biosystems model 431A synthesizer. Site nomenclature (sites P1 to P7) for the SH3 ligands has been described (8). The site nomenclature for the WW ligand places the critical tyrosine at site P3, with the residues preceding it in sequence numbered P2 to P0...

23. Full binding measurements on all peptides and pep- toids are provided in supportive material at www.sciencemag.org/data/983858.sh.

24. Peptoid substitutions were synthesized in the 12- mer background, YEVVPPPVRPRRR, which has a higher affinity than the 7-mer used in Table 1. This peptide from mSos has the highest-affinity natural ligand and both the Sem5 COOH-terminal SH3 domain and the Grb2 NH2-termi- nal SH3 domain. The COOH-terminal peptide portion was synthesized on an Applied Biosystems model 431A synthesizer. Coupling of the peptides was performed by the submonomer method [R. J. Simon et al., Proc. Natl. Acad. Sci. U.S.A. 89, 9367 (1992); G. M. Brown et al., Methods Enzymol. 267, 437 (1996)] with the following modifications: the peptide NH2-terminus was acetylated and reaction with excess volumes of 1 M bromoacetic acid in dichloromethane and 1 M disocar- bodimide in dimethylformamide (twice for 30 min); nu-
Defective T Cell Differentiation in the Absence of Jnk1

Chen Dong,* Derek D. Yang,† Mark Wysk, Alan J. Whitmarsh, Roger J. Davis, Richard A. Flavell†

The c-Jun NH2-terminal kinase (JNK) signaling pathway has been implicated in the immune response that is mediated by the activation and differentiation of CD4 helper T (TTH) cells into TTH1 and TTH2 effector cells. JNK activity observed in wild-type activated TTH cells was severely reduced in TTH cells from Jnk1−/− mice. The Jnk1−/− TTH cells hyperproliferated, exhibited decreased activation-induced cell death, and preferentially differentiated to TTH2 cells. The enhanced production of TTH2 cytokines in Jnk1−/− cells was associated with increased nuclear accumulation of the transcription factor NFATc. Thus, the JNK1 signaling pathway plays a key role in T cell receptor-initiated TTH cell proliferation, apoptosis, and differentiation.

When activated by antigen-presenting cells (APCs), TTH cells undergo clonal proliferation and produce interleukin 2 (IL-2). The activated TTH cells may then become TTH1 or TTH2 effector cells (1), which mediate inflammatory or humoral responses, respectively. The polarization of TTH cell differentiation is, at least in part, determined by the cytokine environment (1). IL-12, produced by activated APCs, induces TTH1 development of naive TTH cells. IL-4, made by T cells, is required for TTH2 differentiation. Thus, early production of IL-4 or IL-12 determines TTH cell lineage commitment and the type of immune response that occurs. Although most attention has focused on the effect of polarizing cytokines on TTH cell differentiation, signals from the T cell receptor (TCR)-CD3 complex and from the costimulatory factor CD28 may also affect cytokine production by mechanisms not yet understood (2).

JNK, also known as stress-activated protein kinase, phosphorylates the transcription factor c-Jun and increases AP-1 transcription activity (3, 4). Other substrates include JunD, ATF2, ATFa, Elk-1, Sap-1, and NFAT4 (3, 4). Signals from both the TCR-CD3 complex and CD28 are required for JNK and AP-1 activation in T cells, and these signals may be integrated in such a way as to mediate T cell activation and the induction of IL-2 transcription (5). Although JNK is implicated in IL-2 gene transcription, JNK may also act to stabilize IL-2 mRNA (6). AP-1 has also been reported to be important for the regulation of TTH1 and TTH2 cytokine genes (7, 8).

To understand the role or roles of JNK in TTH cell activation and differentiation, we generated Jnk1-deficient mice through homologous recombination in embryonic stem cells (9) (Fig. 1A). Targeted disruption of the Jnk1 gene resulted in a null allele, as confirmed by mRNA (10) and protein expression analysis of embryonic fibroblast (Fig. 1B) and T cell (11) extracts. Jnk1-deficient mice were fertile and of normal size. Lymphocyte development appeared normal, with typical ratios of T cells to B cells, CD4 to CD8, and naive to memory T cells in the periphery (10). The absence of apparent developmental defects of Jnk1−/− lymphocytes might be the result of redundancy, because Jnk1 and Jnk2 are coexpressed in lymphoid tissues (4). Therefore, we tested whether JNK1 and JNK2 are activated similarly during the course of TTH cell activation.

Purified CD4 T cells from wild-type or knockout mice were stimulated by antibodies to CD3 (anti-CD3) with or without anti-CD28 (12, 13), and JNK activity was measured using c-Jun as the substrate. During the first 48 hours, induced JNK activity was greatly reduced in the Jnk1−/− TTH cells; moreover, anti-CD28 could not enhance kinase activity (Fig. 1C). Essentially no JNK activity was detected in Jnk1−/− TTH cells stimulated for only 5 min, despite the same JNK2 protein expression (11). Thus, JNK1 appeared to account for most of the JNK activity in newly activated T cells. After 60 hours of stimulation, JNK activity in the Jnk1−/− cells was similar to that in wild-type cells, and in each case this activity was presumably derived from JNK2. In fact, JNK2 represents most JNK activity in TTH1 effector cells (14).

To investigate the role or roles of JNK1 in TTH cell activation and IL-2 production, we stimulated T cells with concanavalin A (Con A), anti-CD3, or anti-CD3 plus anti-CD28 (12, 13). Relative to wild-type cells, Jnk1−/− spleen cells produced the same amount of IL-2 (Fig. 2A) and CD4 T cells produced the same amount of IL-2 mRNA (10) 24 hours after stimulation, despite the lack of JNK activation (Fig. 1C), similar to Jnk2- and c-Jun-deficient T cells (14, 15). Although JNK may therefore not be required for IL-2 expression, it is also possible that JNK1 and JNK2 are redundant for IL-2 regulation. Despite normal IL-2 production, Jnk1−/− splenocytes and CD4 T cells (10) displayed enhanced proliferation (12, 13) (Fig. 2B). In addition, Jnk1-deficient TTH cells had a