



**Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice:
Mutations in *Tlr4* Gene**

Alexander Poltorak, *et al.*

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14. Templates for in vitro synthesis of primary transcripts of tRNA^{Tyr} were made from pxt62 DNA (73) by polymerase chain reaction (PCR) with primers 5'GGAATTCATTTAGGTGACACTATAGAACCGGCTTCGATAGC and 5'-GGCAAGCTTAAAGCGTCTCGAGCCGGAA-T(g, c, a)G(a, g)ACCAGC; lowercase letters represent sequence variants used to generate mutants (T55C, G57T/C) within loop III of mature tRNA^{Tyr}. Template for the precursor of tRNA^{Met} lacking the 3' CCA end (23) was used in PCR to make template for the precursor containing the mature 3' end with primers 5'-GTGAAT-TCTAATCAGCTCACTATAGGG and 5'-CTCTGGATC-CTGGTAGCAGAGGATGGTTTCGAT followed by digestion with Mva I. Unlabeled yeast tRNA^{Phe} was from Sigma. Transcription, purification, injection, and isolation of the RNAs from oocytes were as described (32). Transfer RNA transcripts were coinjected with U1_{sm} and U3 RNAs, serving as controls for export and nuclear injection and dissection, respectively, and low and high amounts of tRNA^{Tyr} primary transcripts (Fig. 1) were 10 to 20 and 80 to 100 fmol per oocyte, respectively. For DNA injections, low and high amounts of the *X. laevis* tRNA^{Tyr} gene were 0.125 and 1.0 ng per oocyte, respectively, of pxt62 plasmid DNA (73); for in vivo labeling, [α -³²P]GTP was used at 0.5 μ Ci/oocyte. For depletion of nuclear RanGTP, 20 to 30 ng of RanT24N [an inhibitor of the guanine-nucleotide exchange factor for Ran, RCC1 (9, 19)] or RanGAP was preinjected into the nucleus; comparable results were obtained with RanT24N or RanGAP (78). For standard RNA analyses under neutral conditions, electrophoresis was in 8% (30:0.8) polyacrylamide, 7 M urea, 0.5 \times TEB (45 mM tris-borate, 1.15 mM EDTA, pH 8.3) gels. For analyses under acid conditions, RNAs were isolated at pH 5.0 and on occasion deacylated at pH 9.0, as described (24), and electrophoresis was in 6.5% (19:1) polyacrylamide, 8 M urea, 0.1 M NaOAc (pH 5.0) gels. Periodate oxidation of RNAs was as described by E. Lund and J. E. Dahlberg [*Science* **255**, 327, (1992)]. For in vivo aminoacylation of tRNA^{Met} (Fig. 3C), oocytes were injected with ³⁵S-methionine (0.2 μ Ci per oocyte), and comigration of the ³⁵S-label and met-tRNA^{Met} was determined by RNA staining before autoradiography of the dried gels (78). For blockage of aminoacylation or protein synthesis, Tyr-AMS (27) was injected to final concentrations of 150 to 300 μ M or cycloheximide was added to 200 μ g/ml of medium; inhibition of protein synthesis was monitored by labeling with ³⁵S-methionine (50 μ Ci/ml of medium).
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21. Human pre-tRNA^{Met} lacking an intron but with a comparable mutation at position 57 also is processed and exported slowly [M. Zaslouf, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6439 (1983); J. A. Tobian, L. Drinkard, M. Zaslouf, *Cell* **43**, 415 (1985); C. Traboni, G. Ciliberto, R. Cortese, *ibid.* **36**, 179 (1984); (23)]. Mutations at position 55 were not tested previously, because they alter the B-box of the tRNA promoter, which is required for producing tRNAs in vivo from injected genes.
22. Mutated tRNAs or end-immature processing intermediates either may not be recognized by the export machinery or may be actively retained by nuclear proteins that bind to such molecules [C. J. Yoo and S. Wolin, *Cell* **89**, 393 (1997); E. Bertrand, F. Houser-Scott, A. Kendall, R. H. Singer, D. R. Engelke, *Genes Dev.* **12**, 2463 (1998)]. At least some form of retention appears likely because wild-type and mutant pre-tRNAs containing 5' m⁷G caps were not exported efficiently (18). These molecules are recognized as having some tRNA character, because they undergo base modifications soon after synthesis (16, 18).
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25. When aminoacylated, both forms of tRNA^{Tyr} migrated during electrophoresis as broad bands (denoted by the brackets in Fig. 3D, lanes 3 and 5) that could be resolved into doublets (lane 5). Deacylation of each form produced a species with a single electrophoretic mobility (lanes 4 and 6), indicating that alternative conformers may form upon aminoacylation; however, aminoacylated tRNA^{Met} migrated as a single band (Fig. 3A), showing that formation of the doublet is not general to all tRNAs.
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Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in *Tlr4* Gene

Alexander Poltorak, Xiaolong He,* Irina Smirnova, Mu-Ya Liu,†
 Christophe Van Huffel,‡ Xin Du, Dale Birdwell, Erica Alejos,
 Maria Silva, Chris Galanos, Marina Freudenberg,
 Paola Ricciardi-Castagnoli, Betsy Layton, Bruce Beutler§

Mutations of the gene *Lps* selectively impede lipopolysaccharide (LPS) signal transduction in C3H/HeJ and C57BL/10ScCr mice, rendering them resistant to endotoxin yet highly susceptible to Gram-negative infection. The codominant *Lps*^d allele of C3H/HeJ mice was shown to correspond to a missense mutation in the third exon of the Toll-like receptor-4 gene (*Tlr4*), predicted to replace proline with histidine at position 712 of the polypeptide chain. C57BL/10ScCr mice are homozygous for a null mutation of *Tlr4*. Thus, the mammalian *Tlr4* protein has been adapted primarily to subserve the recognition of LPS and presumably transduces the LPS signal across the plasma membrane. Destructive mutations of *Tlr4* predispose to the development of Gram-negative sepsis, leaving most aspects of immune function intact.

Conservative estimates hold that in the United States alone, 20,000 people die each year as a result of septic shock brought on by Gram-negative infection (1). The lethal effect of a Gram-negative infection is linked, in part, to the biological effects of bacterial

lipopolysaccharide (endotoxin), which is produced by all Gram-negative organisms. A powerful activator of host mononuclear cells, LPS prompts the synthesis and release of tumor necrosis factor (TNF) and other toxic cytokines that ultimately lead to shock in

sepsis. Nonetheless, it is clear that timely recognition of LPS by cells of the innate immune system permits effective clearance of a Gram-negative infection before it becomes widely disseminated (2, 3).

More than 30 years ago, mice of the C3H/HeJ strain were found to have a defective response to bacterial endotoxin (4–8). Inquiry into the genetic basis of LPS resistance revealed a single locus (*Lps*), wherein homozygosity for a codominant allele (*Lps^d*) was responsible for the endotoxin-unresponsive state. The *Lps^d* mutation arose in mice of the C3H/HeJ substrain and became fixed in the population during the early 1960s (9). In

contrast to C3H/HeJ mice, substrains C3H/HeN and C3H/OuJ (*Lpsⁿ* homozygotes), which diverged from the same stock as C3H/HeJ mice, exhibit vigorous responses to LPS.

A second mutation preventing responses to endotoxin was identified in mice of the strain C57BL/10ScCr (10–12); animals of the control strain C57BL/10ScSn are normally responsive. The allelic nature of the C3H/HeJ and C57BL/10ScCr mutations was indicated by the observation that F₁ animals produced by the cross C57BL/10ScCr × C3H/HeJ are as unresponsive as individuals of the C3H/HeJ parental strain (10). But significantly, heterozygotes produced by the cross C57BL/10ScCr × C57BL/10ScSn are as responsive to LPS as the normal (C57BL/10ScSn) parent (10), indicating that the C57BL/10ScCr allele is not codominant, but is strictly recessive to the common wild-type allele.

Speculations regarding the protein that is affected by mutations of *Lps* have, for the most part, posited that the LPS signal transduction apparatus is disrupted. Ulevitch, Tobias, Wright, and co-workers showed that LPS is concentrated from the plasma by lipopolysaccharide binding protein (LBP), and that the genetically unlinked plasma membrane protein CD14 is the principal receptor for LPS on the surface of mononuclear cells (13–15). Deletion of the CD14 gene substantially increases the concentration of LPS required for a biological response (16). However, because CD14 lacks a cytoplasmic domain, it has been postulated that a coreceptor for LPS must permit transduction of the signal across the plasma membrane. Several protein kinase cascades are known to become activated by LPS (17–21), ultimately leading to the production of TNF and other cytokines that mediate LPS effects (22, 23). However, direct biochemical, immunologic, and expression cDNA cloning approaches have failed to identify the genetic lesions in endotoxin-resistant mice.

In 1978, the *Lps* locus was mapped to mouse chromosome 4 and shown to occupy a position between the *Mup-1* and *Ps* loci (24, 25). Our own genetic and physical mapping

data (26) identified two limiting genetic markers (B and 83.3) that were separated from *Lps^d* by, respectively, four crossovers in a panel of 1600 meioses and three crossovers in a panel of 493 meioses.

A minimal contig, consisting of 20 bacterial artificial chromosome (BAC) clones and one yeast artificial chromosome (YAC) clone, was analyzed by sequencing. Nearly 40,000 reads were obtained from shotgun-cloned genomic DNA, bringing over 1.6 Mb of the central contig to a near-contiguous state and yielding dense coverage of >95% of the entire critical region. BLAST searches (27) performed on masked versions of the sequence disclosed dozens of high-scoring homologies with published expressed sequence tags (ESTs), but these were excluded from consideration as they could not be cloned from macrophage or fetal cDNA libraries of reliable complexity. Several pseudogenes were observed, but were dismissed because they were found to be fragmentary. GRAIL analyses, performed on long, contiguous sequences of the central contig with the program X-GRAIL (28), revealed an abundance of retroviral repeats and scattered non-retroviral exons, many of which proved to be derived from pseudogenes.

Only two authentic genes (a portion of the *Pappa* locus and the complete *Tlr4* locus) were identified in the entire region, each by BLAST analysis and by GRAIL analysis (Fig. 1). *Pappa* encodes a secreted metalloproteinase and is not expressed by primary macrophages or macrophage cell lines (29). These considerations, as well as its extreme proximity to marker B, made it seem a poor candidate. *Tlr4* seemed an excellent candidate, both on the grounds of map position and because the proinflammatory interleukin-1 (IL-1) receptor, like *Tlr4*, is a member of the Toll receptor family. Further, a human mutation causing coresistance to LPS and IL-1 (30) attests to the likelihood that the IL-1 and LPS use structurally related receptors.

Accordingly, we cloned the *Tlr4* cDNAs from C3H/HeJ mRNA and from the mRNA of several LPS-responsive strains of mice (includ-

A. Poltorak, X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, B. Layton, B. Beutler, Howard Hughes Medical Institute and the Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235–9050 USA. C. Galanos and M. Freudenberg, Max-Planck Institute für Immunobiologie, Freiburg, Germany. P. Ricciardi, CNR–Cellular and Molecular Pharmacology Center, Milan, Italy.

*Present address: Northwestern University, 2300 Children’s Plaza, No. 209, Chicago, IL 60614–3394, USA.

†Present address: University of Texas Southwestern Medical Center, Department of Pharmacology, Dallas, TX 75235–9041 USA.

‡Present address: Millennium, Inc., Cambridge, MA 02139–4815, USA.

§To whom correspondence should be addressed at Howard Hughes Medical Institute, 5323 Harry Hines Boulevard, Dallas, TX 75235–9050, USA.

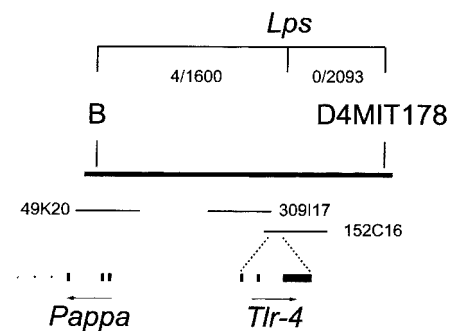


Fig. 1. Diagram of a small portion of the 2.6-Mb contig spanning the *Lps* critical region, described elsewhere in its entirety along with the sequences of primer pairs used to amplify markers (26). Centromere is to the left. Three BACs (49K20, 309117, and 152C16; Research Genetics designations) contain fragments of *Pappa* (49K20) and the complete *Tlr4* gene (309117 and 152C16). Each BAC is ~150 kb long. The orientation and exonic composition of the genes identified is schematically correct, but for clarity, the genes are drawn at far higher magnification than the BACs. Dots to the left of the three *Pappa* exons that were identified in K20 indicate that the gene continues to the left of the contig [other exons were detected in BACs 131M6, 216C14, and 358P4 (29)]. Genetic mapping data (number of crossovers per number of meioses examined) are bracketed above the two genetic markers nearest to the *Lps^d* (B and D4MIT178).

Fig. 2. The *Lps^d* allele represents a missense mutation affecting the cytoplasmic domain of *Tlr4*. Reverse transcription of mRNA isolated from mouse peritoneal macrophages was followed by PCR with the primers TTCTAACTTCCTCTGCGAC and CCTCTTCTCCTTACAGATTAAG, which amplify the entire coding region of the mouse *Tlr4* cDNA, yielding a product 2951 nucleotides (nt) long. The open reading frame of the mouse *Tlr4* cDNA predicts a protein that is 835 amino acids long. The mutation, at position 712, lies in the most conserved portion of the *Tlr4* sequence and is located within the cytoplasmic domain. Dots below the sequence indicate residues that vary between species. The Pro→His substitution that distinguishes *Tlr4* of C3H/HeJ mice is boxed. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

	mouse/rat	712
C3H/HeJ (700) ...RFHLCLHYRDFI	H	GVAIAANIIEGEGFHKS...(730)
C3H/HeN(700) ...RFHLCLHYRDFI	P	GVAIAANIIEGEGFHKS...(730)
rat (700) ...RFQLCLHYRDFI	P	GVAIAANIIEGEGFHKS...(730)
human (702) ...PFQLCLHYRDFI	P	GVAIAANIIEGEGFHKS...(732)

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ing C3H/HeN) by reverse transcriptase-polymerase chain reaction, using primers derived from the genomic sequence. A single mutation (the presence of an A instead of a C) was observed at position 2342 of the C3H/HeJ Tlr4 cDNA sequence (GenBank accession number AF095353). This mutation lies within the coding region: At position 712 (within the cytoplasmic domain), a histidine is predicted to occur in the Tlr4 protein of C3H/HeJ mice, whereas LPS-responsive mice, rats, and humans display a proline in this position (Fig. 2) (31). The same mutation was identified in C3H/HeJ genomic DNA, but not in genomic DNA from C3H/HeN mice or mice of any other strain examined (29).

Although the Tlr4 cDNA was readily amplified by RT-PCR from macrophage RNA derived from C3H/HeJ, C3H/HeN, and C57BL/10ScSn mice, it could not be amplified from macrophage RNA derived from C57BL/10ScCr mice. In contrast, a low-abundance control cDNA (32) could be readily amplified from all strains (Fig. 3A). Moreover, the Tlr4 mRNA could be detected on Northern (RNA) blots prepared with total RNA derived from macrophages of C57BL/10ScSn mice but not C57BL/10ScCr mice (Fig. 3B).

Because a definable mutation exists within *Tlr4* in C3H/HeJ mice, and complete absence of Tlr4 mRNA expression is observed in C57BL/10ScCr mice, it is apparent that *Lps* is identical to *Tlr4*. Certain inferences may thus be drawn from the phenotypes that result from distinct allelic combinations of *Lps*.

The null allele of *Lps* represented in C57BL/10ScCr mice behaves as a recessive mutation (10). Hence, the presence of a single wild-type *Tlr4* allele (*Tlr4^{Lps-n}/θ*) is sufficient to permit normal LPS signal transduction. By contrast, the mutation of C3H/HeJ mice is codominant, in the sense that *Tlr4^{Lps-n}/Tlr4^{Lps-d}* heterozygotes show intermediate levels of endotoxin response (7). Thus, the Pro→His point mutation exerts a dominant negative effect on LPS signal transduction.

A single copy of the *Lps^d* allele (*Tlr4^{Lps-d}/θ*) yields a phenotype as unresponsive as two copies (*Tlr4^{Lps-d}/Tlr4^{Lps-d}*) (10). This fact is consistent with the notion that LPS signal transduction proceeds directly through the Tlr4 molecule, and tends to detract from the alternative hypothesis that Tlr4 undergoes interaction with a second plasma membrane protein that acts, in turn, as an LPS signal transducer.

Tlr4 mRNA is reportedly expressed predominantly in lymphoid tissues (33). Our own data (29) are in agreement with this finding, but in addition (Fig. 4) suggest that the Tlr4 mRNA is strongly and transiently suppressed by LPS in RAW 264.7 cells (34). As such, down-regulation of Tlr4 mRNA may contribute to endotoxin tolerance (35). It remains to be seen whether species-dependent variation in LPS responses,

and modulation of LPS sensitivity by steroids, interferon- γ , and other agents, may be traced to the Tlr4 protein.

LPS signal transduction via Tlr4 has not previously been observed in any experimental system. However, it has recently been reported that human Tlr2 cDNA transfected into 293 cells can promote LPS signal transduction, given coexpression of CD14 (36). The present study excludes an independent role for Tlr2 in LPS signal transduction. The demonstration that *Lps* is identical to *Tlr4* effectively proves that Tlr4 is essential for LPS signaling. And in mice that lack Tlr4 (for example, C57BL/10ScCr animals), endogenously expressed Tlr2 does not contribute appreciably to LPS signal transduction, which fails to occur at measurable levels despite the presumption that the Tlr2 locus is intact. Although Tlr2, like Tlr4, might be required for LPS signaling, the available data

are not sufficient to sustain this conclusion.

In *Drosophila*, the Toll signaling pathway culminates in activation of the drosomycin gene and is required for effective protection against fungal infection (37). Several homologs of the prototypic gene *Toll* exist in *Drosophila*, including *18-wheeler* (38, 39), which facilitates the antibacterial response of flies (40). In mice, *Tlr4* appears to have been retained chiefly to serve the LPS response pathway. Hence, C3H/HeJ and C57BL/10ScCr mice are developmentally and immunologically normal, aside from their inability to respond to LPS and to counter Gram-negative infection. CD14, the best-characterized cell surface receptor for LPS, is also a member of the *Toll* superfamily. It is conceivable that it directly engages Tlr4 upon interaction with LPS, thereby inducing signal transduction through the latter protein.

Hu and co-workers (41) have recently ad-

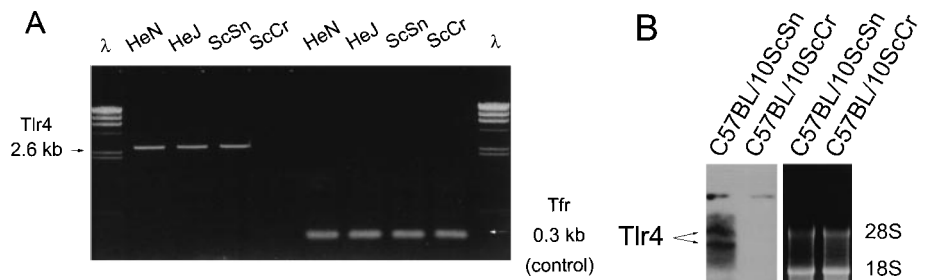
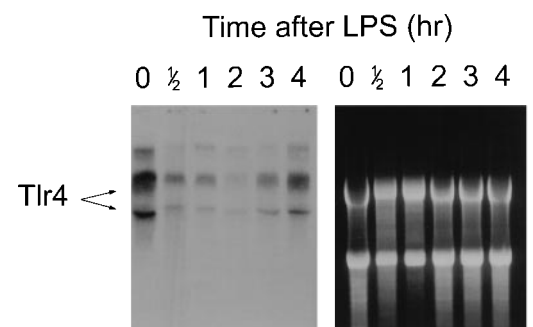


Fig. 3. C57BL/10ScCr mice fail to express Tlr4 mRNA. **(A)** RT-PCR was carried out with the primers TGCCAGGGACTCTGCGCTGCCAC and GTTCTCCTCAGGTCCAAGTTGCCGTTTC, predicted to yield a product 2596 nt long. As a positive control, a fragment from the central portion of the low-abundance, 5.1-kb transferrin receptor (Tfr) mRNA was amplified from the same cDNA preparation, with primers from the Marathon amplification kit (Clontech, Palo Alto, California). Complementary DNA from C3H/HeJ, C3H/HeN, SWR, and C57BL/10ScSn macrophages yielded the expected 2.6-kb Tlr4 amplification product with 35 cycles of amplification, whereas cDNA from C57BL/10ScCr mice did not yield any product. All cDNA samples yielded the expected 0.3-kb product when amplified with Tfr control primers. **(B)** A Northern blot of total macrophage RNA obtained from C57BL/10ScCr and C57BL/10ScSn mice reveals that the non-responder strain produces no detectable Tlr4 mRNA. RNA was separated in a 1.2% agarose gel, transferred to a nylon membrane (Magnagraph; Micron Separations Westborough, Massachusetts), and probed with a genomic DNA fragment from the third exon, corresponding to the region between nt 844 and 2641 of the mouse Tlr4 cDNA sequence (GenBank accession number AF095353). Two bands are consistently detected on Northern blots of control (C57BL/10ScSn) mice (left) and on Northern blots prepared with RNA from mice of other strains (29). C57BL/10ScCr RNA yields no signal, even with prolonged exposure. The ethidium-stained gel is shown in the panel on the right.

Fig. 4. Induction of RAW 264.7 cells by LPS suppresses expression of Tlr4 mRNA. Cells ($\sim 10^6$) were induced with LPS at a concentration of 100 ng/ml for the period indicated. Cells were disrupted by NP40 lysis, nuclei were removed by sedimentation, and cytoplasmic RNA was extracted with SDS and phenol. The Tlr4 mRNA was detected on Northern blot with the 1.5-kb fragment described in Fig. 3. Tlr4 mRNA concentration declined upon LPS stimulation, before approaching preinduction levels. The ethidium-stained gel is shown in the panel on the right.



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.

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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.

Protein-protein interaction domains, such as Src homology 3 (SH3) and WW domains, participate in diverse signaling pathways and are important targets in drug design (1, 2). These domains specifically recognize unique proline-rich peptide motifs but bind them with low affinities ($K_d = 1$ to 200 μ M) compared with other peptide recognition proteins such as antibodies and receptors ($K_d =$ nanomolar to picomolar concentrations). SH3 domains recognize sequences bearing the core element, PXXP (P = proline, X = any amino acid), flanked by other domain-specific residues (3). Identification of compounds that potentially interrupt these interactions has proven difficult: extensive screening of natural and nonnatural combinatorial libraries has not yielded compounds that bind as well as or better

than PXXP peptides (4, 5). Here we show that the essential ligand feature recognized by both SH3 and WW domains is an irregular backbone substitution pattern: N-substituted residues placed at key positions along an otherwise normal C α -substituted peptide scaffold. Prolines are required at these sites, not on the basis of side chain

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

Site	Peptide	K_d mutant/ K_d wild type
	SH3 ligands	X = A A*
Wild type	PPPVP <u>PP</u> R	— —
P ₃	X <u>PP</u> V <u>PP</u> R	1 2
P ₂	P <u>X</u> PPV <u>PP</u> R	>50 3
P ₁	PP <u>X</u> V <u>PP</u> R	2 2
P ₀	PPP <u>X</u> PPR	6 >50
P ₋₁	PPP <u>V</u> X <u>PP</u> R	>50 3
P ₋₂	PPP <u>V</u> PP <u>X</u> R	2 2
	WW ligands	X = A A*
Wild type	GTP <u>PP</u> PPYTVG	— —
P ₋₃	G <u>T</u> XP <u>PP</u> YTVG	1 7
P ₋₂	G <u>T</u> PP <u>PP</u> YTVG	2 >100
P ₋₁	G <u>T</u> PP <u>X</u> PPYTVG	>100 6
P ₀	G <u>T</u> PP <u>PP</u> X ^Y TVG	2 4

J. T. Nguyen, F. E. Cohen, W. A. Lim, Department of Cellular and Molecular Pharmacology, Department of Biochemistry and Biophysics, and Graduate Group in Biophysics, University of California, San Francisco, CA 94143, USA. C. W. Turck, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA. R. N. Zuckermann, Chiron Corporation, Emeryville, CA 94608, USA.

*To whom correspondence should be addressed E-mail: wlim@itsa.ucsf.edu