control over cellular microtubule assembly. Many studies have shown that transcription factors and other regulatory proteins retain the ability to function in heterologous systems across long phylogenetic distances. Our functional test of Hvβt in the *Drosophila* testis reveals that the cytoskeletal proteins that carry out the instructions of the regulatory genes may have acquired much more stringent species-specific restrictions on function.

REFERENCES AND NOTES

1. K. F. Sullivan and D. W. Cleveland, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4327 (1986); K. F. Sullivan, P. S. Machlin, H. Ratrie, D. W. Cleveland, *J. Biol. Chem.* **261**, 13317 (1986); K. F. Sullivan, *Annu. Rev. Cell Biol.* **4**, 687 (1988); R. G. Burns and C. Surridge, *FEBS Lett.* **27**, 1 (1990); in *Microtubules*, J. S. Hyams and C. W. Lloyd, Eds. (Wiley, New York, 1994), pp. 3–31.
2. J. D. Fackenthal, F. R. Turner, E. C. Raff, *Dev. Biol.* **158**, 213 (1993); J. D. Fackenthal, J. A. Hutchens, F. R. Turner, E. C. Raff, *Genetics* **139**, 267 (1995).
3. H. D. Hoyle and E. C. Raff, *J. Cell Biol.* **111**, 1009 (1990); H. D. Hoyle, J. A. Hutchens, F. R. Turner, E. C. Raff, *Dev. Genet.* **16**, 148 (1995).
4. A. Gasch, U. Hinz, D. Leiss, R. Renkawitz-Pohl, *Mol. Gen. Genet.* **211**, 8 (1988); M. Kimble, J. P. Incardona, E. C. Raff, *Dev. Biol.* **131**, 415 (1989); M. Kimble, R. W. Dettman, E. C. Raff, *Genetics* **126**, 991 (1990); R. W. Dettman, F. R. Turner, E. C. Raff, *Dev. Biol.* **177**, 117 (1996).
5. M.-T. B. Davis and S. G. Miller, *Insect Biochem.* **18**, 389 (1988).
6. C. D. Silflow, *Protoplasma* **164**, 9 (1991).
7. E. C. Raff, in *Microtubules*, J. S. Hyams and C. W. Lloyd, Eds. (Wiley, New York, 1994), pp. 85–109.
8. A. D. Tates, *Cytodifferentiation During Spermatogenesis in* Drosophila melanogaster*: An Electron Microscope Study* (Pasmans, The Hague, 1971); L. E. LaChance and G. Olstad, *Ann. Entomol. Soc. Am.* **81**, 292 (1988); J. D. Fackenthal, thesis, Indiana Univ. (1993).
9. Even when the synthesis rate was the same, less Hvβt protein accumulated than β2; the moth protein is thus less stable than the endogenous isoform. However, testes of sterile males carrying multiple inserts of the Hvβt transgene accumulated Hvβt in amounts equivalent to those of β2 that we have shown to be sufficient to support microtubule function. Thus, the functional deficit of the moth isoform is not attributable simply to its relative instability in *Drosophila* cells. The total failure of Hvβt to support microtubule function contrasts with ectopic expression of *Drosophila* β3 in the male germ cells; β3 can support wild-type function of one class of cytoplasmic microtubules, even though it cannot assemble axonemes, spindles, or other microtubule arrays (*3*).
10. In a given spermatid, the threshold may be lower; we did not detect Hvβt in motile sperm in males in which the amount of Hvβt was sufficiently low as to allow fertility.
11. C. Savage *et al.*, *Genes Dev.* **3**, 870 (1989).
12. M. M. Mogensen and J. B. Tucker, *J. Cell Sci.* **88**, 95 (1987); _____ and H. Stebbings, *J. Cell Biol.* **108**, 1445 (1989).
13. L. Evans, T. Mitchison, M. Kirschner, *J. Cell Biol.* **100**, 1185 (1985).
14. Vertebrate isoforms are designated by the organism, followed by the isotype class to which the sequence shown belongs (*1*): class II, major neuronal; class III, minor neuronal; class IVb, predominant testis; class VI, hematopoetic. COOH-termini of isoforms of the same class in other species are identical or very similar to those shown. Widely expressed class I and V isotypes also lack the axoneme motif. β-Tubulins in other *Drosophila* species appear to be identical to those in *D. melanogaster* [(*4*); F. Michiels *et al.*, *Chromosoma* **95**, 387 (1987)].
15. *Aspergillus nidulans* has two β-tubulin genes; benA also lacks the axoneme motif.
16. The *Tetrahymena thermophila* axoneme motif is identical to *Paramecium*. *Giardia* represents the deepest group in eukaryotic taxa, and *Chlamydomonas* groups with plants [M. L. Sogin, H. G. Morrison, G. Hinkle, J. D. Silberman, *Microbiol. Semin.* **12**, 17 (1996)]. β-Tubulins in higher plants, which do not have ciliated cells, lack axoneme motifs.
17. H. B. Diaz, M. A. Conine, E. C. Raff, *J. Cell Biol.* **111**, 412 (1990); H. Diaz and E. Raff, in preparation.
18. To generate the Hvβt transgene, we generated cloning sites 29 base pairs (bp) 5′ and 27 bp 3′ of the Hvβt coding sequence, and the resulting fragment was inserted between 2.1 kb of the 5′ β2 genomic sequences and 1.5 kb of the 3′ β2 genomic sequences previously shown to be sufficient to drive expression of heterologous proteins in the postmitotic male germ cells with correct developmental specificity and at the same level of expression as wild-type β2 (*2, 3*) (J. Hutchens, H. Hoyle, F. R. Turner, E. C. Raff, *Mol. Biol. Cell,* in press). Intronless and intron-containing versions were constructed; in the latter, an oligonucleotide matching the 59-bp β2 intron sequence was inserted between Hvβt codons 73 and 74. Transgenes were inserted into the CaSpeR vector [V. Pirrotta, *Biotechnology* **10**, 437 (1988)] and introduced into the *Drosophila* genome by *P* element–mediated transformation (*2, 3*). Multiple transgenic lines were obtained and testis tubulins analyzed on two-dimensional gels as described previously (*2, 3*); the level of Hvβt expression depended on the site of insertion and presence of the intron. We obtained wild-type β2-like levels of expression (as in Fig. 2) only with an intron-containing insert, suggesting that splicing may be important in normal β2 expression. All transgenic lines exhibited the same suite of defects in spermatogenesis; thus, the phenotype is attributable solely to expression of the moth β-tubulin.
19. Electron microscopy and tannic acid staining were done as previously described (*2, 3*).
20. The morphology of doublet microtubules and the central pair is the same in moths, flies, and transgenic flies. Doublets have a 13-pf A-tubule and a 10-pf shared-wall B-tubule; central pair microtubules are 13-pf. Accessory microtubules in fly axonemes are 13-pf, but 16-pf in moth. Most accessory microtubules in transgenic males are 13-pf, but the abnormal large-diameter accessory microtubules are 16-pf.
21. Accessory microtubules begin as a projection of a protofilament sheet from the B-tubule of each doublet, but completed accessory microtubules are no longer physically associated with the doublet. Completed accessory microtubules in immature axonemes of flies and moths were of a slightly larger diameter than in mature axonemes; thus, adjacent protofilaments in the walls of the accessory microtubules appear to "tighten up" as they form.

22. We thank M.-T. Davis and S. Miller for providing us with the *H. virescens* testis-specific β-tubulin cDNA clone; C.-S. Hong and M. Martin for their enthusiastic participation and contributions as undergraduate research students in the early parts of this study; and W. Saxton and R. Raff for critical reading of the manuscript. This work was supported by a grant from National Institute of Child Health and Human Development (of NIH) to E.C.R.

19 August 1996; accepted 30 October 1996

Recognition of Unique Carboxyl-Terminal Motifs by Distinct PDZ Domains

Z. Songyang,* A. S. Fanning, C. Fu, J. Xu, S. M. Marfatia, A. H. Chishti, A. Crompton, A. C. Chan, J. M. Anderson, L. C. Cantley

The oriented peptide library technique was used to investigate the peptide-binding specificities of nine PDZ domains. Each PDZ domain selected peptides with hydrophobic residues at the carboxyl terminus. Individual PDZ domains selected unique optimal motifs defined primarily by the carboxyl terminal three to seven residues of the peptides. One family of PDZ domains, including those of the Discs Large protein, selected peptides with the consensus motif Glu-(Ser/Thr)-Xxx-(Val/Ile) (where Xxx represents any amino acid) at the carboxyl terminus. In contrast, another family of PDZ domains, including those of LIN-2, p55, and Tiam-1, selected peptides with hydrophobic or aromatic side chains at the carboxyl terminal three residues. On the basis of crystal structures of the PSD-95-3 PDZ domain, the specificities observed with the peptide library can be rationalized.

Many cytosolic signaling proteins and cytoskeletal proteins are composed of modular units of small protein-protein interaction domains that allow reversible and regulated assembly into larger protein complexes. Examples are SRC homology 2 (SH2) and SH3 domains and phosphotyrosine-binding (PTB) domains (*1*). PDZ domains have been observed in more than 40 cytosolic proteins, many of which are located at specific regions of cell-cell contact, such as tight junctions, septate junctions, and synaptic junctions. The name PDZ derives from three proteins that contain repeats of this domain: mammalian postsynaptic density protein, PSD-95; *Drosophila* disc large tumor suppressor, Dlg; and the mammalian tight junction protein, ZO1 (*2–5*).

Certain PDZ domain–containing proteins bind directly to the last several (COOH-terminal) residues of transmembrane proteins. For example, the second PDZ domain of PSD-95 binds the *N*-methyl-D-aspartate receptor through interaction with the COOH-terminal Ser/Thr-Xxx-Val se-

quence (6). PDZ domains of PSD-95 and Dlg bind similar COOH-terminal sequences on Shaker-type K^+ channels, and PDZ domains may be necessary for the clustering of these channels on the cell surface (7).

These results have raised several interesting questions about PDZ domains: (i) Do these domains (like SH2 and SH3 domains) recognize internal sequences on proteins, or can they bind only to the free COOH-terminus of the target protein? (ii) Do individual members of the more than 80 PDZ domains defined to date recognize unique linear sequences? (iii) What is the structural basis for protein or peptide binding to PDZ domains?

The crystal structures of the third PDZ domains of hDlg (hDlg-3) alone (8) and PSD-95 (PSD-95-3) bound to a peptide (9) provide a starting point from which to answer these questions. The side chain of the COOH-terminal Val of the associated peptide is buried in a deep hydrophobic pocket. The free carboxylate of the valine interacts with amide nitrogens from a loop between two β structures in a sequence (Gly-Leu-Gly-Phe) that is highly conserved in most PDZ domains. However, the COOH-terminus is not deeply buried, raising the possibility that a free COOH-terminus may not be necessary for binding to all PDZ domains.

To address these questions, we used an oriented peptide library approach (*10*, *11*). A soluble mixture of peptides of the same length and some common internal fixed residue or residues was passed over a column containing the domain of interest, and the subgroup of peptides retained by the column was sequenced to obtain a consensus motif (*12*). A library of peptides in which the COOH-terminal eight positions had degenerate amino acids (*11*) was used to investigate the binding specificities of nine PDZ domains (Table 1). In general, the PDZ domains bound preferentially to peptides that terminated in a hydrophobic amino acid (usually Val or Ile). In addition, most (but not all) of the PDZ domains selected for peptides with either Ser, Thr, or Tyr located two residues from the COOH-terminus (−2 position). To increase the fraction of peptides with high affinity, we constructed a library with the degeneracy at the −2 position restricted to Ser, Thr, or Tyr. This method increased our ability to determine selectivities at other positions (Table 1). Results with this library were consistent with those obtained with the fully degenerate library. Additional selection specificity was observed out to the −8 position for some domains (Table 1).

To determine whether an internal sequence could be recognized by PDZ domains, we used a library with a fixed internal Tyr that was nine residues from the COOH-terminus (MAXXXXYXXXXAKKK-NH_2, where X indicates positions with degeneracy of all amino acids except Cys, Trp, Tyr, Ser, and Thr; total degeneracy ~2.5 billion). The fixed COOH-terminus of this library (AKKK-NH_2) is predicted not to bind to PDZ domains on the basis of the results with the other libraries (Table 1). Consistent with the idea that PDZ domains do not bind to internal sequences, none of the PDZ do-

Table 1. PDZ domain specificity deduced through the use of oriented peptide libraries (*10*) and known binding sites of PDZ domains. Either a peptide library with the sequence KNXXXXXXX-COOH or a library with the sequence KNXXXXXX(S/T/Y)XXCOOH was screened with the indicated GST-PDZ domain. X indicates all amino acids except Cys or Trp. Selectivities greater than 1.5 are shown. Values larger than 2.0 are indicated in bold. On the basis of peptide library studies on other protein modules, a value greater than 1.5 indicates significant selection, and a value greater than 2.0 indicates strong selection. Underlined residues indicate the position where the degeneracy is limited to Ser, Thr, and Tyr. ND, not determined. 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.

PDZ domain	Position									Known binding sites	Protein
	−8	−7	−6	−5	−4	−3	−2	−1	0 (COOH)		
						Group I					
mDlg-1	–	X	X	X	X	E	**T** **S**	D	**V** **I**	ND	
mDlg-2	–	X	X	X	K	**E**	**T** **S**	D	**V**	AVETDV KIFTDV YLVTSV	Kv1.4 channel Kv1.3 channel APC
mDlg-1/2	–	X	X	X	X	E	**S** T	D E	L V	Same as above	
S/T/Y library	K	K	**K** P Q	**K** **P**	**K** Q	**E** Q H	**T** **S**	D Q E	**V** **I**		
mDlg-3 S/T/Y library	**K**	**K**	**K**	**K** P Q	**K** P Q	**E** Q K	**T** **S**	K	**V** **I** M	**ND**	
PTPbas-3	–	X	X	X	E	E V	**S** **T**	X	**V** **I** L		
S/T/Y library	D K	K D	**D** K	D N Q E	**P** Q E D	**E** D	**S** **T**	**N** D	**V** **I**	EIQSLV	Fas antigen
PTPbas-5	–	X	X	X	E	I Y V	**Y**	**Y** K	**V** K I	**ND**	
						Group II					
p55	–	X	X	X	X	X	F	X	X		
S/T/Y library	X	X	X	X	X	Y	**Y**	Y	F	RKEYFI	Glycophorin C
LIN-2	–	X	X	X	X	X	**F**	F	V F A	KDEYYV	Neurexin
Tiam-1	–	X	X	X	X	I	**Y** F	Y H	F A	ND	
S/T/Y library	X	X	X	X	X	X	**Y**	Y	**A**		
AF-6	–	X	X	X	Y	E A	F I M Y	**Y** F	**V** F I L	**ND**	
S/T/Y library	Y P	F	L	Q	X	A P E	**Y**	**Y** F	**V** **I**		

Z. Songyang, J. Xu, L. C. Cantley, Division of Signal Transduction, Beth Israel Hospital, and Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA.
A. S. Fanning and J. M. Anderson, Departments of Internal Medicine and Cell Biology, Yale University School of Medicine, New Haven, CT 06520, USA.
C. Fu and A. C. Chan, Howard Hughes Medical Institute, Center for Immunology, Department of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA.
S. M. Marfatia and A. H. Chishti, Laboratory of Tumor Cell Biology, Department of Biomedical Research, St. Elizabeth's Medical Center, Tufts University, Boston, MA 02135, USA.
A. Crompton, ONYX Pharmaceuticals, 3031 Research Drive, Building A, Richmond, CA 94806, USA.
*To whom correspondence should be addressed. Present address: 68-380, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

mains investigated retained specific peptides from this third library (*13*).

The PDZ domains in Table 1 can be divided into two major groups on the basis of the amino acid selected at the −2 position. One group, including the third and fifth PDZ domains of the phosphotyrosine phosphatase PTPbas/FAP-1 (PTPbas-3 and PTPbas-5) (*14*) and the three PDZ domains of murine hDlg (mDlg) (*12*), selected peptides with amino acids containing hydroxyl groups (Ser, Thr, or Tyr) at position −2 (Table 1). However, another group, including the PDZ domains of p55 (*15*), human LIN-2 (*16*), Tiam-1 (*17*), and AF-6 (*18*), selected peptides with hydrophobic amino acids at −2 (Table 1). Most members of this latter group preferred Phe at −2, although Tyr was also selected at this position. This result was surprising because previous studies led to speculation that a hydroxyl group at position −2 is important for binding to PDZ domains (*7*, *9*).

The peptides from the library that appeared to be optimal for binding to the mDlg, PTPbas-3, and Tiam-1 PDZ domains were synthesized and investigated for their abilities to bind to each of these domains (Fig. 1). As measured by BIAcore, the estimated dissociation constants (K_d's) for mDlg-2 and PTPbas-3 PDZ domains to their optimal peptides were 42 nM (K_{on} = 2.4×10^4 M^{-1} s^{-1} and K_{off} = 1.0×10^{-3} s^{-1}) and 154 nM (K_{on} = 1.3×10^4 M^{-1} s^{-1} and K_{off} = 2.1×10^{-3} s^{-1}), respectively. These affinities are in the same range as those observed for binding of optimal peptides of these sizes to SH2 and SH3 domains (*19*, *20*). Moreover, the mDlg-2 PDZ domain (at 0.5 μM) failed to bind to the PTPbas peptide, whereas the PTPbas-3 PDZ bound only weakly to the Dlg peptide (K_d ~ 1.5 μM) (Fig. 1, A and B). None of the three PDZ domains, when added at 0.5 μM, bound significantly to the optimal Tiam-1 peptide on the basis of the BIAcore technique (*21*). One reason for this result could be either that the cross-linking procedure interfered with binding or that the affinity of this peptide is out of the sensitivity range of BIAcore analysis. Nevertheless, the specificity of the Tiam-1 peptide could be demonstrated by competitive binding experiments in which a PDZ domain precipitation approach was used (Fig. 1C). Whereas 50% maximal displacement of Tiam-1 PDZ domain binding occurred at 10 μM of the Tiam-1 optimal peptide, no significant competition for binding was observed at 50 μM of the peptides designed for the other two PDZ domains. These results indicate that the peptides predicted by the library approach have relatively high affinity for their respective PDZ domains and that distinct PDZ domains bind to distinct optimal sequences.

The optimal peptides predicted by the peptide library approach can be rationalized on the basis of the recent crystal structure of the PSD-95-3 PDZ domain bound to a high-affinity peptide (*9*). The PDZ domain of PSD-95 is composed of two α helices (designated αA and αB), six β strands (designated βA to βF), and several loop regions, with the loop between βA and βB interacting with the carboxylate group of the peptide's COOH-terminal residue. In Fig. 2, we have highlighted the residues of the PSD-95-3 PDZ domain that are predicted to interact with the side chains of the associated peptide. Residues forming each pocket on PSD-95-3 are also listed in Table 2. Corresponding residues on the other PDZ domains were deduced by alignment of their primary sequences with PSD-95-3 and labeled according to their positions in the crystal structures of PSD-95-3 (for example, the first residue in helix αB is named αB1). To emphasize the nature of the binding pockets in Fig. 2 and Table 2, we show basic, acidic, hydrophobic, and hydrophilic (uncharged) residues in different colors. In particular, we have focused on His-αB1 (His^{372} of PSD95-3), which coordinates the hydroxyl group of the Thr at postion −2; Glu-BC2, which is near the −4 (Lys) side

Table 2. Position of residues of the PDZ domain peptide-binding pockets that are predicted to interact with the side chains of the associated peptide. Color designation: basic residues (blue), acidic residues (red), hydrophobic residues (black), and hydrophilic (uncharged) residues (green). Ec-Htra (*26*) and Ss-Ctpa (*27*) are bacterial and plant proteases, respectively.

	Pocket					0	Pocket					-1	Pocket			-2	Pocket				-3
Residue	AB6	βB1	βB3	αB5	αB8		AB7	βB1	βB2	βC5	CA1		αB1	αB2	αB5		βB2	βB4	βC4	βC5	
Group 1A																					
PSD95-3	L	F	I	A	L	VAL*	G	F	N	F	L	SER*	H	E	A	THR*	N	V	S	F	GLN*
mDlg-3	L	F	I	A	L	VAL/ILE	G	F	N	F	L	LYS	H	E	A	THR/SER	N	V	S	F	GLU
mDlg-1	L	F	I	V	L	VAL/ILE	G	F	S	K	I	ASP	H	S	V	THR/SER	S	A	T	K	GLU
mDlg-2	L	F	I	V	L	VAL/ILE	G	F	S	K	I	ASP	H	E	V	THR/SER	S	A	T	K	GLU
PTPbas-3	L	I	V	V	L	VAL/ILE	G	I	S	A	I	ASN	H	K	V	SER/THR	S	T	K	A	GLU
PTPbas-5	L	F	V	V	L	VAL	G	F	T	D	I	TYR/LYS	H	T	V	TYR	T	T	H	D	ILE/TYR
LIN-7	L	F	V	V	L	?	G	F	N	R	I	?	H	E	V	?	N	M	S	R	?
Group 1B																					
LCF	L	F	L	W	I	?	G	F	S	R	F	?	R	F	W	?	S	E	N	R	?
ZO1-3	V	I	L	V	L	?	G	I	R	G	L	?	R	E	V	?	R	A	A	G	?
PICK-1	I	I	I	A	I	?	G	I	S	Q	F	?	K	V	V	?	S	G	V	Q	?
Group 2A																					
p55	M	I	L	Q	M	PHE	G	I	T	R	L	F	V	D	Q	F/Y	T	K	A	R	XXX
LIN-2	M	I	L	Q	L	VAL/PHE	G	I	T	R	M	F	V	E	Q	F	T	K	A	R	XXX
PTPbas-6	L	F	L	N	L	?	G		S	D	N	?	L	E	N	?	S	C	S	D	?
Ss-Ctpa	V	L	I	A	L	?	G	L	Q	A	L	?	L	D	A	?	Q	N	M	A	?
Ec-Htra	I	F	I	Q	T	?	G	F	A	Q	L	?	A	L	Q	?	A	P	S	Q	?
Group 2B																					
Tiam-1	Y	F	L	S	L	PHE/ALA	G	F	S	S	K	TYR	D	A	S	TYR/PHE	S	S	N	S	ILE
INAD	F	I	I	I	I	?	G	I	C	G	V	?	E	Q	I	?	C	V	K	G	?
AF-6	M	L	I	A	M	VAL/PHE	G	L	S	S	V	TYR	Q	E	A	PHE	S	V	K	S	GLU
DSH	L	I	I	V	I	?	G	I	S	S	M	?	N	D	V	?	S	V	G	S	?

*Sequence from PSD95-3/pep cocrystal rather than optimal sequence.

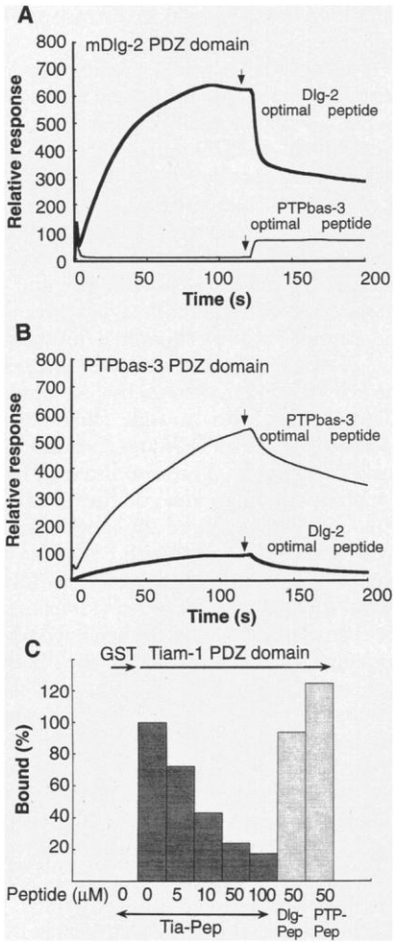

Fig. 1. Specific binding of predicted optimal peptides to PDZ domains of Dlg, PTPbas, and Tiam-1. Optimal peptide for mDlg-1/2 (Dlg-Pep: KKKKETDV-COOH) or PTPbas-3 (PTP-Pep: KDDQESNV-COOH) was coupled to BIAcore sensor CM5 chips (Biosenser). The binding of PDZ domains to these peptides was monitored with the use of BIAcore 2000. Time 0 to 120 s indicates the association phase where GST-PDZ domain fusion proteins were injected. The arrows indicate injection of 20 μM competitor peptides. To determine K_{on} and K_{off} rates, we injected various concentrations of GST-PDZ fusion proteins and analyzed data with the BIAevaluation 2.1 software (Biosensor). (**A**) Relative response (binding) when GST-mDlg-2 PDZ domain (0.5 μM) was passed through surfaces coated with Dlg-Pep or PTP-Pep. (**B**) Relative response when GST–PTPbas-3 PDZ domain (0.5 μM) was passed through surfaces coated with Dlg-Pep or PTP-Pep. (**C**) Tiam-1 optimal peptide (Tia-Pep: SSRKEYYA-COOH) was coupled to cyanogen bromide–activated Sepharose beads (Sigma). The beads were then incubated with GST or GST PDZ domain fusion proteins (4 μg/ml) with various concentrations of peptides (0 to 100 μM) in TSN buffer containing BSA (1 mg/ml) and DTT (1 mM). Bound proteins were washed three times with TSN buffer, separated by SDS–polyacrylamide gel electrophoresis, and visualized by protein immunoblotting with antibody to GST (Transduction Lab). Relative binding was quantified with the use of an AGFA Scanner.

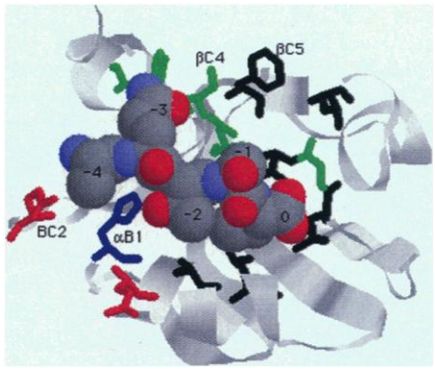

Fig. 2. COOH-terminal peptide recognition by PSD95-3 PDZ domain. The peptide binding site of PSD95-3 PDZ domain was presented with the PDZ domain backbone shown in ribbon and peptide KQTSV-COOH shown in sphere structures (*9*). Residues that form various binding pockets are indicated in color tubes. Color designation: basic residues (blue), acidic residues (red), hydrophobic residues (black), and hydrophilic (uncharged) residues (green).

chain; and Ser-βC4, which coordinates the Gln at position −3. Phe-βC5, also labeled, does not directly contact residues on the associated peptide but is at a position that could affect binding of residues with large side chains at the −1 or −3 positions, or both. These residues vary among the PDZ domains that we have studied (Table 2).

The first residue in the αB helix (αB1) correlates strongly with peptide selectivity at position −2. We have therefore grouped PDZ domains into subgroups (Table 2). The domains in group 1A with His at the αB1 position selected peptides with Ser, Thr, or Tyr at position −2, in agreement with His-αB1 coordinating the hydroxyl group (*9*). Consistent with this prediction, LIN-7 binds directly to LET-23, which has the sequence SQKETCL at the COOH-terminal (*22*). Those PDZ domains that lack a basic residue at αB1 (group II), however, selected hydrophobic or aromatic amino acids at position −2. The Tiam-1 PDZ domain has an Asp at αB1, and although it still selects Tyr, it is less selective, with Phe being the second best. It is possible that the Asp carboxylate can also weakly coordinate the hydroxyl group of Tyr. In contrast, the PDZ domains of p55 (Val-αB1), LIN-2 (Val-αB1), and AF-6 (Gln-αB1) selected for Phe over Tyr at position −2. Thus, the residue at αB1 is a good indicator of the selectivity at the −2 position and therefore determines the selectivity subgroups in Table 1.

Specificity at the other positions can also be rationalized. The residues that make up the P0 pocket are hydrophobic and relatively highly conserved (*9*). All the domains studied selected hydrophobic residues at the 0 position, and in most instances, Val was optimal. Small differences in the residues of the pocket are likely to explain the selectivities for Phe or Ala of Tiam-1 and p55 at this position.

Considerable variability in selectivity at the −1 position was observed, although in no instance was the selectivity at this position very strong (compared to the 0 and −2 positions). This selectivity might be explained by residue βC5. Although this residue does not directly contact the −1 Ser in the PSD-95-3 structure (Fig. 2), the ε amino group of a Lys at βC5 could be close enough to the −1 position to explain the selection for Asp at this location by mDlg-1/2. In contrast, the PTPbas-5 PDZ domain has an Asp at βC5 and selects weakly for Lys at position −1. Finally, basic residues at βC5 and βC4 might also help explain the selectivity of several PDZ domains for peptides with Glu at the −3 position.

The optimal motifs predicted by the peptide library are in good agreement with known binding sites of PDZ domains (Table 1). For mDlg-2 and PTPbas-3 PDZ domains, their known binding sites on channel proteins and Fas antigen are consistent with the optimal binding motifs. In addition, p55 and LIN-2 were demonstrated to bind glycophorin C and neurexin, respectively (Table 1) (*23*, *24*). In agreement with our peptide library prediction, the last three residues of these proteins are hydrophobic and contain aromatic residues at both the −2 and −1 positions (Table 1).

One possible function of PDZ domains is their role in cytoskeletal and membrane organization. Many PDZ domain–containing molecules (for example, LIN-7, Dlg, p55, and PTP-meg) not only associate with the membrane, but also carry sequence motifs for cytoskeleton localization (*5*, *22*). The clustering function of PDZ domains can be achieved through network binding of PDZ domains, because many proteins carry multiple copies of PDZ domains. In addition to their ability to bind COOH-terminal sequences, some PDZ domains can also dimerize with each other (*25*). The three PDZ domains of Dlg were shown to recognize similar motifs, whereas PDZ domains of PTPbas recognize different motifs (Table 1). Thus, PDZ domains may cooperate to enhance the binding to their common targets, or in the case of PTPbas, they may help to bind simultaneously to multiple, different targets.

REFERENCE AND NOTES

1. G. B. Cohen, R. Ren, D. Baltimore, *Cell* **80**, 237 (1995); T. Pawson, *Nature* **373**, 573 (1995).
2. D. F. Woods and P. J. Bryant, *Cell* **66**, 451 (1991); *Mech. Dev.* **44**, 85 (1993).
3. K. O. Cho, C. A. Hunt, M. B. Kennedy, *Neuron* **9**, 929 (1992); M. B. Kennedy, *Trends Biochem. Sci.* **20**, 350 (1995).

4. M. Itoh *et al.*, *J. Cell Biol.* **121**, 491 (1993); E. Willott *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7834 (1993).
5. C. P. Ponting and C. Phillips, *Trends Biochem. Sci.* **20**, 102 (1995).
6. H. C. Kornau, L. T. Schenker, M. B. Kennedy, P. H. Seeburg, *Science* **269**, 1737 (1995).
7. E. Kim, M. Niethammer, A. Rothschild, Y. N. Jan, M. Sheng, *Nature* **378**, 85 (1995).
8. J. H. M. Cabral *et al.*, *ibid.* **382**, 649 (1996).
9. D. A. Doyle *et al.*, *Cell* **85**, 1067 (1996).
10. Z. Songyang *et al.*, *ibid.* **72**, 767 (1993).
11. A primary peptide library, KNXXXXXXXX-COOH, where X indicates all amino acids except Cys and Trp, was first used to screen peptides that bind specifically to the glutathione-S-transferase (GST)–PDZ domains. All the peptides in the library end with free carboxylate, therefore orienting all binding pockets. The peptides that bound were sequenced as a mixture, and the selectivities for amino acids at a given position were determined by comparison to the sequence of control experiments with GST alone (*10*). Arg was not included in the calculation because of buffer contamination during sequencing. A secondary library, KNXXXXXX(S,T,Y)XX-COOH, where the −2 position was fixed with Ser, Thr, and Tyr, was used to further define the preference of some PDZ domains.
12. Peptide library synthesis was as described (*10*). Individual PDZ domains were expressed and purified as GST fusion proteins: murine hDlg PDZ-1 (186–282), PDZ-2 (281–377), PDZ-3 (428–518), and PDZ-1/2 (281–518); murine PTPbas PDZ-3 (1351–1445) and PDZ-5 (1758–1848); murine Tiam-1 PDZ; human LIN-2 PDZ (422–507); human erythroid p55 PDZ (1–164); and human AF-6 PDZ (983–1102). Glutathione beads (50 to 60 μl) saturated with GST-PDZ proteins were mixed with the peptide library (1 mg) in 300 μl of TSN buffer [40 mM triethylamine (pH 7.6), 150 mM NaCl, and 0.01% NP-40] containing bovine serum albumin (BSA, 1 mg/ml) and 1 mM dithiothreitol (DTT). After 45 min of constant shaking at 4°C, the beads were washed with TSN buffer. The peptides retained were eluted with 30% acetic acid, lyophilized, resuspended in distilled water, and sequenced on a Bio-Applied 477A sequencer.
13. Z. Songyang and L. C. Cantley, unpublished data.
14. T. Sato, S. Irie, S. Kitada, J. C. Reed, *Science* **268**, 411 (1995).
15. P. Ruff, D. W. Speicher, A. Husain-Chishti, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6595 (1991).
16. R. Hoskins, A. F. Hajnal, S. A. Harp, S. K. Kim, *Development* **122**, 97 (1996); A. Brecher *et al.*, in preparation.
17. G. G. Habets *et al.*, *Cell* **77**, 537 (1994).
18. R. Prasad *et al.*, *Cancer Res.* **53**, 5624 (1993).
19. G. Payne, S. E. Shoelson, G. D. Gish, T. Pawson, C. T. Walsh, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4902 (1993).
20. H. T. Yu *et al.*, *Cell* **76**, 933 (1994).
21. Z. Songyang *et al.*, data not shown.
22. J. S. Simske, S. M. Kaech, S. A. Harp, S. K. Kim, *Cell* **85**, 195 (1996).
23. S. M. Marfatia *et al.*, *J. Biol. Chem.*, in press.
24. V. Hata, S. Butz, T. C. Sudhof, *J. Neurosci.* **16**, 2488 (1996).
25. J. E. Brenman *et al.*, *Cell* **84**, 757 (1996).
26. B. Lipinska, M. Zylicz, C. Georgopoulos, *J. Bacteriol.* **172**, 1791 (1990).
27. S. V. Shestakov *et al.*, *J. Biol. Chem.* **269**, 19354 (1994).
28. We thank M. Berne for peptide synthesis and sequencing, R. Mackinnon for structural coordinates of PSD-95-3, W. Boll and A. Nguyen for technical assistance, A. Couvillon for antibodies to GST, M. Oishi and T. Woodford-Thomas for the PTPbas cDNA, and A. Brecher for human LIN-2 PDZ. C.F. is a Lucille Markey Fellow. Supported by grants from American Cancer Society and Lucille P. Markey Charitable Trust (L.C.C.), NIH grants CA66263 and DK34989 (J.M.A. and A.S.F), Pew Scholars Program (A.C.C.), and NIH grant CA66263 (A.H.C. and S.M.M.).

22 July 1996; accepted 23 October 1996

The Inverse Association Between Tuberculin Responses and Atopic Disorder

Taro Shirakawa, Tadao Enomoto, Shin-ichiro Shimazu, Julian M. Hopkin*

Human immune responses are heterogeneous and may involve antagonism between T helper (T_H) lymphocyte subsets and their cytokines. Atopy is characterized by immediate immunoglobulin E (IgE)–mediated hypersensitivity to agents such as dust mites and pollen, and it underlies the increasingly prevalent disorder asthma. Among Japanese schoolchildren, there was a strong inverse association between delayed hypersensitivity to *Mycobacterium tuberculosis* and atopy. Positive tuberculin responses predicted a lower incidence of asthma, lower serum IgE levels, and cytokine profiles biased toward T_H1 type. Exposure and response to *M. tuberculosis* may, by modification of immune profiles, inhibit atopic disorder.

Atopy is a state of allergic response, mediated by IgE, to largely innocuous, common environmental antigens (allergens) such as those derived from house dust mites and plant pollens (*1*); it underlies the clinical diseases of asthma, hay fever, and eczema (*2*). Atopy can be recognized by allergen-specific IgE in serum or by immediate-type hypersensitivity reactions to allergens upon intradermal skin testing. Heterogeneous genetic and environmental factors interact in the development of atopy (*3*); a set of cytokines—interleukin-4 (IL-4), IL-10, and IL-13 derived from the T_H2 subset of T lymphocytes—is central in mediating IgE production and the development of immediate hypersensitivity (*4*).

In recent decades there has been an increase in severity, and probably in prevalence, of atopic disorders in developed countries (*5*). Studies on migrants from developing to developed countries support the importance of etiological environmental changes associated with "Westernization" (*6*). The nature of these environmental changes is obscure, but speculation has focused on increased air pollution or other toxins in the environment, increased indoor exposure to dust mite antigens in less ventilated modern homes, and dietary changes (*7*). One factor temporally associated with the rise of atopy is the decline of many infectious diseases in developed countries as the result of improved living standards and immunization programs (*8*). Data on the risk of atopy according to sibship size and birth order (*9*) also support the possibility that diminished exposure to infection might, in some way, promote atopic responses. Childhood respiratory infections that might strongly modify the developing immune system, both systemically and within the lung, include measles, whooping cough, and tuberculosis. Some of these infections cultivate a T_H1 immunological environment with IL-12, interferon-γ (IFN-γ), and tumor necrosis factor (TNF) as predominant cytokines (*10*); because these cytokines inhibit T_H2 cytokine functions (*11*), the absence of such infections might release T_H2 immune mechanisms and thus promote atopic disorder.

In the case of tuberculosis, an important marker of T_H1-mediated acquired immunity (not synonymous with protection) is the development of delayed-type hypersensitivity. This can be tested by observing the reaction, after 48 hours, to the intradermal injection of tuberculin protein (*12*). There is likely a "J-shaped" relation between the degree of delayed hypersensitivity and the risk of tuberculous disease, in which people with moderate hypersensitivity are at least risk (*13*).

To test for clinical evidence of antagonism between delayed hypersensitivity to tuberculin and immediate atopic responses, we conducted an epidemiologic survey in a county of the Wakayama prefecture in southern Honshu, Japan, where there has been a long-established program of tuberculin testing and immunization with attenuated bovine *M. tuberculosis* vaccine [bacillus Calmette-Guérin (BCG)] after birth and at 6 and 12 years of age (*14*). From a population of approximately 1000 12- to 13-year-old schoolchildren attending the 18 junior high schools of the county in 1995, we studied 867 children with complete retrospective records of their tuberculin responses. We administered a

T. Shirakawa and J. M. Hopkin, Lung Research Laboratory, Osler Chest Unit, Churchill Hospital, Oxford OX3 7LJ, UK.
T. Enomoto, Department of Otolaryngology, Japanese Red Cross Society, Wakayama Medical Center, Wakayama, Japan.
S. Shimazu, Department of Pediatrics, National Wakayama Hospital, Wakayama, Japan.
*To whom correspondence should be addressed. E-mail: jhopkin@immsvr.jr2.ox.ac.uk