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A Chemical Approach to the Identification of Tensin-Binding Proteins

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ABSTRACT Many protein–protein interactions are mediated by small modular domains, which recognize short peptide motifs in their partner proteins. However, for the great majority of these domains, the identity of their partner proteins remains unknown. In this work, a chemical/bioinformatics approach has been developed to identify phosphotyrosyl (pY) proteins that bind to tensin, a protein involved in the formation of actin cytoskeleton and signal transduction. A pY peptide library was chemically synthesized and screened against the Src homology 2 (SH2) domain of tensin to identify the peptide motifs that bind to the SH2 domain. Next, protein databases were searched for proteins containing the SH2 domain-binding peptide motifs. Finally, the potential tensin-binding proteins were confirmed (or disproved) by *in vitro* pull-down and coimmunoprecipitation assays. This procedure identified phosphoinositide-dependent kinase-1 and downstream of tyrosine kinase 2 as novel tensin-binding proteins. In addition, a cell-permeable pY peptide was designed as tensin SH2 domain inhibitor, which caused the disruption of actin filaments in NIH 3T3 cells. This method should be generally applicable to other modular domains that recognize small peptide motifs.

Protein–protein interactions provide a major part of the organization and regulation in a living cell. Many of these interactions are mediated by a growing number of protein modular domains that recognize short peptide motifs in their target proteins (*1*). The Src homology 2 (SH2) domain was one of the first such domains identified, and the human genome encodes 116 SH2 domains in 106 proteins (*2*). The SH2 domain consists of ~100 amino acids and binds to specific phosphotyrosine (pY)-containing peptides. The affinity and specificity of the SH2 domain–pY peptide interaction are dictated by the pY residue and two to three residues immediately N- and C-terminal to the pY residue (*2*–*4*). For many of the modular domains, including the SH2 domain, the structural basis for their interaction with peptide ligands has been well-established. In contrast, their cellular protein partners remain largely unknown.

In recent years, major efforts have been made to systematically map the protein–protein interaction network in a living cell (or the "interactome"). The most commonly used method for identification of new protein–protein interactions is the yeast two-hybrid system (*5*). Unfortunately, this method is less effective with modular domains that require post-translational modification for binding (*e.g.*, SH2, phosphotyrosine binding (PTB), and tryptophan-containing WW domains). A second method is coimmunoprecipitation/affinity pull-down of binding partners coupled with mass spectrometric (MS) identification (*6*). This method, however, is generally limited to the identification of the most abundant and tight-binding proteins. A third approach was recently reported by MacBeath and coworkers (*7*); in this approach, all 116 human SH2 domains are displayed on a single microchip and tested simultaneously for binding to a fluorescently labeled pY peptide. This

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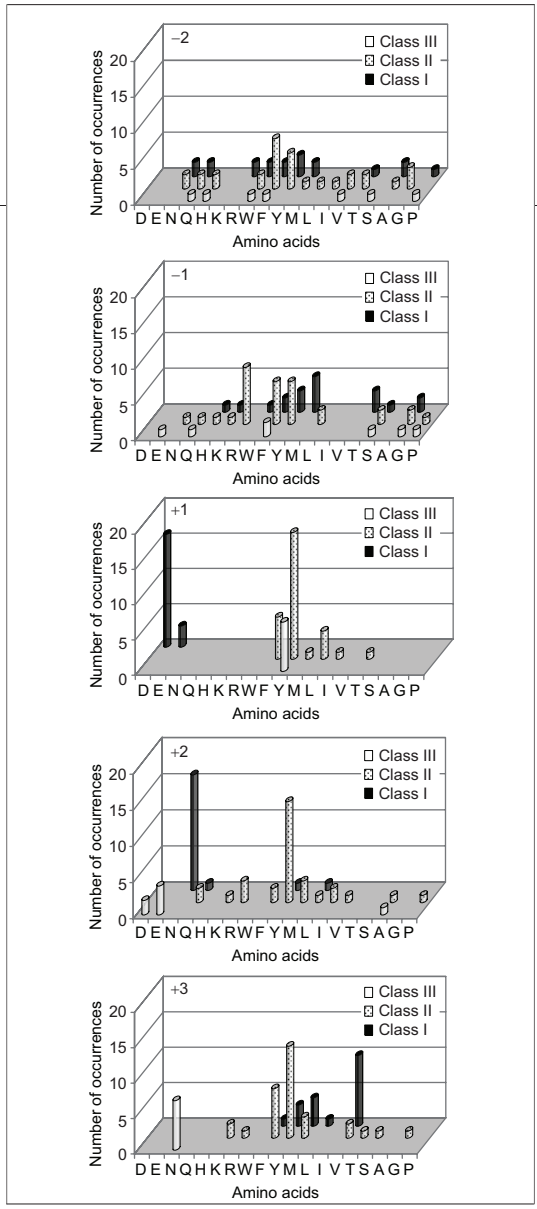

Figure 1. Sequence specificity of tensin SH2 domain. Displayed are the amino acids identified at each position from −2 to +3 relative to pY (position 0). Number of occurrences on the *y*-axis represents the number of selected sequences that contain a particular amino acid at a certain position. Key: M, norleucine.

method has the advantage of providing quantitative binding data for each interaction pair, but requires a known pY motif beforehand, the kind of information that is currently lacking for the majority of human proteins. Finally, we and others have been pursuing a combinatorial peptide library approach (*8*). Since the modular domains recognize short peptide motifs in their partner proteins, in principle, one should be able to identify the peptide motifs that bind to a modular domain by screening a peptide library and then search the protein/genomic databases for its potential partner protein(s). We have recently developed a combinatorial library method that allows the rapid identification of peptide ligands recognized by any modular domain (*4*). Herein, we have applied this methodology to identify the interacting partners of tensin, a cytoplasmic protein that bridges focal adhesion to actin filaments (*9*). In addition to its structural function, tensin also plays a role in signal transduction (*10*). Tensin contains a single SH2 domain, which is critical for actin cytoskeleton organization and tensin-regulated cell migration (*11*–*13*). The tensin SH2 domain was reported to bind to a large number of pY proteins, two of which have been identified as phosphoinositide 3-kinase (PI3-kinase) and p130Cas (*11, 14, 15*). Identification of the other tensin-interacting pY proteins should help scientists understand the mechanism by which tensin regulates cell migration, cytoskeletal organization, and signal transduction.

RESULTS AND DISCUSSION

Identification of the binding partners of a modular domain by our method entails three steps. First, the peptide motifs recognized by the modular domain are determined by screening a combinatorial peptide library. Next, potential partner proteins of the domain are identified by searching protein and genomic databases with the binding sequence motif(s). Finally, the candidate proteins are tested for binding to the modular domain by *in vitro* pull-down and coimmunoprecipitation assays. In addition, cell-permeable inhibitors can be designed from the binding motifs and used to specifically probe the physiological function of the interaction between the modular domain and its protein target(s).

Sequence Specificity of Tensin SH2 Domain. Because SH2 domains typically interact with the pY residue and two to three residues flanking the pY residue (*2*), a pY peptide library containing five randomized positions, TAXXpYXXXLNBBRM-resin [where X represents L-norleucine (Nle) or any of the 18 proteinogenic amino acids excluding cysteine and methionine; B is β-alanine], was designed. The invariant LNBBRM linker was added to facilitate peptide cleavage from the resin (after methionine with CNBr) and MS analysis (positively charged arginine) (*4*). The theoretical diversity of the library is 19^5 or 2.5×10^6. Screening a total of 200 mg of the library (~572,000 beads) against the tensin SH2 domain produced 63 positive beads, which were manually removed from the library and individually sequenced by partial Edman degradation/MS (PED/MS) (*16*) to give 57 complete sequences. Inspection of the selected sequences (Supplementary Table 1) reveals that the tensin SH2 domain can bind to three distinct classes of pY peptides of the consensuses pY(D/E)N(V/M/Y/L) (class I), pY(Y/F/L)ϕ(Y/F/M) (class II), and pYY(E/D)N (class III) (where ϕ represents hydrophobic amino acids) (Figure 1). By using a pooled sequencing method, Auger *et al.* previously reported a consensus sequence of pY(E/D)N(I/V/F) for the tensin SH2 domain (*14*), which corresponds to the class I sequences in this work. The class II and III sequences have not previously been observed. For all three classes of peptides, the tensin SH2 domain exhibits little selectivity on the N-terminal side of pY, indicat-

TABLE 1. Dissociation constants (K_d, μM) of selected peptides toward tensin SH2 domain[a]

Peptide	Peptide sequence	K_d MS (μM)
1	NN-MYpYDNV-LNR	1.0 ± 0.1
2	NN-QQpYYEN-LNR	1.6 ± 0.2
3	NN-GFpYYYF-LNR	0.7 ± 0.1
4	SQLpY^{9}DAVPI (PDK-1)	>6
5	EDCpY^{373}GNYDN (PDK-1)	1.9 ± 0.2
6	YGNpY^{376}DNLLS (PDK-1)	0.27 ± 0.03
7	NELpY^{139}SSAVT (Dok-2)	>6
8	DSPpY^{271}SRPHD (Dok-2)	No binding
9	EGEpY^{299}AVPFD (Dok-2)	>6
10	DHIpY^{345}DKPEG (Dok-2)	>6
11	GTEpY^{402}DNVVL (Dok-2)	0.44 ± 0.05
12	RALpY^{12}DNVPE (Cas-L)	0.19 ± 0.02
13	MAPpY^{775}DNYVP (PDGFR-β)	0.55 ± 0.07
14	RALpYVNVPE	0.71 ± 0.04

[a]Each peptide contains a biotin-miniPEG linker at the N-terminus.

ing that this SH2 domain has the canonical SH2 domain–pY peptide binding mode (*2*). To confirm the screening results, a representative peptide was selected from each consensus group (Table 1, peptides 1−3), chemically synthesized, and tested for binding to the tensin SH2 domain by surface plasmon resonance (SPR). All three peptides bound to the tensin SH2 domain with high affinity (K_d = 0.7–1.6 μM).

We note that other investigators have used different library methods to define the sequence specificity of SH2 domains (comprehensively reviewed in ref 17). These other methods generally provide information on the relative preference of certain amino acid(s) at a given position but do not give individual peptide sequences. They cannot detect sequence covariance and are therefore ineffective with domains that recognize more than one type of consensus sequences. Furthermore, in the previous methods, positive peptides are selected on the basis of both their affinity to the modular domain and their abundance in the library. Consequently, a high-affinity peptide may be overlooked if it has low abundance in the library. Our method does not suffer from these problems. It provides the actual, individual binding sequences, from which a consensus sequence(s) may be derived. Our library screening is based solely on the affinity between the protein domain and its peptide ligands, and therefore, a high-affinity peptide is selected regardless of its abundance in the library. The improved quality of our sequence specificity data is crucial for the success of the combinatorial library approach to partner identification, by producing a smaller number of hits from database searches.

Database Search of Potential Tensin-Binding Proteins. The class I motif of the tensin SH2 domain [pY(D/E)N(V/M/Y/L)] was used as a query to search three protein databases on the Internet for potential tensin-binding proteins (http://phospho.elm.eu.org, www.phosphosite.org, and http://pir.georgetown.edu). After removal of false positives on the basis of subcellular localization, a total of 29 proteins were identified as potential tensin-binding proteins (Table 2). One of these proteins, p130Cas, has previously been reported to be a *bona fide* tensin-binding protein (*10, 15*).

Confirmation of Predicted Tensin-Binding Proteins. To gauge the number of pY proteins that can bind to the tensin SH2 domain, a maltose-binding protein–tensin SH2 fusion (MBP-SH2) was used to pull down pY proteins from cell lysates. Raw 264.7 murine cells, treated and untreated with the protein tyrosine phosphatase inhibitor pervanadate, were lysed and incubated with MBP-SH2 protein. The SH2 domain and the proteins associated with it were precipitated by the addition of amylose-coated beads and analyzed by SDS-PAGE. Immunoblotting with an anti-pY antibody (4G10) revealed that over a dozen pY proteins were associated with the SH2 domain (Figure 2, panel a). Next, the precipitated proteins were similarly separated by SDS-PAGE but immunoblotted with antibodies specific for the following proteins: phosphatases SHP-1 and SHP-2, PDGFR-β, LAT, CD-22, 3-phosphoinositide-dependent protein kinase 1 (PDK-1), downstream of tyrosine kinase 2 (Dok-2), and Cas-L. These proteins are among the predicted tensin-binding proteins, and each contains a pY motif that matches the class I consensus sequence of tensin SH2 domain (Table 2). They were chosen for further binding tests because specific antibodies against them were available. The p85 subunit of PI3-kinase was also included in the tests as a positive control. This analysis showed that PI3-kinase (data not shown) and PDK-1 were precipitated by the tensin SH2 domain (Figure 2, panel b). Dok-2 was also precipitated by the tensin SH2 domain; however, its signal overlapped with that of

TABLE 2. Potential tensin-binding proteins from database search

Potential binding proteins	Potential binding motifs
Actin-binding LIM protein 3	YENL
Angiomotin	YENL
Ankyrin	YENL
CRK-associated substrate-related protein (Cas-L)	YDNV
B-lymphocyte antigen (CD19)	YENM
CD22	YENV
CDC42-binding protein kinase	YENL
Contactin 6	YDNL
Downstream of protein kinase 3 (Dok-3)	YENL
Downstream of protein kinase 2 (Dok-2)[a]	YDNV
Focal adhesion kinase (FAK-1)	YENV
Granulocyte colony-stimulating factor receptor precursor (GCSFR)	YENL
Linker for activation of T-cells family member 1 (LAT)	YENL
MAPK/ERK kinase	YDNV
Nephocystin-2	YDNL
p130Cas[a]	YDNV
β-platelet derived growth factor receptor (PDGFRβ)	YDNY
3-phosphoinositide-dependent protein kinase-1 (PDK-1)[a]	YDNL
PDZK1-interacting protein 1	YENV
Protein tyrosine kinase 2 (PTK2)	YENV
Focal adhesion kinase 2 (PYK2, FAK2)	YLNV
Rho-related GTP-binding protein	YDNV
Serine/threonine-protein kinase LATS1	YENV
SHP-1 (Tyrosine-protein phosphatase, non-receptor type 6)	YENL
SHP-2 (Tyrosine-protein phosphatase, non-receptor type 11)	YENV
SREC-5	YENV
Tenascin-M1	YDNV
Tensin 3	YENL
Tweety 2	YENV

[a]Proteins that have been confirmed to bind to tensin *via* its SH2 domain.

MBP-SH2 because of their similar molecular masses (56 and 53 kDa, respectively) (data not shown). Therefore, Dok-2 was immunoprecipitated from whole cell lysates in the presence of a histidine-tagged tensin SH2 domain (H_6-SH2), and Western blotting was performed with an antibody against the histidine tag. The results show that the tensin SH2 domain was bound to Dok-2 when the cells were treated with pervanadate, whereas no interaction was detected without pervanadate treatment (Figure 2, panel c). In a control experiment, anti-goat IgG did not pull down the tensin SH2 domain. Finally, PDK-1 and Dok-2 were tested for binding to the intact tensin protein by coimmunoprecipitation assays. PDK-1 and Dok-2 were immunoprecipitated from human THP1 monocytes and then blotted with anti-tensin antibody. THP1 cells were utilized in this experiment because the anti-tensin antibody gave a relatively clean signal in these cells. In both cases, tensin was coprecipitated when cells were treated with pervanadate, but not in the absence of the phosphatase inhibitor or when anti-rabbit or anti-goat IgG was used in coimmunoprecipitation (Figure 3). These results indicate that PDK-1 and Dok-2 associate with tensin in a phosphorylation-dependent manner. The other tested proteins did not show any interaction with the SH2 domain of tensin under our experimental conditions.

Determination of pY Sites for Tensin SH2 Domain Association. Three phosphotyrosine sites have been reported for PDK-1 at pY9DAV, pY373GNY, and pY376DNL (*18*), whereas Dok-2 protein contains five pY sites at pY139SSA, pY271SRP, pY299AVP, pY345DKP, and pY402DNV (*19*–*23*). To determine which pY motif is responsible for binding to tensin and confirm our prediction on the basis of the library screening results, peptides corresponding to the eight pY motifs were synthesized and tested for binding to the tensin SH2 domain by SPR analysis (Table 1, peptides 4−11). Among the three PDK-1 peptides, pY376DNL had the highest affinity (K_d = 0.27 μM), whereas the other two peptides bound with at least 7-fold lower affinity (Table 1 and Figure 4, panel a), consistent with our prediction of pY376DNL as the *in vivo* binding site for tensin. Similarly, pY402DNV of Dok-2 bound to the tensin SH2 domain with the highest affinity (K_d = 0.44 μM) and is likely the *in vivo* binding site, whereas the other four pY motifs either showed no binding or bound very weakly (K_d > 6 μM) (Table 1 and Figure 4, panel b). We also measured the binding affinity of the tensin SH2 domain for the pY12DNV motif in Cas-L and the pY775DNY motif of PDGFRβ chain, because these two motifs

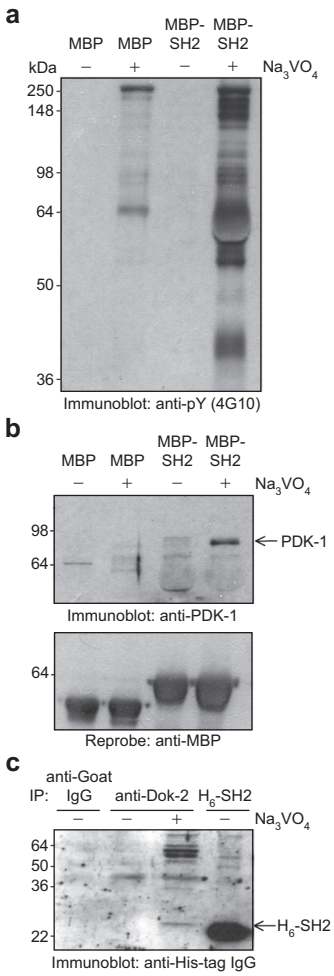

Figure 2. Pull-down assay of the interaction between tensin and PDK-1, and Dok-2. a) Anti-pY Western blot showing the proteins precipitated from Raw 264.7 murine cell lysate by MBP-tensin SH2 domain. b) Anti-PDK-1 Western blot showing the specific interaction between PDK-1 and MBP-tensin SH2 domain. Reprobe with anti-MBP antibody showed roughly equal amounts of MBP or MBP tensin SH2 in each lane. c) Western blot analysis of the interaction between Dok-2 and tensin SH2 domain.

closely match the class I consensus sequence (Table 1, peptides 12 and 13). Both peptides bound to the tensin SH2 domain with high affinity (K_d values of 0.19 and 0.55 μM, respectively). The strong binding affinity between the peptide motifs and the tensin SH2 domain suggests that these interactions might take place inside a cell. Our failure to observe them by the pull-down assay might be due to inefficient phosphorylation of the tyrosyl residues under the assay conditions (*e.g.*, pervanadate treatment).

Design, Synthesis, and Testing of Tensin SH2 Domain Inhibitor. The sequence specificity data may be utilized to develop specific inhibitors to modulate protein–protein interactions in a cellular system. As a proof of principle, we designed a peptide inhibitor against the tensin SH2 domain by using the peptide motif derived from Cas-L, RALpYDNVPE, which has the highest affinity to the tensin SH2 domain among all of the pY peptides tested (K_d = 0.19 μM). An octaarginine (R_8) sequence was added to its N-terminus to make the peptide permeable through the cell membrane (*24*). To visualize the peptide inside a cell, fluorescein isothiocyanate (FITC) was coupled with its N-terminus *via* a flexible linker (miniPEG). A control peptide with the same sequence but containing a tyrosine in replacement of the pY (Y peptide) was also synthesized.

The ability of the inhibitor peptide to disrupt the association between tensin and its binding partners was assessed by coimmunoprecipitation assays. An NIH 3T3 cell lysate was treated with the anti-PDK-1 antibody in the presence and absence of the pY peptide (or Y peptide). The precipitated proteins were analyzed by Western blot with the anti-tensin antibody. Tensin coprecipitated with PDK-1 in the absence of the peptides (Figure 5, panel a). However, the addition of 10 μM pYDNV peptide largely abolished the association between tensin and PDK-1. The corresponding Y peptide also reduced the amount of precipitated tensin, though less effectively than pYDNV. This may be due to partial phosphorylation of the Y peptide by a kinase(s) in the cell lysate. Peptide pYDNV also disrupted the interaction between tensin and PI3-kinase, which binds to tensin *via* its p85 subunit (*14*). *In vitro* pull-down assays performed in the presence of the peptide inhibitors gave similar results (Supplementary Figure 2). While peptide pYDNV inhibited the interaction between tensin and PDK-1, peptide YDNV and pY peptide TITpYSLLKH, which do not bind to tensin SH2, had

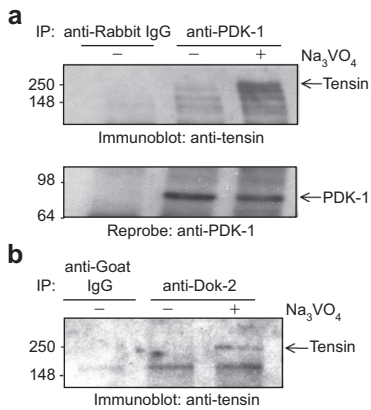

Figure 3. Coimmunoprecipitation of PDK-1 and Dok-2 with tensin. a) PDK-1 was precipitated from THP1 cells, and the membrane was probed with anti-tensin antibody (top panel). Reprobe of the membrane with anti-PDK-1 antibody shows that an equal amount of PDK-1 was present in each lane (bottom panel). b) Same experiment as in panel a) except that immunoprecipitation was carried out with anti-Dok-2 antibody.

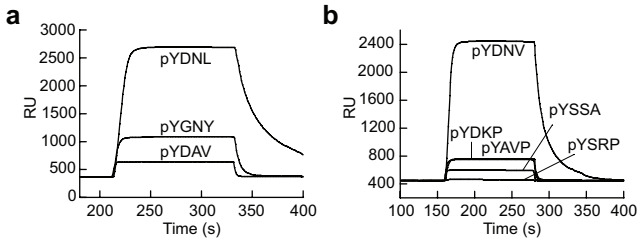

Figure 4. SPR sensograms showing the binding of tensin SH2 domain (2 μM) to a) immobilized PDK-1 and b) Dok-2 pY motifs. RU = Relative units.

little effect. Because peptide RALpYDNVPE matches the binding consensus of Grb2 SH2 domain (*25*), we synthesized peptide RALpYVNVPE, which should be an excellent Grb2 ligand but a poorer ligand to the tensin SH2 domain. Indeed, this peptide bound to the tensin SH2 domain with a 4-fold lower affinity than pYDNV (Table 1, compare peptides 12 and 14). Accordingly, in the pull-down experiment, the pYVNV peptide was less effective in disrupting the PDK-1–tensin SH2 interaction (17% and 30% inhibition for pYVNV and pYDNV, respectively).

The peptides were next tested for their effect on the actin skeletal structure in NIH 3T3 cells. Tensin is required for the formation of actin cytoskeletal structures (*26*). Overexpression of the N-terminal fragment of tensin (which does not contain the SH2 domain) causes the disruption of actin filaments (*13*). Treatment of NIH 3T3 cells with 5 μM cell-permeable peptides (pYDNV and YDNV) resulted in intense green fluorescence inside the cells, indicating that both peptides were efficiently taken up by the cells (Figure 6, panel a). Flow cytometry analysis showed that, at peptide concentrations of 5–10 μM, >90% of the cells became fluorescent after 3 h (Supplementary Figure 3). The cells were then washed and stained with rhodamine-phalloidin and Hoechst reagent to visualize the actin filaments and the nuclei, respectively. In the presence of the Y peptide (5 μM), actin filaments were readily visible in >65% of the cells (Figure 6, panels b–e). However, upon treatment with 5 μM pYDNV peptide, only ~40% of the cells displayed visible actin filaments. The retention of actin skeletal structures in this cell population may be due to poorer peptide uptake by these cells (Supplementary Figure 3) and increased peptide degradation (*e.g.*, by phosphatases and proteases). Treatment with 10 μM pYDNV peptide resulted in loss of actin filaments in a higher percentage of cells but also caused significant cell death (data not shown). Peptide pYVNV was more effective than the Y peptide but less efficient than peptide pYDNV in disrupting the actin structures. Control peptide pYSLL had no visible effect. Taken together, our data suggest that peptide pYDNV disrupted actin filaments by inhibiting the interaction between the tensin SH2 domain and a yet unknown pY protein(s).

Implications of Tensin Function in Cell Signaling. The SH2 domain of tensin is known to be critical for tensin function, for example, in regulating cell migration (*12*). The SH2 domain was shown to bind to a variety of pY proteins (*11*), but only p130Cas and PI3-kinase had been identified previously. Our finding of PDK-1 and Dok2 as tensin-binding proteins should help elucidate the signaling pathways through tensin. PDK-1 is a member of the AGC superfamily of protein serine/threonine kinases. Ubiquitously expressed in human tissues, it is a key enzyme in multiple signaling pathways. PDK-1 functions downstream of PI3-kinase and phosphorylates Akt/PKB and a number of other kinases, including p70S6-kinase and protein kinase C (*27, 28*). PDK-1 contains an N-terminal kinase domain, a linker region, and a C-terminal pleckstrin homology (PH) domain (*29*). The PH domain of PDK-1 binds to phophatidylinositol-3, 4-biphosphate (PIP_2) and phophatidylinositol3, 4, 5-triphosphate (PIP_3) and translocates to

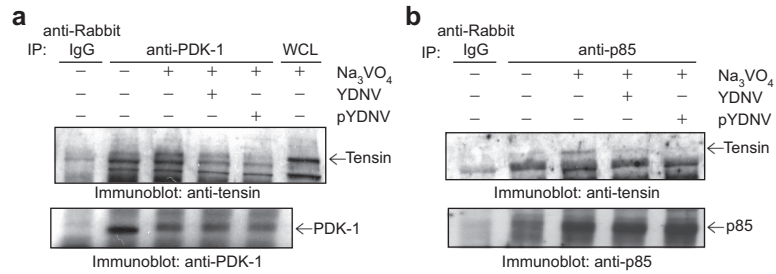

Figure 5. Inhibition of tensin-PDK-1 and tensin-PI3-kinase interaction by pY peptide. a) Anti-tensin Western blot of proteins precipitated by anti-PDK-1 antibody in the presence of the indicated peptides (10 μM). Reprobe with anti-PDK-1 antibody showed roughly equal amounts of PDK-1 in each lane. b) Same experiment as in panel a) except that immunoprecipitation was carried out with anti-p-85 antibody.

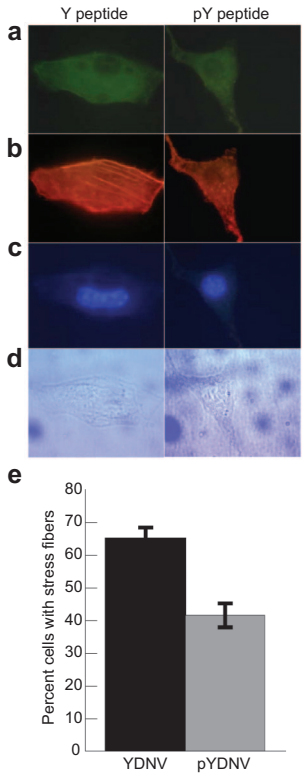

Figure 6. Disruption of actin skeleton by tensin SH2 domain ligand. a) FITC fluorescence showing the uptake of peptides by NIH 3T3 cells. b) Actin filaments visualization with rhodamine-phalloidin, showing the disruption of actin filaments in the presence of pYDNV. c) Nuclear visualization with Hoechst reagent. d) Normal light. e) Percentage of cells with intact actin filaments in the presence of 5 μM Y or pY peptide. Data were compiled with 3 sets of 100 NIH 3T3 cells. Key: Y peptide, FITC-miniPEG-(R_8)-RALYDNVPE; pY peptide, FITC-miniPEG-(R_8)-RALpYDNVPE.

the plasma membrane (*30*). Inositol lipid signaling is known to influence the remodeling of actin skeleton (*31*), and PI3-kinase has previously been shown to interact with the tensin SH2 domain (*14*), a result also confirmed by this work. Thus, the colocalization of PI3-kinase and PDK-1 to the focal adhesion sites seems to be quite fitting, although the detailed mechanism remains to be determined.

Dok-2 belongs to the Dok family of proteins, which act as negative regulators of a number of signaling pathways (*32, 33*). Like PDK-1, Dok-2 contains an N-terminal PH domain, followed by a PTB domain and a proline- and tyrosine-rich carboxyl-terminal tail. Dok-2 has been shown to be critical in regulating actin dynamics (*34*). Master *et al.* (*35*) have shown that Dok-2 binds directly to c-Abl, resulting in an increase in c-Abl tyrosine phosphorylation and kinase activity. The activated c-Abl phosphorylates p130Cas (*36*), another known interacting partner of tensin, and associates directly with the cytoskeleton (*37*). Thus, upon phosphorylation, Dok-2 may serve as a docking scaffold for assembling other signaling molecules, including tensin. Our data suggest that Dok-2 and PDK-1 bind directly to the tensin SH2 domain. Because none of the known pY motifs in PI3-kinase match any of the three consensus sequences for tensin SH2, we agree with the previous suggestion that the interaction between PI3-kinase and tensin is indirect, perhaps through PDK-1 (or Dok-2).

Advantage of the Chemical/Bioinformatics Approach. As described above, each of the existing methods for the identification of new protein–protein interactions has it own limitations. Our method provides a complementary approach, which is ideally suited for modular domains that recognize small peptide motifs. The requirement of post-translational modification for binding actually facilitates our method, as it reduces the number of potential partner proteins from database searches. Our method should be effective with low-abundance proteins, because a candidate protein is identified by *in silico* searches (which are not affected by low abundance), and its confirmation is achieved by Western blot analysis (which is highly sensitive and specific). Unlike any other methods, our method provides not only the identity of an interacting partner, but also the molecular basis of the interaction (*e.g.*, the binding motif). Finally, peptide motifs identified from the libraries can be converted into potent inhibitors to probe the physiological function of the protein–protein interaction pair in question. It is often possible to identify peptide ligands that bind to a modular domain with higher affinity than the physiological protein target (*4, 38*). Because our library is chemically synthesized, it is also possible to incorporate unnatural amino acids or peptidomimetics into the ligands to improve their pharmacological properties.

Conclusion. We have developed a powerful chemical/bioinformatics approach for the identification of the binding partners of modular domains. This approach should be readily applicable to any modular domains that recognize short peptide motifs. Even for protein–protein interactions that involve larger surface contacts and secondary structures, it may be possible to find small peptide inhibitors, which provide useful pharmacological tools to elucidate the physiological function of the protein–protein interactions.

METHODS

Expression and Purification of Tensin SH2 Domain. The DNA sequence coding for the tensin SH2 domain (amino acids 1460−1576) was isolated by polymerase chain reaction (PCR) from the Marathon-Ready human spleen cDNA library (Clontech) using the following primers: 5′-CGGAATTCTCTAAGTATTGGTACAAGCCTGAGATCT-3′ and 5′-CCCAAGCTTTTATGTGGGGTCTCGGTTTGGAATGA-3′. The PCR product was digested with restriction endonucleases *Eco*RI and *Hin*dIII and ligated into the corresponding site of plasmid pMAL-c2 (New England Biolabs). This procedure resulted in the fusion of the SH2 domain to the C-terminus of MBP. The SH2 domain was also constructed in its isolated form containing an N-terminal six-histidine tag. In this case, the digested PCR product was ligated into plasmid pET-28a (Novagen). The authenticity of the DNA constructs was confirmed by dideoxy sequencing. Expression, purification, and biotinylation of SH2 domains were performed as previously described (*4*). Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

Synthesis and Screening of pY Peptide Library. The pY peptide library was synthesized on TentaGel S-NH_2 resin (90 μm, 0.3 mmol g^{-1}) and screened as described previously (*4*). In a typical screening experiment, 50 mg of the pY library placed in a micro-BioSpin column (0.8 mL) (Bio-Rad) was extensively washed with dichloromethane, methanol, ddH_2O, and HBST buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, and 0.01% Tween 20). The resin was then blocked for 1 h with HBST buffer containing 0.1% gelatin. The library was incubated with varying concentrations of the biotinylated MBP-SH2 protein (10−50 nM) at 4 °C for 4−16 h with gentle mixing. The library was subsequently treated with a streptavidin–alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indoyl-phosphate. Beads with the most intense turquoise color were picked manually under a dissecting microscope and sequenced using PED/MS (*16*). Control experiments with biotinylated MBP produced no colored beads under identical conditions.

Synthesis of Individual pY Peptides. Individual pY peptides were synthesized on 50−100 mg of CLEAR-amide resin with a loading capacity of 0.46 mmol g^{-1} (Peptides International) using standard Fmoc/HBTU/HOBt chemistry. Fmoc-8-amino-3,6-dioxooctanoic acid (Peptides International) was added to the N-terminus of each peptide to provide a flexible linker, prior to the addition of biotin. For cell-permeable peptides, a fluorescein molecule was added to their N-termini by treating the resin-bound peptides with 4 equiv of FITC (Sigma) for 2 h at RT. Peptide cleavage from the resin and side-chain deprotection were carried out using a modified reagent K as previously described (*4*). The product was precipitated in cold diethyl ether and purified by reversed-phase HPLC on a C_{18} column (Varian Dynamax 300 Å, 250 × 10 mm). The identity of each peptide was confirmed by matrix assisted laser desorption/ionization time-of-flight MS analysis. The concentration of the cell-permeable peptides was determined by the absorbance of fluorescein at 495 nm.

Determination of Dissociation Constants by SPR. SPR analysis was performed on a BIAcore 3000 instrument, with N-terminally histidine-tagged SH2 domain (not MBP fusion) and biotinylated pY peptides immobilized onto a streptavidin-coated SA_5 biosensor chip. Binding assays were performed in HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20) at RT. Each pY peptide was loaded onto the chip by passing the peptide solution (~20 μM) over the chip at a flow rate of 15 μL min^{-1} until a constant response unit was reached (typically 400−500 RU). Varying concentrations of the tensin SH2 domain (0.05−5.0 μM) were passed over the chip for 2 min at a flow rate of 15 μL min^{-1}. A blank flow cell (no immobilized pY peptide) was used as a control. Between two runs, the sensorchip was regenerated by injecting a strip buffer (10 mM NaOH, 200 mM NaCl, and 0.05% SDS) for 5−10 s at a flow rate of 100 μL min^{-1}. The equilibrium response unit (RU_{eq}) at a given SH2 protein concentration was obtained by subtracting the response of the blank flow cell from the flow cells containing the pY peptide. The dissociation constant (K_d) was obtained by nonlinear regression fitting of the data to the equation:

$$RU_{eq} = RU_{max}[SH2]/(K_d + [SH2])$$

where RU_{eq} is the measured response unit at a given SH2 domain concentration and RU_{max} is the maximum response unit.

***In Vitro* Pull-Down Assay.** Raw 264.7 murine macrophage cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated for 1 h in incomplete RPMI (no FBS added) on ice, before being stimulated with 1 mM pervanadate at 37 °C for 15 min. The cells were then lysed in TN1 lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, and 10 μg mL^{-1} each of aprotinin and leupeptin), and the nuclei were removed by centrifugation (17,000*g*) for 10 min at 4 °C. Equal amounts of protein from each sample were incubated overnight at 4 °C with 10 μg of MBP (as a negative control) or MBP-SH2, and amylose beads (20 μL). The beads were washed three times with TN1 buffer and boiled in SDS sample buffer for 5 min. The eluted proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% dried skim milk in phosphate buffered saline (PBS) containing 0.05% Tween 20 and probed with the relevant antibody overnight at 4 °C. After washing, the blot was incubated with horseradish peroxidase-conjugated secondary antibody, washed again, and briefly incubated in ECL (Amersham) for chemiluminescent detection on X-ray films. Anti-PDK-1 polyclonal antibody was from Cell Signaling, while other antibodies were from Santa Cruz Biotechnology, Inc. The assays were performed in triplicates.

THP1 cells (human monocytes) were cultured in RPMI supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin/streptomycin. Cell activation and lysis were carried out as described above. Equal amounts of protein from each sample were incubated overnight at 4 °C with 10 μg of histidine-tagged tensin SH2, protein G beads, and either anti-goat or anti-Dok-2 antibodies. The beads were washed, and the bound proteins were eluted by heating in SDS loading buffer. After separation by SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane, which was probed with anti-His tag antibody (Cell Signaling) overnight at 4 °C. Chemiluminescent detection was performed as described above. For pull-down assays in the presence of peptide competitors, the same procedure was used except for the following modifications. MBP-SH2 protein, cell lysate, and amylose beads were incubated overnight at 4 °C, and the competing peptide (10 μM) was then added. The resulting mixture was incubated for an additional 2 to 3 h at 4 °C prior to Western blot analysis.

Coimmunoprecipitation Assays. THP1 cells were cultured, activated, and lysed as described above. The lysate was incubated overnight at 4 °C with anti-rabbit antibody or anti-PDK-1 polyclonal antibody (or anti-goat antibody and anti-Dok-2 polyclonal antibody for the Dok-2 assay) and protein G-agarose beads. The beads were washed twice with TN1 buffer and boiled in SDS sample buffer. The eluted proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the membrane was probed with anti-tensin polyclonal antibody overnight at 4 °C. Chemiluminescent detection

was performed as described above. For coimmunoprecipitation assays in the presence of competing peptides, anti-PDK-1 (or anti-p85 PI3K) antibody was incubated overnight at 4 °C with the cell lysate and protein G-agarose beads, and the peptide competitor (10 μM) was added. The mixture was incubated at 4 °C for an additional 2 to 3 h before Western blot analysis. The experiments were performed in triplicates.

Fluorescence Assay. NIH 3T3 cells were cultured in Dulbecco's modified eagle's medium supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin/streptomycin. The cells were grown on coverslips coated with poly(L-lysine) (50 μg mL^{-1} in PBS) and treated with the cell-permeable peptides (5 μM) for 3 h at 37 °C. The treated cells were washed three times with PBS, fixed with 3.7% paraformaldehyde for 10 min, and washed again with PBS. The cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and stained with rhodamine-phalloidin (Molecular Probes) for 30 min. The cells were washed three times with PBS (5 min each time) and stained with 1:10,000 Hoechst trihydrochloride trihydrate (Molecular Probes) in PBS for 5 min. The coverslips were mounted with fluorescence mounting medium (DakoCytomation) and analyzed using an Olympus BX40 microscope equipped with a 100× or 40× objective lens.

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Supporting Information Available: This material is free of charge *via* the Internet.

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