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An Improved Method for the Synthesis of Cellulose Membrane-Bound Peptides with Free C Termini Is Useful for PDZ Domain Binding Studies

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Summary

SPOT synthesis permits parallel synthesis and screening of thousands of cellulose membrane-bound peptides to study protein-protein interactions in a proteomic context. Recognition of C-terminal residues is one of the most common binding features of PDZ domains. Unfortunately, most solid support-bound peptide libraries lack a free C terminus due to C-terminal fixation on the solid support. To overcome this restriction, we developed a robust methodology based on our previous strategy for generating peptides with authentic C termini. To validate this improved method, we screened a human peptide library of 6223 C termini with the syntrophin PDZ domain. Furthermore, using the same library, new peptide ligands derived from membrane proteins and receptors were found for the ERBIN PDZ domain. Finally, we identified the protein kinase breakpoint cluster region, which is known as a negative regulator of cell proliferation and oncogenic transformation, as an ERBIN ligand.

Introduction

SPOT synthesis [1] (reviewed in [2–4]) opens up opportunities to both synthesize and screen large arrays of synthetic peptides on planar cellulose supports. This is the remarkable advantage of the SPOT concept, since compounds can be rapidly generated and subsequently screened in parallel, directly on the solid support. In recent years, peptide arrays prepared by the SPOT technique have become popular tools for studying protein-protein interactions [5].

Some protein domains, such as PDZ domains (reviewed in [6, 7]), require a free carboxyl terminus (C terminus) for ligand recognition. Unfortunately, peptides synthesized according to the standard SPOT synthesis protocol [8] lack free C termini due to their C-terminal fixation to the cellulose support. In principle, free C termini can be obtained after standard SPOT synthesis by reversing the peptide orientation (inverted peptides). Methods for synthesizing free C-terminal resin-bound [9–11] and cellulose-bound [12] peptides have been published. Nevertheless, using our previously reported protocol [12], we have to concede that yields and purities of the inverted peptides are generally low, that generation of the inverted peptide arrays is extremely time consuming, and that screening the arrays for PDZ interactions is often unsuccessful. We therefore initiated this work with the goal of developing a more robust and efficient protocol for the preparation of cellulose membrane-bound inverted peptide arrays that could be used for extensive experimental projects mapping different PDZ domain interactions.

PDZ domains (named after the proteins *P*ost-synaptic density-95, *D*iscs large, and *Z*onula occludens 1) [13–15] are globular protein interaction modules known to bind C termini of membrane-integrated receptors or channels [14, 16]. These domains are conserved protein sequences of approximately 90 amino acids and play a central role in organizing signal transduction complexes, especially in submembranous compartments. Based on their target sequence, specific PDZ domains have been classified in class I and class II, recognizing peptides with the consensus x(S/T)xϕ_{COOH} and xϕxϕ_{COOH}, respectively [17] (peptide motifs and consensus sequences below are given according to the modified Seefeld Convention 2001 nomenclature [18]). The residues at positions 0 to −3 of the peptide (position 0 referring to the C-terminal residue) play a critical role in the specificity and affinity of the PDZ:ligand interaction.

To demonstrate the applicability of the improved method, we used the α-1-syntrophin (SYNA1) and the ERB2 interacting protein (ERBIN) PDZ domains as models. SYNA1 is a cytoplasmic, peripheral membrane protein and a component of the dystrophin-associated protein complex (DAPC) [19, 20]. The PDZ domain of SYNA1 recognizes the C termini of various proteins, including muscle sodium channel protein type IV α subunit [21] and the guanylate cyclase soluble, α-2 chain [12]. ERBIN was originally identified as a protein interacting with the receptor protein tyrosine kinase ERB2 involved in cell proliferation and differentiation [22, 23]. In addition, ERBIN is known to bind δ-catenin, the armadillo repeat protein deleted in velo-cardio-facial syndrome (ARVCF), and p0071 in a PDZ-specific manner [24–26].

Here, we describe an improved protocol for preparing inverted peptide arrays using the SPOT synthesis concept and demonstrate its applicability for PDZ binding assays. The improved protocol allows the rapid and robust synthesis of large peptide arrays with free C termini. Short reaction times, together with high coupling efficiencies at each step during SPOT synthesis, fewer side reactions, and the possibility of automating the SPOT synthesis process are the considerable advantages of our methodology. Based on Hoffmüller et al. [12], we screened a similar peptide library with the

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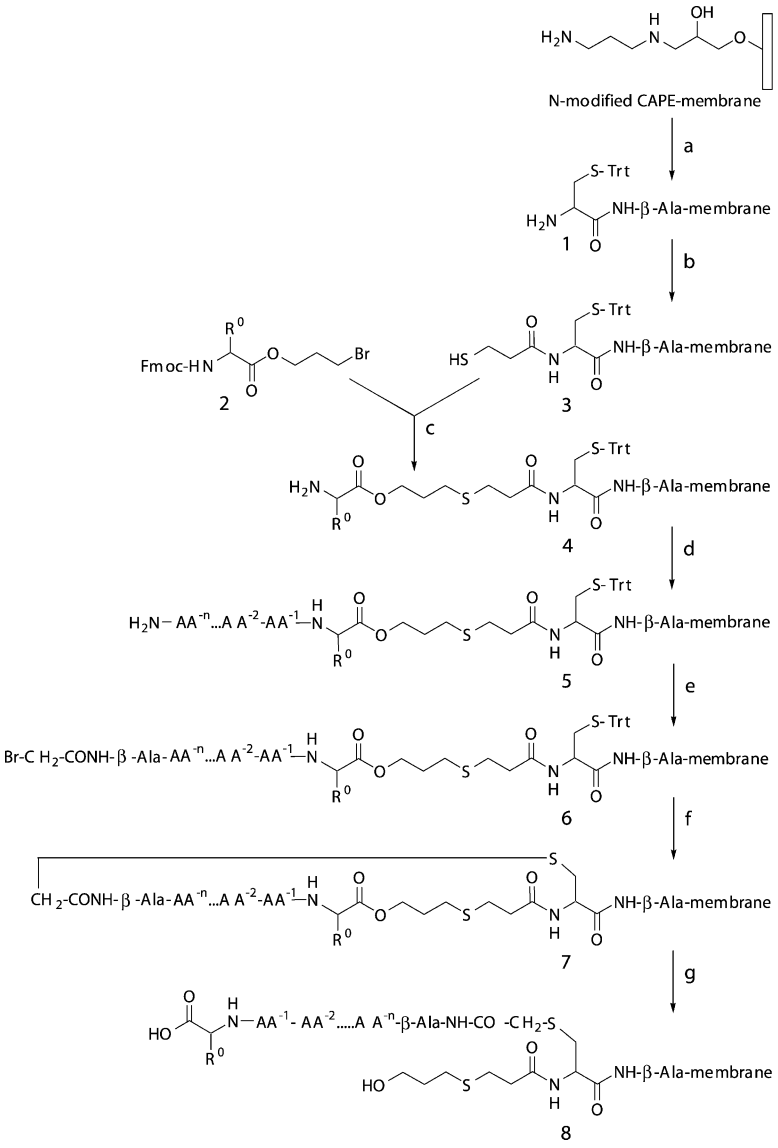


Figure 1. Reaction Scheme for the Synthesis of Inverted Peptides on Cellulose Membranes

Reaction conditions were as follows: a, Fmoc-β-alanine-Opfp in DMSO, then piperidine followed by Fmoc-cysteine-(Trt)-Opfp in NMP and again followed by piperidine for Fmoc cleavage; b, Mmt-S-CH_2-CH_2-COOH in DMF preactivated with HATU and NMI and, subsequently, dichloroacetic acid, TFA, TIBS, DCM; c, aqueous solution of Cs_2CO_3, then a Fmoc-amino acid 3-bromopropyl ester (**2**) in DMF and piperidine; d, peptide synthesis using the standard SPOT synthesis protocol [8]; e, Fmoc-β-alanine-Opfp in NMP, then piperidine and 2,4-dinitrophenyl-bromoacetate in NMP; f, TFA, TIBS, DCM, and, subsequently, aqueous solution of Cs_2CO_3; g, saturated aqueous solution of Li_2CO_3.

SYNA1 PDZ domain, which enabled us to validate our method. Encouraged by the coincident results, we used a new synthesized library containing the same peptide sequences and found new peptide ligands for the ERBIN PDZ domain, i.e., the calcium-transporting ATPase plasma membrane, isoform 1 (ATB1), and the voltage-gated potassium channel proteins Kv1.4 (CIK4) and Kv1.5 (CIK5). Furthermore, we identified a PDZ-specific interaction between the breakpoint cluster region (BCR) protein kinase and ERBIN, which was validated by co-precipitation of endogenous ERBIN and BCR.

Results and Discussion

SPOT Synthesis of Cellulose Membrane-Bound Inverted Peptides

The reaction scheme for SPOT synthesis of cellulose membrane-bound inverted peptides is shown in Figure 1. In contrast to the standard SPOT synthesis protocol [8], synthesis of inverted peptides was performed on a cellulose membrane carrying a stable N-functionalized anchor (N-modified cellulose-amino-hydroxypropyl ether membrane [N-CAPE]) [27, 28], which retained the inverted peptides (**8**) (the numbers **1-8** in bold refer to Figure 1). The inverted and N terminally fixed peptides (**8**) display a free C terminus resulting from reversal of the peptide orientation and achieved by successive thioether-cyclization/ester cleavage (Figure 1, f–g). Key compounds in the synthesis are the Fmoc-amino acid 3-brompropyl esters (Fmoc-aa-OPBr) (**2**), the membrane-bound mercaptopropionyl cysteine adduct (**3**), the matrix-bound amino acid ester derivative (**4**), and the cyclic peptide (**7**). Critical reaction steps are the formation of both the cleavable ester bond and the cyclic peptide (Figure 1, c and f, respectively).

The decisive step during the synthesis of the key compound (**3**) is the selective removal of the 4-methoxytriphenyl (Mmt) protection group (Figure 1, b). The acidic mixture used in the Mmt-deprotection step has to be adjusted carefully in order to prevent cleavage of the

Table 1. Selective Cleavage of S-Mmt Group

Acidic Conditions[a]	Protecting Group[b] Mmt	Trt
<0.5% TFA in DCM	−	−
0.5%–1% TFA in DCM	+	−
≥1% TFA in DCM	+ +	+
<8% dichloracetic acid in DCM	−	−
8%–15% dichloroacetic acid in DCM	+	−
≥15% dichloroacetic acid in DCM	+ +	+
50% acetic acid	−	−
10% dichloroacetic acid, 0.5% TFA in DCM[c]	+ +	−

[a] Mixtures contain additional 5% TIBS; % = v/v.
[b] −, no cleavage; +, cleavage; + +, immediate and fast cleavage.
[c] For details, see Experimental Procedures.

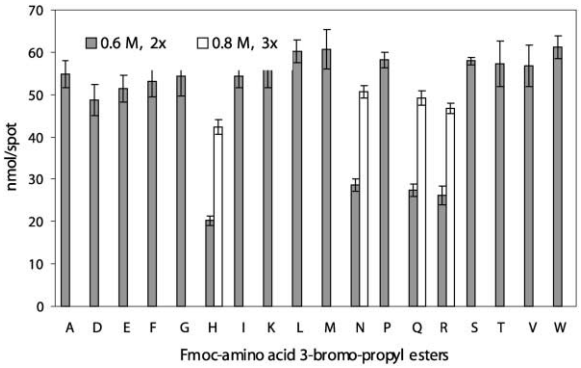


Figure 2. Coupling Efficiency of Fmoc-Aino Acid 3-Bromo-Propyl Esters (2)

Fmoc-amino acid 3-bromopropyl esters were checked for coupling to membrane-bound cesium thiolate (3) (Figure 1, c). Fifty-seven spots of the cesium thiolate (3) (spot area: 0.25 cm^2) on a noncleavable membrane were treated with Fmoc-amino acid 3-bromopropyl esters (2) in DMF. Three spots were used for each bromopropyl ester derivate (19 bromopropyl esters were tested; cysteine-adduct was omitted). Each spot was treated with 1 μl of the respective Fmoc-amino acid 3-bromopropyl esters (2) solution (one-letter code for the amino acids is used). Gray bars indicate a double coupling approach using 0.6 M solutions. White bars indicate the use of 0.8 M solutions and a three times coupling approach. Coupling yields were determined by measuring the UV absorbance of the released Fmoc group [28] and are given as the mean loading of one spot (nmol/spot).

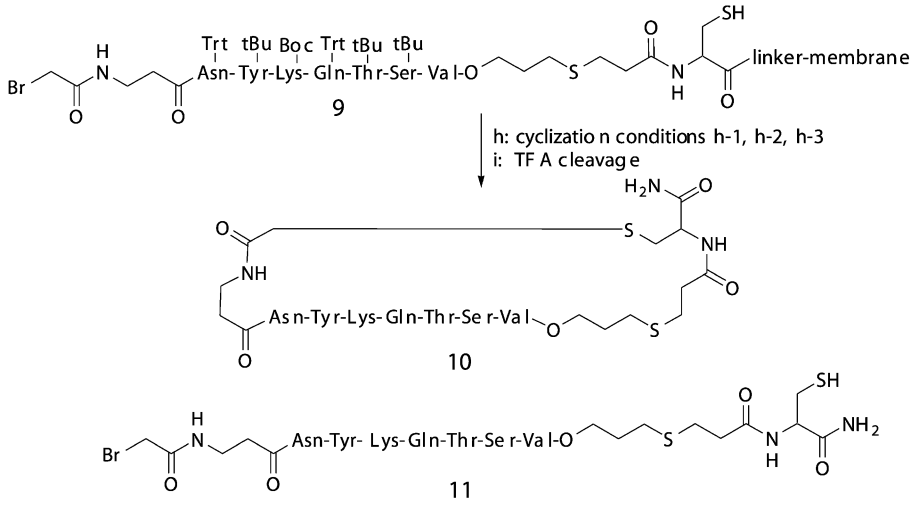
triphenylmethyl (Trt) protection group of the cysteine residue. As shown in Table 1, selective removal of the Mmt group is achieved by applying a mixture of 10% dichloroacetic acid and 0.5% trifluoroacetic acid (TFA) followed by the three-times repeated incubation of 10% dichloroacetic acid, 0.5% trifluoroacetic acid, and 5% triisobutylsilane (TIBS) in dichloromethane (DCM). The key compound (4) was synthesized by cesium-salt-supported S-alkylation of (3) with a Fmoc-aa-OPBr (2).

Compounds (2) (except the arginine derivative) were prepared in high yields by O-acylation of 1-bromo-3-propanol with Fmoc-amino acid fluorides [29] using a "one-pot" reaction (see Supplemental Table S1 and Supplemental Experimental Procedures in the supplemental data available with this article online). The reaction is characterized by its lack of side reactions. Only the corresponding Fmoc-amino acids were observed as impurities in the range of 10%–25%. Racemic products could not be detected, which is in good accordance with the literature [30]. The Fmoc-arginine 1-bromo-3-propanol ester was synthesized according to standard esterification [31] (Table S1). Yields of the prepared Fmoc-aa-OPBr were determined by analyzing the crude reaction products using reversed-phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization-mass spectrometry (ESI-MS) as shown in Table S1. During the reaction between the Fmoc-aa-OPBr (2) and the membrane-bound mercaptopropionyl cysteine adduct (3) (Figure 1, c), the C-terminal amino acid of the peptide and the ester cleavage site, necessary to reverse the peptide orientation, were incorporated simultaneously. Due to problems arising from the ubiquitous cellulose hydroxyl functions, we chose the S-alkylation reaction for selective formation of the ester linkage into a growing peptide chain. Coupling efficiencies of the Fmoc-aa-OPBr (3) are shown in Figure 2. Low coupling efficiencies were found for the Fmoc-aa-OPBr derivatives of histidine, asparagine, glutamine (Trt-protected side chains), and arginine (Pbf-protected side chain), probably due to sterical hindrance from the bulky protection groups. However, increasing concentrations of the critical amino acid derivatives up to 0.8 M in dimethylformamide (DMF) and repeated coupling (3×) significantly increased the coupling efficiency (Figure 2). Furthermore, the thiolate formation of compound (3) using 10% cesium carbonate is stable after drying the cellulose membrane, and we observed no significant decrease in coupling efficiency even when the S-alkylation reaction starts up to 3 hr later. This result is particularly important, since one coupling step with the SPOT robot-supported preparation of large peptide arrays may require up to 3 hr.

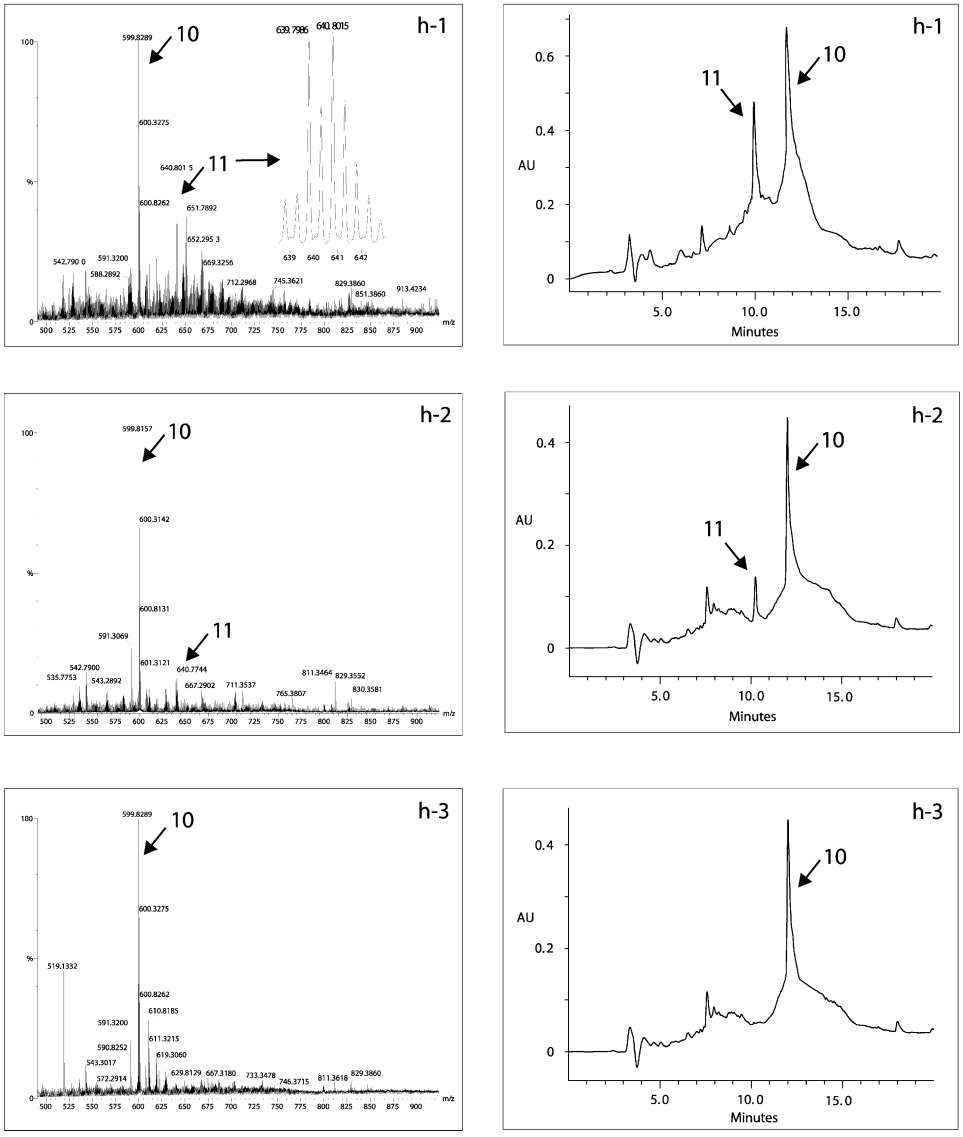
Before starting preparation of the key compound (7), the completed peptide (5) was N terminally elongated with Fmoc-β-alanine pentafluorophenyl ester (Pfp) and bromoacetic acid 2,4-dinitrophenyl ester, respectively, and subsequently its side chains were deprotected (Figure 1, e). Cyclization of the resulting linear peptide (6) involving the formation of a thioether bond (Figure 1, f) was performed by incubating the membrane with a dilute aqueous solution of cesium carbonate. The formation of cyclic peptides based on thioether has been reported to show high tolerance for various peptide sequences and to deliver cyclic peptides in high yields and excellent purity [32]. Finally, the generation of peptides with reverse sequence orientation (8) was achieved by cleaving the ester bond of the cyclic compound (7) using a saturated aqueous solution of lithium carbonate (Figure 1, g) as described [12].

The formation of cellulose membrane-bound inverted peptides depends on the rate of peptide thioether cyclization. Therefore, we chose the epitope peptide NYKQT SV_{COOH} of the PSD-95 PDZ3 domain [33] to test the efficiency of thioether cyclization. The bromoacylated peptide (9) (numbers **9–11** in bold refer to Figure 3) was synthesized on several cleavable spots (cleavability is conferred by incorporating the Fmoc-Rink linker). Three conditions were used for cyclization: one array was incubated with DMF (Figure 3, h-1), the second was first

A


Br
Trt tBu Boc Trt tBu tBu
Asn-Tyr-Lys-Gln-Thr-Ser-Val-O
linker-membrane
9
h: cyclization conditions h-1, h-2, h-3
i: TFA cleavage
Asn-Tyr-Lys-Gln-Thr-Ser-Val
10
Asn-Tyr-Lys-Gln-Thr-Ser-Val-O
11

B


h-1
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treated with a dilute aqueous solution of cesium carbonate and then incubated with DMF (Figure 3, h-2), and the third array was incubated with a solution of cesium carbonate (1 mM) in DMF (Figure 3, h-3). After 3 hr, the peptide products h-1, h-2, and h-3 were cleaved from the spots using TFA and analyzed by HPLC and ESI-MS (Figure 3B). Analysis of the reaction products clearly demonstrated the efficiency of the thioether-cyclization reaction using condition h-3. To determine the influence of the peptide sequence on the cyclization/cleavage step, we synthesized two further membrane-bound bromacylated peptides: EFHAALGSYV$_{COOH}$ and QHIDSQK KA$_{COOH}$. Cyclization using a dilute aqueous solution of cesium carbonate (5%, 12 hr) and subsequent analysis by ESI-MS showed, in both cases, as with h-3, quantitative and reproducible thioether cyclization (data not shown).

Taken together, only successfully cyclized peptides will be reversed in their sequence orientation. The uncyclized peptides are released during the ester-cleavage procedure, due to the sensitivity of the ester bound. In addition, side reactions between neighboring peptide sequences that perhaps occur during the cyclization step, e.g., crosslinking via a disulfide bridge or thioether crosslinking, do not disturb the reversal of peptide orientation. Disulfide crosslinked compounds were cleaved from the membrane, and thioether crosslinked products also resulted in inverted peptides.

Evaluation of the Novel Strategy

To assess the novel method for synthesizing cellulose membrane bound, inverted peptides, we screened a library of 6223 C termini (11-mers) of human proteins (SWISS-PROT database, release 40). This library (Figure S1) was incubated with SYNA1 PDZ domain (Q61234), and the results were compared with previously published data (incubation of 3514 C termini [7-mers] of human proteins [SWISS-PROT database, release 34] with SYNA1 PDZ domain) [12]. Due to the greater numbers of peptide sequences, it is essential that the new method is less time consuming and more adapted for automatic SPOT synthesis. To compare both binding experiments accurately, we determined the signal intensity of each spot of the library of 3514 C termini (7-mers) of human proteins (called 3514-Humlib) and the library of 6223 C termini (11-mers) of human proteins (called 6223-Humlib) using Boehringer Light Units (BLU). To deduce the peptide sequences that could be counted as PDZ domain binders, we determine the signal intensity of the background using 20 spots randomly located on the cellulose membrane. Peptide sequences showing a signal intensity higher than the background intensity plus the double standard deviation were defined as PDZ domain binders (3514-Humlib: background $= 3 \times 10^3$ BLU; standard deviation $= 1 \times 10^3$ BLU; PDZ domain binder $\geq 5 \times 10^3$ BLU; 6223-Humlib: background $= 9 \times 10^3$ BLU; standard deviation $= 1 \times 10^3$ BLU; PDZ domain binder $\geq 11 \times 10^3$ BLU) (A.A. Weiser and R.V.-E., unpublished data). Taken together, we obtained signal intensities for peptide sequences that bind to the SYNA1 PDZ domain with values ranging between 5×10^3–28×10^3 BLU for the 3514-Humlib and between 11×10^3–519×10^3 BLU for the 6223-Humlib. The first 30 spots of each library with the highest intensities were chosen for further investigation: 3514-Humlib with values ranging between 12×10^3–28×10^3 BLU and 6223-Humlib between 237×10^3–519×10^3 BLU (Table S2). We observed that the new method produced a 20-fold increase in signal intensity but only a 3-fold increase in the background signal. Altogether, we obtained a total of 60 peptide sequences where 3 identical sequences and 17 sequences from the 6223-Humlib were not represented in the 3514-Humlib. Of the 40 comparable sequences, all strong binders from the 3514-Humlib were also found as binders in the 6223-Humlib and vice versa. Compared to the 3514-Humlib, the 6223-Humlib is characterized by a 2-fold increase in peptide sequence number, by a 20-fold increase in signal intensity, and by different incubation/detection procedures. Due to these facts, it is difficult to directly compare only the 30 strongest spots. Comparing all sequences defined as SYNA1 PDZ binders, we obtain a good correlation between both libraries. The only exception was the rhombotin-1 peptide, which was detected as a strong binder (261×10^3 BLU) in the 6223-Humlib but showed no signal in the 3514-Humlib. This may by an artifact of large-scale screening that would have to be excluded by additional experiments, such as substitutional analysis or Biacore measurements.

In accordance with previously published results, we detected strong signals for the peptide derivates from the guanylate cyclase soluble α-2 chain (3514-Humlib: 28×10^3 BLU; 6223-Humlib: 359×10^3 BLU) [12], for muscle sodium channel protein type IV α subunit (3514-Humlib: 16×10^3 BLU; 6223-Humlib: 197×10^3 BLU) [21], for the stress-activated protein kinase-3 (3514-Humlib: sequence not represented; 6223-Humlib: 186×10^3 BLU) [34], for glutaminase-L (3514-Humlib: sequence not represented; 6223-Humlib: 485×10^3 BLU) [35], and a weaker signal for aquaporin-4 (3514-Humlib: 7×10^3 BLU; 6223-Humlib: 72×10^3 BLU) [36]. This confirms that the new method enables us to screen the binding specificities of PDZ domains in a proteomic context.

Analysis of the ERBIN PDZ Domain Binding Specificity

The new synthesized 6223 C termini library of human proteins was incubated with the ERBIN PDZ domain

Figure 3. Synthesis and Analysis of the Peptides (**10** and **11**)

(A) The cyclic peptide (**10**) was generated according to Figure 1 with β-alanine replaced by the Fmoc-Rink linker (for details, see Experimental Procedures). Conditions used for cyclization were as follows: h-1, DMF, 3 hr; h-2, cesium thiolate, DMF, 3 hr; h-3, cesium carbonate in DMF (1 mM), 3 hr; i, TFA, water, TIBS, DCM, 3 hr.

(B) ESI mass spectrometry (left) and RP-HPLC (right) of the crude products resulting from the conditions h-1, h-2, and h-3 used in cyclization of the membrane-bound peptide (**9**). HPLC and MS analysis of the crude reaction product h-3 indicates that cyclization is quantitative using this condition. Conditions h-1 and h-2 led to incomplete cyclization of (**9**). Depending on the conditions used, various amounts of the linear peptide (**11**) were detected by HPLC and MS analysis.

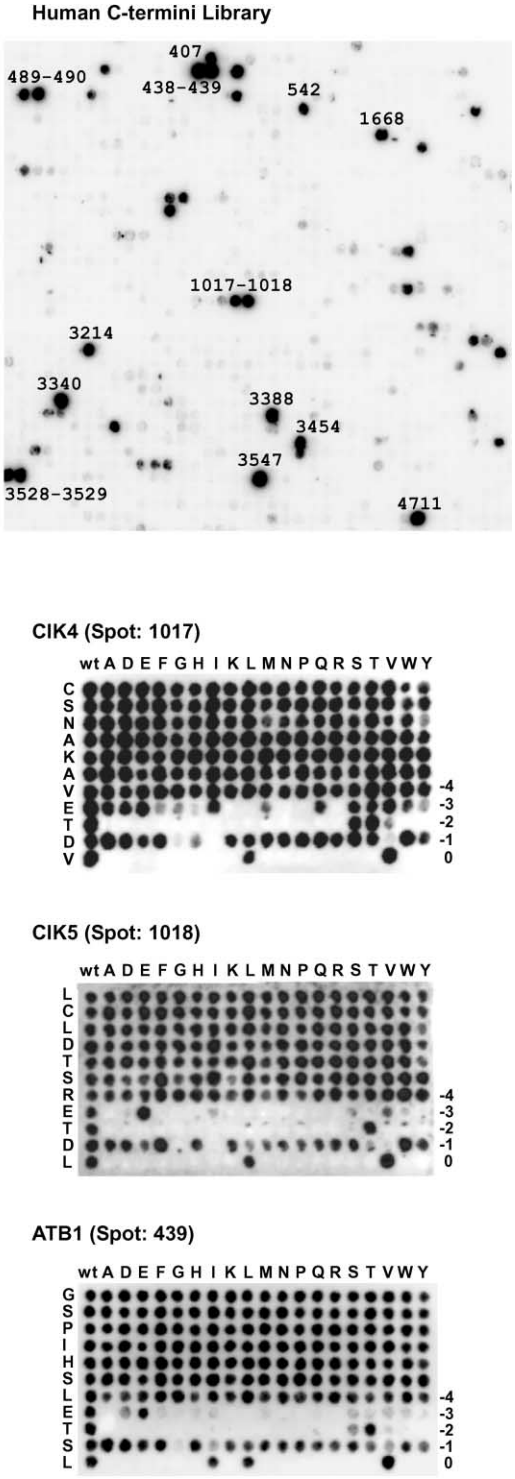

Figure 4. Inverted Peptide Arrays on Cellulose Membranes for Protein Binding

(A) Extract from the library of 6223 C termini (11-mers) of human proteins from the SWISS-PROT database incubated with GST-labeled ERBIN PDZ domain (cysteine replaced by serine). The 30 strongest binders are listed in Table 2.

(B–D) Substitutional analyses of the peptide derivates from the voltage-gated potassium channel protein Kv1.4 (CIK4), (B), from the voltage-gated potassium channel protein Kv1.5 (CIK5) (C) and from the calcium-transporting ATPase plasma membrane, isoform 1 (ATB1) (D) incubated with GST-ERBIN-PDZ. Each residue of the peptide ligand was substituted by 19 gene-encoded L-amino acids (cysteine omitted). All spots in the left column are identical and represent the wild-type peptide (wt). All other spots are single substitution analogs, with rows defining the substituted sequence position and columns defining the amino acid used as a replacement.

[Q9NR18] (Figure 4A). The PDZ binders were determined analog to the SYNA1 incubation of the 6223-Humlib by measuring the background intensity with 20 randomly located spots on the cellulose membrane (background, 4×10^4 BLU; standard deviation, 1×10^4 BLU; PDZ domain binder $\geq 6 \times 10^4$ BLU; signal range, 6×10^4–676×10^4 BLU). The sequences of the 40 strongest interacting peptides are given in Table 2 (310×10^4–676×10^4 BLU). The list reveals many membrane proteins and receptors including the well-known ERBIN PDZ domain interaction with ARVCF (O00192, spot no. 407) [26]. Surprisingly, we found an interaction with the muscle sodium channel protein type IV α subunit (P35499, spot no. 1027) and with the guanylate cyclase soluble α-2 chain (P33402, spot no. 1351), which are both described as SYNA1 PDZ domain ligands [12, 21]. For further investigations, we selected the voltage-gated potassium channel protein Kv1.4 (CIK4 [P22459] peptide $CSNAKAVETDV_{COOH}$ [Figure 4B]), the voltage-gated potassium channel protein Kv1.5 (CIK5 [P22460] peptide $LCLDTSRETDL_{COOH}$ [Figure 4C]), the plasma membrane Ca(2+) ATPases, isoform 1 (ATB1 [P20020] peptide $GSPIHSLETSL_{COOH}$ [Figure 4D]), and the breakpoint cluster region protein (BCR [P11274] peptide $KRQSILFSTEV_{COOH}$ [Figure 5A]). Substitutional analysis, in which each residue of the ligand is substituted by all gene-encoded L-amino acids (cysteine omitted), enabled us to identify the key residues of the ligand. The observed substitutional patterns for these chosen peptides were generally very similar to each other and typical for the canonical PDZ:peptide interaction. In all cases, the ERBIN PDZ domain shows a clear preference for valine in ligand position 0, but also tolerates leucine or isoleucine. Position -1 shows less restriction, but some amino acids, such as glycine, isoleucine, and valine, are not tolerated. For position -2, the ERBIN PDZ domain prefers threonine and serine, classifying this PDZ domain as a class I PDZ domain. Nevertheless, ligands such as the receptor protein tyrosine kinase ERB2 (peptide $NPEYLGLDVPV_{COOH}$), with a hydrophobic amino acid at position -2 (class II), can also interact with this PDZ domain [22]. In position -3, ERBIN prefers a negatively charged amino acid such as glutamate or aspartate, but also tolerates substitutions with small or hydrophobic amino acids. The substitutional analysis showed less selectivity for ligand positions beyond residue -3 (positions -4 to -10). Normally, residues N terminal to ligand position -3 do not contribute substantially to affinity or specificity of the PDZ domain, although several studies have shown their participation in PDZ domain-ligand interactions [37–39].

In addition, dissociation constant (Kd) values for selected peptides (spot nos. 407, 438, 439, 542, 1018, 1027, 1300, and 1351) derived from the 40 strongest ERBIN PDZ domain-interacting ligands were deter-

Table 2. ERBIN PDZ Binding Peptides from Human Carboxyl Termini

Spot	Sequence	Accession No.	Kd [μM]	Protein Description
102	FSTALYGESDL	P12814		alpha-actinin 1, cytoskeletal isoform
103	FSSALYGESDL	P35609		alpha-actinin 2, skeletal muscle isoform
116	LQDEKVKESYV	O95477		ATP-binding cassette, sub-family A, member 1
131	KRNTLYFSTDV	Q12979		active breakpoint cluster region-related protein
220	SSLREMETFVS	P50052		type-2 angiotensin II receptor (AT2)
336	RHSGSYLVTSV	P25054		adenomatous polyposis coli protein (APC protein)
407	DAKPQPVDSWV	O00192	8.0 ± 3.1	armadillo repeat protein deleted in velo-cardio-facial syndrome [26]
438	GSPLHSLETSL	P20020	24.5 ± 5.0	plasma membrane calcium-transporting ATPase, isoform 1
439	GSPIHSLETSL	Q01814	54.2 ± 4.8	plasma membrane calcium-transporting ATPase, brain isoform 2
441	DSSLQSLETSV	P23634		plasma membrane calcium-transporting ATPase, isoform 4
489	NVDFPPKESSL	P27037		activin receptor type II precursor
490	NVDLPPKESSI	Q13705		activin receptor type IIb precursor
505	GCLREWCDAFL	O75531		barrier-to-autointegration factor
542	KRQSILFSTEV	P11274	36.0 ± 5.2	breakpoint cluster region protein
788	FKLLDQMETPL	P08311		cathepsin G precursor
1017	CSNAKAVETDV	P22459		voltage-gated potassium channel protein KV1.4
1018	LCLDTSRETDL	P22460	79.4 ± 2.8	voltage-gated potassium channel protein KV1.5
1027	TVRPGVKESLV	P35499	82.7 ± 1.3	sodium channel protein, skeletal muscle alpha-subunit
1168	KINLSQKETSI	P53618		coatomer beta subunit
1253	NSRPHTNETSL	P23508		colorectal mutant cancer protein
1284	APQWVPVSWVY	O14936		peripheral plasma membrane protein CASK
1300	SNQLAWFDTDL	P35222	53.1 ± 0.5	beta-catenin
1351	IGTMFLRETSL	P33402	115.5 ± 9.2	guanylate cyclase soluble, alpha-2 chain
1668	SPQWVPVSWVY	Q00013		55 kDA erythrocyte membrane protein (p55)
1958	KQASSQSWVPG	O43524		forkhead protein O3A
3214	SQKVAVYSTCL	P07942		laminin beta-1 chain precursor
3340	TLDSQIQETSI	P27816		microtubule-associated protein 4
3388	VKRMRMDAWVT	Q02078		myocyte-specific enhancer factor 2A
3454	LGARVSKETPL	P53778		mitogen-activated protein kinase 12
3528	EPQWVPVSWVY	Q14168		MAGUK p55 subfamily member 2
3529	DTHWVPVSWVR	Q13368		maguk p55 subfamily member 3
3547	PSTLTIFETAL	O15439		multidrug resistance-associated protein 4
3807	WRRISSLESEV	Q14957		glutamate [NMDA] receptor subunit epsilon 3 precursor
3848	LLSDMYKSSDI	Q9Y466		orphan nuclear receptor NR2E1
3882	GLIAGEKETHL	P48065		sodium- and chloride-dependent betaine transporter
4711	QLNGTFESQVQ	P25800		rhombotin-1
5027	DGGRDQQETNL	Q92911		sodium/iodide cotransporter
5028	SSTCILQETSL	Q9Y289		sodium-dependent multivitamin transporter
5840	QSFLQTETSVI	P41587		vasoactive intestinal polypeptide receptor 2 precursor
5956	MNDPAWDETNL	Q14155		hypothetical protein KIAA0142

Accession numbers refer to the SWISS PROT database; cysteine replaced by serine.

mined by surface plasmon resonance (Biacore) measurements (Table 2). We obtained Kd values of 8.0 ± 3.1 μM for AVRCF and 115 ± 10 μM for CYG4, which are typical for PDZ domain interactions.

To confirm that the BCR peptide interaction observed in vitro corresponds to protein interactions between BCR and ERBIN in mammalian cells, we performed a pull-down assay by using the PDZ domain of ERBIN fused to GST. The GST-ERBIN PDZ and the GST protein alone as a control were incubated with lysates of HEK293 cells overexpressing BCR_{WT} and a C-terminal mutant BCR_{V1271A}, both as HA-tagged fusions. As predicted, BCR_{WT} but not the mutant BCR_{V1271A} bound to the ERBIN PDZ domain (Figure 5B, lanes 1 and 2), whereas GST protein alone did not bind (Figure 5B, lanes 3 and 4). We extended this study to substantiate the biological relevance of the interaction between full-length ERBIN and BCR as endogenous proteins. To test this, BCR and ERBIN were analyzed in MKN-7 cells, where both proteins are well expressed (Figure 5C). Indeed, ERBIN coprecipitated with BCR (Figure 5C, lanes 4 and 6). Competition of the BCR and the ERBIN antibody with their respective antigen reduced immunoprecipitation, supporting the specificity of the interaction (Figure 5C, lanes 3, 5, and 7).

Our results, namely the in vitro interactions between the ERBIN PDZ domain and the three C-terminal peptides derived from the voltage-gated potassium channel proteins Kv1.4 and Kv1.5 and the plasma membrane Ca(2+) ATPases, isoform 1, could be relevant interactions with regard to a decisive role of the ERBIN PDZ domain in a neuronal context [40]. Further in vivo experiments will be needed to determine the biological relevance of such protein interactions and the ERBIN PDZ domain itself.

ERBIN is the founding member of the leucine-rich repeat (LRR) and PDZ domain (LAP) family, which is characterized by 16 LRRs at the N terminus and 1–4 PDZ domains at the C terminus [41]. Besides the PDZ-specific interactions with ERB2, δ-catenin, ARVCF, or

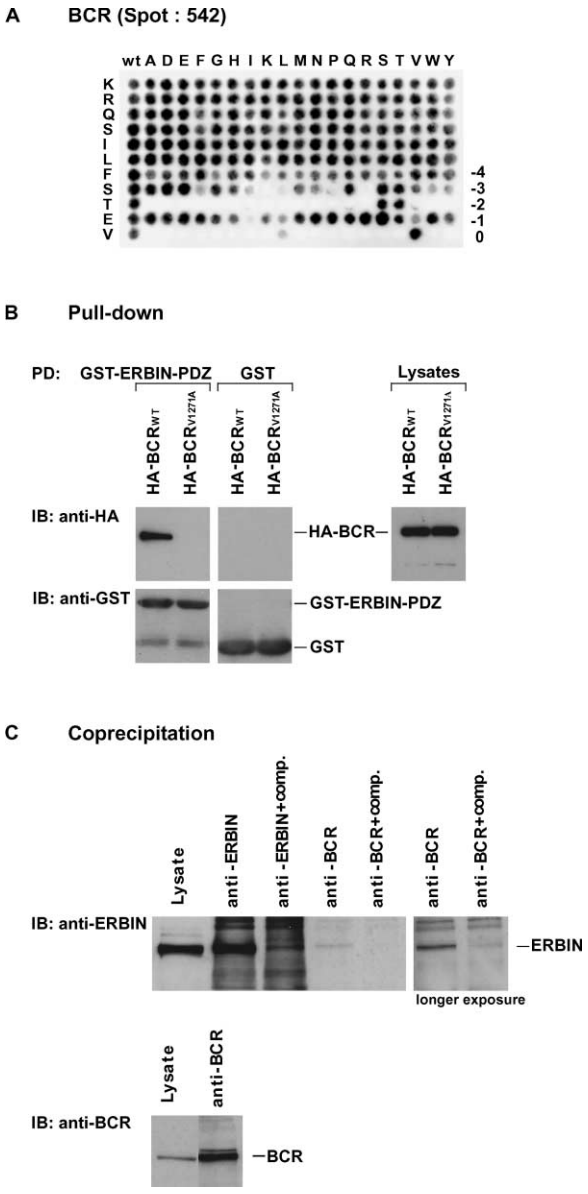

Figure 5. BCR Is a Ligand of the ERBIN PDZ Domain

(A) Substitutional analysis of the C-terminal peptide derived from the breakpoint cluster region (BCR).

(B) Pull-down (PD): HEK293 cells transfected with plasmids encoding HA-BCR_{WT} and HA-BCR_{V1271A} were lysed and incubated with GST-ERBIN-PDZ bound to GSH-agarose beads. Proteins bound to GST fusion proteins were fractioned by SDS-PAGE and immunoblotted (IB) with anti-HA antibody and anti-GST antibody, respectively. As control for the expression of the BCR proteins, direct lysates of the 293 cells were immunoblotted with anti-HA antibody.

(C) Coprecipitation of endogenous ERBIN and BCR in MKN-7 cells. The lysate of MKN-7 cells was incubated with anti-BCR antibody in the absence (top panel, lanes 4, 6) or presence of BCR peptide (+ comp.) (top panel, lanes 5, 7) followed by immunoblotting with anti-ERBIN. Expression of endogenous ERBIN and BCR was verified by immunoprecipitation with anti-ERBIN (top panel, lane 2) with anti-BCR (bottom panel, lane 2), and by analysis of the direct lysate with anti-ERBIN (top panel, lane 1) and anti-BCR (bottom panel, lane 1).

p0071 [22, 24–26], ERBIN is know to interact with the small GTPase Ras and Rho through the LRRs [42, 43]. Our novel interacting partner of ERBIN, BCR, is a multidomain protein containing a serine/threonine protein kinase domain, a guanine nucleotide exchange factor (GEF) function, and a GTPase-activating protein (GAP) domain. The GEF and GAP domains can modulate the activity of Rho-type GTPases [44, 45]. We suggest that ERBIN is a scaffold-like protein that links BCR and Rho-type GTPases into a single complex.

Significance

The SPOT synthesis concept has been widely used to prepare peptide arrays for proteomic studies. Our overall goal is to extend the application range of the SPOT synthesis concept by creating N terminally fixed inverted peptide arrays, enabling free C-terminal display on planar cellulose supports. In the context of proteomic studies, a reliable and robust technology is required, and this is satisfied by our enhanced synthetic method. We demonstrate here that new ligands of PDZ domains can be identified in a proteomic context by our extended SPOT synthesis concept. This could be applied to nonredundant statistical libraries or libraries based on database sequences. With the improved and robust methodology, particulary with regard to automatic, robot-assisted SPOT synthesis, we were able to screen a large range of divergent peptide sequences. In addition, the extended SPOT synthesis concept can provide better insights into PDZ:ligand interactions due to the various screening options such as substitutional analyses, combinatorial libraries, length analyses, and integration of unnatural building blocks or other chemical entities.

Our screen of 6223 C termini of human proteins revealed four new peptide ligands for the ERBIN PDZ domain. The voltage-gated potassium channel proteins Kv1.4 and Kv1.5 and the plasma membrane Ca(2+) ATPases, isoform 1, could be potential ERBIN PDZ domain ligands in neuronal context. The biological relevance of the ERBIN:breakpoint cluster region protein interaction was substantiated by pull-down and coprecipitation studies using full-length and/or endogenous proteins. Our results strengthen the suggestion that ERBIN acts as a scaffold-like protein, linking BCR and Rho-type GTPases into a single complex via its PDZ domain and LRRs, respectively.

Taken together, these data suggest that the improved method for generating cellulose membrane-bound inverted peptides may be a powerful tool to screen proteomic databases on a large scale and to find new ligands for PDZ domains.

Experimental Procedures

Synthesis of Membrane-Bound Inverted Peptide Arrays

The peptides were synthesized on N-modified CAPE membranes [28] and prepared semiautomatically using a SPOT robot (INTAVIS Bioanalytical Instruments AG, Cologne, Germany). Array design was performed using the in-house software LISA 1.571. The synthesis started with the spot definition using a standard protocol [8] followed by the coupling of a 0.3 M solution of Fmoc-cysteine-(Trt)-Opfp in N-methyl-pyrolidone (NMP) (double coupling, 15 min each). After

Fmoc cleavage with 20% piperidin in DMA, Mmt-mercaptopropionic acid was dissolved in DMF (1 M solution), activated with 1 equiv. HATU and 2 equiv. NMI, and directly spotted on the membrane; after 15 min this step was repeated. The membrane was washed with DMA (3 × 3 min) and DCM (3 × 3 min). The Mmt group was removed using treatments of 10% (v/v) dichloroacetic acid and 0.5% (v/v) TFA in DCM for 1 × 5 min followed by 10% (v/v) dichloroacetic acid, 0.5% (v/v) TFA, and 5% (v/v) triisobutylsilane (TIBS) in DCM for 3 × 5 min. The membrane was washed with DCM (1 × 3 min), EtOH (2 × 3 min), water (2 × 3 min), 10% aqueous solution of cesium carbonate for forming the cesium thiolate (1 × 2 min), water (1 × 30 s), EtOH (2 × 30 s), diethyl ether (2 × 30 s), and finally air dried. Solutions of the Fmoc amino acid 3-bromopropyl esters in DMF were spotted on the membrane (0.6 M solutions; 0.8 M solutions for C, H, N, Q, and R; triple coupling, 15 min each). The Fmoc group was removed from the spots, and the sequences of the peptides were completed using the standard SPOT synthesis protocol [8] and followed by a N-terminal tag with β-alanine. For standard SPOT synthesis, Fmoc-aa-Opfp were used with the following side-chain protections: E-, N-(O*t*Bu); S-, T-, Y-(*t*Bu); K-, W-(Boc); N-, Q-, H-(Trt); R-(Pbf). For thioether cyclization, all peptides were N-acylated with bromoacetic acid 2,4-dinitrophenyl ester in NMP (1 M), double coupling, 15 min each. The membrane was washed with DMA (3 × 3 min), DCM (3 × 3 min), and dried. Side chain protection groups were removed using one treatment of 90% TFA and 3% TIBS in DCM for 30 min without shaking, followed by washing steps including DCM 3 × 3 min, DMA 3 × 3 min, EtOH 3 × 3 min, and diethyl ether 2 × 3 min, followed by 50% TFA and 3% TIBS in DCM for 2.5 hr without shaking. The membrane was washed with DCM (3 × 3 min), DMA (3 × 3 min), EtOH (2 × 3 min), 0.1 M phosphate buffer pH 7.4 (2 × 3 min) and water (2 × 3 min). The membrane-bound peptides were cyclized by incubating the membrane with a 5% aqueous solution of cesium carbonate for approximately 12 hr. The membrane was washed five times with water, and the membrane-bound cyclized peptides were inverted by ester-hydrolysis, using one treatment with a saturated aqueous solution of lithium carbonate. Finally, the membrane was washed with water (3 × 3 min), 1% hydrochloric acid (1 × 3 min), water (2 × 3 min), EtOH (2 × 3 min), diethyl ether (2 × 3 min), and air dried.

SPOT Synthesis and Chemical Characterization of the Peptides (10, 11)

Synthesis of peptides was performed by manually pipetting 1 μl of each reagent solution onto the spots (spot area: 0.25 cm^2). Three arrays were generated, each consisting of eight identical spots. Cleavability of the prepared peptides and spot definition were achieved by spot-wise treatment of the N-modified CAPE membrane with a TBTU-activated 0.3 M solution of the Fmoc-Rink linker (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) in DMF (1 equiv. Fmoc-Rink linker, 1 equiv. TBTU, and 2 equiv. DIEA). After 15 min, the procedure was repeated using a freshly prepared reagent solution. Synthesis of the peptides (**9**) by coupling Fmoc-cysteine-(Trt)-Opfp, Mmt-mercaptopropionic acid, and standard SPOT synthesis was performed according to the protocol described above. Removal of the cysteine Trt group was achieved by treatments of 2% (v/v) TFA in DCM for 1 × 5 min and 2% (v/v) TFA, 5% (v/v) TIBS in DCM for 3 × 5 min. The membrane was washed with DCM (3 × 3 min), air dried, and the three arrays were separated by cutting the membrane. For cyclization, each part was treated in a different manner: h-1, incubation with DMF for 3 hr; h-2, the membrane was washed with EtOH (2 × 3 min) and water (2 × 3 min), then incubated with a 5% aqueous solution of cesium carbonate for 1 min, washed with water (6 × 1 min), EtOH (1 × 3 min), DMF (2 × 3 min), and incubated with DMF for 3 hr; and h-3, the membrane was washed with DMF (2 × 3 min) and incubated with a 1 mM solution of cesium carbonate in DMF for 3 hr. Subsequently, all membranes were washed with DMF (2 × 3 min), DCM (3 × 3 min), and dried. The spots were punched out from each membrane and transferred to eppendorf tubes. Cleavage from the solid support and amino acid side chain deprotection were performed simultaneously by adding a solution (300 μl) of 90% TFA, 5% water, and 3% TIBS in DCM to each tube. The tubes were shaken for 3 hr, the cellulose membranes were taken out, and the resultant solution was concentrated in vacuo. Peptide precipitation was achieved by the addition of 100 μl diethyl ether and completed by centrifugation. After separation, the pellet was washed five times with diethyl ether and finally dried. The compounds were dissolved in 50% aqueous acetonitrile (200 μl), and purity was checked by analytical HPLC (Waters, Eschborn, Germany) on a Vydac C 18 column. Cyclic compounds (**10**) were identified using ESI mass spectrometry.

Cyclic Peptide (10), $C_{50}H_{79}N_{13}O_{17}S_2$

ESI-MS: mass calculated (monoisotopic): 1197.5158, mass found: 1197.6578 (found as m/z: 599.7657 $[M+2H]^{2+}$); HPLC: retention time: 12.01 min.

Linear Peptide (11), $C_{50}H_{80}BrN_{13}O_{17}S_2$

ESI-MS: mass calculated (monoisotopic): 1277.4437, mass found: 1277.5972 (found as m/z: 639.7986 $[M+2H]^{2+}$); HPLC: retention time: 10.11 min.

PDZ Preparation

DNA fragments encoding residues 1280 to 1371 of ERBIN and 81 to 164 of SYNA1 were cloned into pGEX6p2 and pGAT2 expression vectors, respectively. *E. coli* BL21(DE3) cultures harboring the expression plasmid were grown at 37°C to mid-log phase (OD_{600} = 0.5). Protein expression was induced by addition of 1.0 mM IPTG, and cells were harvested 3.5 hr later by centrifugation at 7800 × g for 10 min at 4°C. The pellet was washed and resuspended in 20 mM Tris, Complete (protease inhibitor cocktail tablets, Roche, Mannheim, Germany) (pH 8.0) and stored at −80°C.

The cells were lysed using a French press (ThermoSpectronic, Rochester, NJ), centrifuged for 30 min at 75,800 × g, and the supernatant was loaded onto a SP-Sepharose column (Amersham Biosiences, Freiburg, Germany). The column was washed with PBS (pH 7.4), and then the GST-tagged protein was eluted with 50 mM Tris (pH 8.0), 10 mM reduced glutathione. Fractions containing the protein of interest were pooled, concentrated, and used for cellulose incubation.

Binding Studies of Cellulose-Bound Peptides

The nonredundant library of 6223 C termini (11-mers) of human proteins from the SWISS-PROT database (release 40) and all substitution analogs of ligands ("substitutional analysis of ligands") were generated by a semiautomated SPOT robot [1, 8] (Abimed, Langenfeld, Germany; LISA, in-house software) using the novel strategy.

The human C termini library and the substitutional analysis were prewashed once with EtOH (1 × 10 min), with Tris-buffered saline (TBS) (pH 8.0) (3 × 10 min), then blocked for 4 hr with blocking buffer (blocking reagent [Sigma-Genosys, Cambridge, UK] in TBS [pH 8.0] containing 5% sucrose). The membranes were incubated with PDZ-GST (10 μg/ml) in blocking buffer overnight at 4°C and washed with TBS (pH 8.0) (3 × 10 min), followed by a second incubation with anti-GST-HRP (Amersham Biosiences, Freiburg, Germany) (1 μg/ml) in blocking buffer for 3 hr at room temperature. To remove excess antibody, the membrane was washed with TBS pH 8.0 (3 × 10 min). A chemiluminescence system (Pierce Biotechnology, Rockford, IL) was applied for detection using a LumiImager (Boehringer Mannheim GmbH, Mannheim, Germany). The signal intensities were recorded as Boehringer light units (BLU) using the LumiAnalyst software.

Affinity Measurements

The binding affinity (Kd) was measured using the BIACORE X system (Uppsala, Sweden).

PDZ-GST was immobilized on a CM5 chip using the GST coupling procedure, according to the supplier's instructions. The amount of covalently coupled protein corresponded to a signal increase of approximately 500 resonance units (RU). An appropriate amount of GST was coupled to flowcell 1 on the same chip as a reference.

All binding, experiments were performed at 20°C with a flow rate of 5 μl/min (injection volume 10 μl). Peptides were used at various concentrations between 1 μM and 1 mM in HBS buffer (10 mM HEPES with 0.15 M NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 [pH 7.4]). Complete regeneration was obtained after dissociation without regeneration buffer. Transformation of data and analysis were performed with the BIA-evaluation software, version 3.0. The control sensorgram (flowcell 1) was subtracted from the sen-

sorgrams obtained with flowcell 2. The steady-state values of the binding equilibrium were plotted versus the different peptide concentrations and fitted using the implemented steady-state evaluation, resulting in the Kd for the PDZ-peptide complexes.

Pull-Down and Coimmunoprecipitation Assays

The full-length BCR_{WT} and the mutant BCR_{V1271A} were described elsewhere [46]. Pull-down and immunoprecipitation of BCR and HA-BCR were performed with rabbit polyclonal anti-BCR C20 (Santa Cruz Biotechnologie) for BCR and Mab 12CA5 (Roche diagnostics) for HA-BCR. GST-ERBIN PDZ and ERBIN were analyzed with anti-GST (Amersham Pharmacia Biotech) for GST-ERBIN and anti-ERBIN (in-house production, Radziwill, Swizerland) for ERBIN, respectively.

Human embryonic kidney 293 (HEK293) and human gastric adenocarcinoma (MKN7) cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U penicillin/ml, and 100 μg of streptomycin/ml. Transfection was performed based on standard calcium-phosphate methods, and pull-down and coimmunoprecipitaion were performed as described [46].

Supplemental Data

Supplemental data, including experimental procedures for the synthesis of the Fmoc-amino acid 3-bromopropyl esters, a figure of the SYNA1 PDZ domain screen, and the derived table, which contains the evaluation of the SYNA1 PDZ domain interactions, is available at http://www.chembiol.com/cgi/content/full/11/4/449/DC1.

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