Atypical Recognition Consensus of CIN85/SETA/Ruk SH3 Domains Revealed by Target-assisted Iterative Screening*

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Target-assisted iterative screening applied to random peptide libraries unveiled a novel and atypical recognition consensus shared by CIN85/SETA/Ruk SH3 domains, PX(P/A)XXR. Confirmed by mutagenesis and in vitro binding experiments, the novel consensus allowed for the accurate mapping of CIN85 SH3 binding sites within known CIN85 interactors, c-Cbl, BLNK, Cbl-b, AIP1/Alix, SB1, and CD2 proteins, as well as the prediction of CIN85 novel-interacting partners in protein databases. Synaptotagmin 1, PAK2, ZO-2, and TAFII70, which contain CIN85 SH3 recognition consensus sites, were selectively precipitated from mouse brain lysates by CIN85 SH3 domains in glutathione S-transferase pull-down experiments. A direct interaction of synaptotagmin 1 and PAK2 with CIN85 SH3 domains was confirmed by Far Western blotting.

One of the initial approaches to study a novel gene product is the identification of its interacting partners. As increasingly more proteins become functionally characterized it is more and more probable that the identification of interacting partners of a novel protein will suggest a possible function for this protein by associative reasoning and, thus, provide a framework for its characterization. Using this approach, a novel CIN85/CMS adaptor protein family was functionally implicated in a remarkably wide spectrum of different cellular processes such as the down-regulation of receptor tyrosine kinases and endocytosis (through the association of CIN85 with Cbl/Cbl-b and endophilins) (1, 2), apoptosis (through the association of CIN85 with p85-phosphatidylinositol 3-kinase and AIP1/Alix) (3, 4), and adhesion phenomena (through the association of CMS with p85-phosphatidylinositol 3-kinase and cortactin (1, 3, 7, 9, 10). The SH3 domains of CIN85 bound c-Cbl, Cbl-b, BLNK, SB1, CD2, and AIP1/Alix proteins (4, 9–13). Unexpectedly, attempts to map the binding sites of CIN85 SH3 domains within the Cbl-b and of CD2AP/CMS SH3 domains within the c-Cbl proteins by mutagenesis of the canonical SH3 binding motifs PXXP failed, suggesting unusual recognition properties of these SH3 domains (12, 14).

The SH3 domain is a prototype of the protein interaction modules that mediate transient and specific protein associations underlying the assembly of functionally diverse multi-protein complexes (15, 16). The overwhelming majority of the studied SH3 domains binds to linear proline-rich peptide sequences featuring the XPXXP motif (17–19). Variable ligand residues flanking the XPXXP core define the specificity of individual domains within the SH3 family (17, 18, 20). Structural and mutagenesis studies revealed that upon binding, peptide ligands adopt a poly-proline type II helix conformation with the conserved prolines facing the interaction interface. On the complementary side of this interface, the SH3 domain interaction surface features two parallel consecutive grooves, named XP binding grooves, followed by the specificity pocket that accommodates variable residues flanking the XPXXP ligand core (18, 21–23). The XPXXP motif has been canonized as a bona fide SH3 binding site with few exceptions reported (24–26). In the presented work a novel screening format, target-assisted iterative screening (TAIS),1 was applied to random peptide libraries displayed on M13 and T7 bacteriophages to isolate peptide ligands of the CIN85 SH3 domains (27).

At least five discrete protein interaction modules can be inferred from the analysis of the CIN85 amino acid sequence. These are three consecutive SH3 domains at the N terminus followed by a central proline-rich region and a coiled-coil domain situated at the C terminus (Fig. 1). CIN85 was shown to homodimerize through its coiled-coil domains, and a number of proteins interacting with the CIN85 proline-rich region were identified including Grb2, p190RhoA, endophilins, p65-phosphatidylinositol 3-kinase, and cortactin (1, 3, 7, 9, 10). The SH3 domains of CIN85 bound c-Cbl, Cbl-b, BLNK, SB1, CD2, and AIP1/Alix proteins (4, 9–13). Unexpectedly, attempts to map the binding sites of CIN85 SH3 domains within the Cbl-b and of CD2AP/CMS SH3 domains within the c-Cbl proteins by mutagenesis of the canonical SH3 binding motifs PXXP failed, suggesting unusual recognition properties of these SH3 domains (12, 14).

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1 The abbreviations used are: TAIS, target-assisted iterative screening; GST, glutathione S-transferase; BSA, bovine serum albumin; RT, room temperature; TBS-T, Tris-buffered saline with Tween 20; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropanesulfonic acid; PVDF, polyvinylidene difluoride; CMS, Cus ligand with multiple SH3 domains.
sites within its reported interacting partners as well as the prediction of novel putative interactors from protein databases. Putative SH3 domain-dependent interactions of CIN85 with synaptotagin 1, PAK2, ZO-2, and TAF170 were predicted by homology searches with the CIN85 SH3 recognition consensus and confirmed in GST pull-down and Far Western blotting experiments performed with individual CIN85 SH3 domains. The results presented and those reported in the literature regarding recognition properties of CIN85/SETA/Ruk SH3 domains and places a biochemical foundation for the rapid characterization of the CIN85 protein interaction sub-network.

**EXPERIMENTAL PROCEDURES**

**TAIS**—A detailed description of the TAIS method is given in Kurakin and Breeden (27). The TAIS flowchart and protocols can be found on the Internet (www.buckinstitute.org/TAIS). In brief, 30 μg of a GST-SH3 domain fusion immobilized on Sepharose beads were blocked in 0.5% bovine serum albumin (BSA) in TBS-T (Tris-buffered saline, pH 7.4, plus 0.1% Tween 20) and incubated with a random peptide library aliquot (~10^9–10^10 plaque-forming units). After 90 min of incubation at room temperature (RT) the beads were thoroughly washed with TBS-T, and bound phages were eluted with 200 μl of 1% SDS for 15 min at RT. After the phages were immediately mixed with a molten agarose containing host cells and plated onto two pre-warmed 150-mm agar plates. When phage plaques became visible, the plates were cooled down for 30 min at 4 °C and overlaid with 132-mm nitrocellulose membranes (Schleicher & Schuell) for 5 min. After plaque lift, the membranes were blocked in 1% BSA in TBS for 1 h at RT and incubated overnight in 25 ml of TBS-T on a rocker at 4 °C with 10 μg of the same SH3 domain that had been cleaved from the GST moiety, biotinylated, and complexed with streptavidin-alkaline phosphatase at a ratio of 4:1. After extensive washing with TBS-T, positive plaques were developed on the membranes with insoluble alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Individual positive plaques were identified on T7 select plates, and phages from these plaques were propagated separately in the appropriate host for production of phage DNA. T7 phage DNA was prepared for sequencing with the λ DNA Wizard kit from Promega. M13 single-stranded DNA was added to the ELISA plate wells and incubated for 1 h at RT. After incubation, unbound phages were washed away with TBS-T, and the amount of retained phages was determined with phage-specific antibodies. Monoclonal anti-M13 antibodies conjugated to horseradish peroxidase were used for detection of M13 phages. Rabbit polyclonal anti-T7 antibodies are a generous gift from Dr. F. William Studier, Brookhaven National Laboratory. Donkey anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences) were used for detection of anti-T7 antibodies. ELISA readings were taken on a SpectraMAX190 plate reader (Molecular Devices) at 405 nm. To ensure reproducibility, all phage ELISA experiments presented were repeated at least three times for triplicate samples each time.

**Alanine-scanning Mutagenesis**—The CIN85 SH3-2 domain peptide ligand WSDPRPLPRTIL and the CIN85 SH3-3 peptide ligand TTG-PFPVRRSLS were selected by the respective SH3 domains in a TAIS experiment from the Xθ peptide library displayed on M13 phage. The last S of the peptides, shown in italics, denotes a serine residue originating from the cloning site in the M13 display vector. This last residue was mutagenized in all alanine-substituted peptides, potentially contributing to the interaction. Each amino acid in the indicated peptide ligands was successively substituted by an alanine residue, and the mutagenized peptides were displayed on the M13 surface. To display a particular alanine-substituted peptide sequence, a couple of complementary oligonucleotides coding for the alanine-substituted peptide were chemically synthesized, annealed, and ligated into the XhoI/Xhol-linearized M13 pHII display vector (29). The following oligonucleotides coding for the ASDPRPLPTILS peptide alanine mutant are shown as an example.

5′–TCGACGGCTTCCAGCCCCCGCTCCGGCGGATACATGCTGT3′

3′–GGCGAAGCGGCGCGCGCGCGGCGCGGCGGTAGCGAGCATG–5′

**SEQUENCES 1**

Thirteen alanine-substituted peptides for each of the indicated peptide ligands were displayed on M13 phages and tested for binding to their cognate CIN85 SH3 domains in a phage ELISA assay. The only peptide ligand of CIN85 SH3-1 domain that was successfully displayed in the M13 format was SRVTPYPAPKPPFS (see “Results”). Two alanine-substituted mutants of this sequence were generated as described above to test the critical requirements of the invariable proline and arginine residues of the CIN85 SH3 recognition core for binding to GST-PVAPRPPFS and SRVTPYPAPKPPFS. All constructs were verified by sequencing.

**GST Pull Downs**—Mouse brain extracts were prepared by homogenization of one-half of a brain from adult mouse in 2 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) in the presence of a protease inhibitor mixture (Roche Applied Science, tablets). After homogenization on ice, insoluble fraction was pelleted by centrifugation at 13,000 rpm at 4 °C for 5 min. Thirty μg of individual SH3-GST fusions immobilized on Sepharose beads were separated by SDS-PAGE and transferred to polycarbonate PVDF membranes (Schleicher & Schuell) for 5 min. After incubation, unstreptavidin-coated plates were blocked by adding 150 μl of 1% BSA in TBS for 1 h for a GST pull-down assay. bead was used as a negative control. After incubation, beads were washed 6 times with 1 ml of TBS-T, and the retained proteins were eluted into loading buffer at 70 °C for 10 min. Precipitated protein complexes were separated by SDS-PAGE (pre-cast 4–12% Bis-Tris gel/ MOPS buffer, Novex), blotted to a PVDF membrane, blocked in 1% BSA, and developed with specific antibodies. Eighty μg of total brain protein was used as input. Horseradish peroxidase-conjugated secondary antibodies, the ECL system (Amersham Biosciences), and BioMax film (Kodak) were used for protein visualization. Goat polyclonal antibodies for PAK2 and ZO-2 were purchased from Santa Cruz Biotechnology, Inc. Mouse anti-T7 antibodies were a generous gift from Dr. F. William Studier, Brookhaven National Laboratory. Donkey anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences) were used for detection of anti-T7 antibodies. ELISA readings were taken on a SpectraMax190 plate reader (Molecular Devices) at 405 nm. To ensure reproducibility, all phage ELISA experiments presented were repeated at least three times for triplicate samples each time.

**Far Western Analysis**—Nineteen mg of total mouse brain protein in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) complemented with protease inhibitors were incubated with 8 μg of anti-PAK2 antibodies or 19 μg of anti-synaptotagin 1 antibodies for 90 min at 4 °C. After incubation, 90 μl of protein A/G PLUS-agarose beads (Sigma, Cat. No. 17-061-1) was added to the samples for an additional 60 min while tumbling at 4 °C. Beads were subsequently washed 6 times with lysis buffer and eluted into 160 μl of SDS-PAGE loading buffer. Twenty-five μl of the eluate per lane of gel were used to resolve immunoprecipitated protein complexes. After SDS-PAGE (pre-cast 4–12% Bis-Tris gel/MOPS buffer, Novex), proteins were transferred to a PVDF membrane and blocked with 1% BSA in TBS-T, and incubated overnight at 4 °C on a rocker with 13 μg of each individual biotinylated CIN85 SH3 domain conjugated to streptavidin-alkaline phosphatase at 4:1 ratio in 20 ml of TBS-T. The biotinylated WW domain of Yes-associated protein (YAP) complexed to streptavidin-
alkaline phosphatase was used as a negative control. After overnight incubation, the PVDF membranes were thoroughly washed with TBS-T and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

RESULTS

TAIS of CIN85 SH3 Domains—We recently described a novel screening method for phage-displayed cDNA and peptide libraries, target-assisted iterative screening (TAIS) (27). TAIS sequentially utilizes phage display and expression library screening formats. Briefly, in a first, pre-selection step a protein target is immobilized on a solid support such as Sepharose beads and incubated with a phage-displayed peptide library in solution. This step aims to retain phages displaying on their surfaces interacting partners of the target on the solid support. After washing, the retained phages are eluted and plated on a bacterial lawn. In the second step, the pre-selected peptide library plaques are transferred onto a nitrocellulose membrane by blotting the bacterial lawn with the membrane. The protein target tagged with a reporter such as alkaline phosphatase is used then as a one-step detection reagent to screen for interacting plaques on the membrane. The identities of the displayed peptides that interact with the target are deduced by sequencing of the corresponding DNA inserts of the phages from individual positive plaques. Omitting a competition between individual phages altogether, TAIS eliminates a loss of weaker binders and propagation biases inherent to classical phage display panning as a result of competition between individual phages during repetitive selection-amplification cycles.

Two random peptide libraries, X12 and X16, displayed respectively on M13 and T7 phages, were screened by TAIS. Each one of the three CIN85 SH3 domains was used as a target in separate screening experiments. DNA inserts of the phages randomly picked up from individual positive plaques were sequenced to infer identities of the displayed peptides that bound each CIN85 SH3 domain. Specific binders were readily identified for SH3-2 and SH3-3 domains from both libraries (Fig. 1). However, the SH3-1 domain failed to select peptides from the M13 library. The application of a classical panning procedure to the same target was equally unsuccessful, suggesting problems other than idiosyncrasies of a particular methodology. An incompatibility of the M13 display format, which requires the efficient secretion of displayed peptides, with the amino acid sequences of the peptides preferred by SH3-1 domain might be a possible explanation of the failure. Indeed, attempts to display SH3-1 binders on the M13 phage were unsuccessful for 7 of 8 different peptides that were selected from the T7 library (not shown). This fact is indicative of the limitations of the phage display format that relies on bacterial secretion and may explain failures to select peptides from filamentous phage display libraries for occasional targets.

Analysis of Recognition Properties of CIN85/SETA/Ruk SH3 Domains—Sequences of peptides selected by individual SH3 domains and their deduced recognition consensuses are shown in Fig. 1. The following distinct features characterize these peptide sequences: 1) The XPXXPX motif is present only in 2 of a total of 28 different peptides selected by CIN85 SH3 domains despite a relatively high proline content of the peptides averaged at 17% or 2.6 proline residues per sequence; 2) All of the 28 selected
peptides feature a PX/P/A/XXR motif, whose length fits well the core SH3 domain footprint such as PX/P/XR of Src or PX/P/XKR of Crk SH3 domains as examples (27, 29); 3) The amino acid composition of the selected peptides is significantly biased toward positive residues and proline, whereas negatively charged residues are underrepresented.

The minimal recognition motif present in all selected peptides can be readily inferred as PX/P/A/XXR and appears to be shared by all three CIN85 SH3 domains (Fig. 1). There are slight but noticeable variations in the amino acids that are tolerated in X positions within and around this core between the three different SH3 domains, suggesting overlapping but not identical specificities of the domains. As an example, the position preceding arginine of the core is occupied exclusively by proline or valine in the peptides selected by the SH3-3 domain but tolerates charged and polar residues in the case of SH3-1 and SH3-2 domains, respectively. The hypothesis of overlapping but distinct specificities of CIN85 SH3 domains is supported by two sets of experimental data. First, the analysis of cross-reactivity of individual SH3 domains toward peptides selected by the other two domains clearly indicates that although CIN85 SH3 domains largely cross-react, there are unique peptide sequences that bind to some of the domains but only weakly or not at all to others (Fig. 2). In cross-reactivity experiments the SH3-2 domain displayed the largest degree of promiscuity, and the SH3-3 was the least cross-reacting. Second, alanine-scanning mutagenesis performed on the two peptide ligands for SH3-2 and SH3-3 domains indicates that alanine substitution of residues other than the conserved proline and arginine of the recognition core can drastically affect the binding of the mutated peptides, indicating the existence of additional specificity determinants that may be potentially unique for individual domains (Fig. 3).

The recognition consensus of CIN85 SH3 domains, PX/P/A/XXR, inferred from the analysis of selected peptides does not contain a conventional SH3 recognition motif PX/PX (Fig. 1). Therefore, alanine-scanning mutagenesis of two peptide ligands of SH3-2 and SH3-3 domains was performed in an attempt to gain a better understanding of the recognition properties of the respective domains (Fig. 3). The mutagenesis experiments confirmed a critical requirement for proline and arginine flanking the peptide ligand binding core, PX/P/A/XXR. In addition, alanine scanning suggested that certain other amino acid positions of the ligands might bear specificity determinants as well (positions 10 and 12 for the SH3-2 ligand and positions 13 and 14 for the SH3-3 ligand, according to the numbering in Fig. 3). Even though the corresponding positions do show somewhat restricted tolerance to various amino acids in the selected peptides, additional and comprehensive mutagenesis experiments are required to prove and accurately characterize these putative specificity determinants. Substitution of the flanking proline and arginine in the SH3-1 peptide ligand abolished the interaction of the ligand with the CIN85 SH3-1 domain as well, indicating a critical role of these residues for all three CIN85 SH3 domains (not shown).

Analysis of Binding Sites of CIN85 SH3 Domains within Known CIN85 Interactors—In previous reports, mutagenesis of PAXP motifs within the proline-rich fragments of Cbl-b and c-Cbl proteins failed to abrogate binding of CIN85 and CD2AP/CMS SH3 domains, respectively, to the mutated fragments (12, 14). In the case of Cbl-b, the interaction fragment was only 36 amino acids long, and it was concluded that CIN85 SH3 binding to this fragment was PXXP-independent (12). Fig. 4 shows that a point mutation in the arginine flanking the unique putative recognition site of CIN85 SH3 domains, PX/P/A/XXR, within the Cbl-b fragment in question abolished binding of all three CIN85 SH3 domains to this fragment, thus confirming the conclusion of the previous report and demonstrating the validity of predictions based on the CIN85 SH3 recognition consensus revealed by TAI5.

An analysis of the amino acid sequences of the known CIN85 interactors readily allows one to map putative interaction sites within the respective proteins using the newly defined CIN85 SH3 recognition consensus: P22681 c-Cbl (human) (545–550 PPPPRR; 580–585 PVPSRR, 924–929 PKPFPFR; NP 057446.1 BNK (human) (242–247 PSPLPR, 275–280 PIAEA, 307–312 PIPLPR); NP 037506.2 AIP1/Alix (human) (740–745 TPTAPR, 752–757 FQPPAR); Q13191 Cbl-b (human) (486–490 PLAQR, 547–552 PPPLLPR, 556–561 PPPLLPR, 777–782 PLPPAR, 924–929 PPPPRR, 968–973 PLPPAR, 968–973 PKPFPFR); NP 619617.1 SB1 (mouse) (155–160 PAPVRR, 609–614 PLLSR, 618–623 PSPPVR, 648–653 PQAER, 678–683 TPTAPR); NP 085568.2 CD2 antigen (mouse) (320–325 PLPPRR).

We emphasize that the sequences indicated above are only likely to constitute the cores of binding sites of CIN85 SH3 domains, and mutagenesis of these sequences within the context of native proteins is required to reach more definite conclusions. In addition we expect that the interaction of CIN85 SH3 domains with their ligands might be regulated by phosphorylation in vivo, because a serine residue is statistically over-represented in the selected peptides (Fig. 1 and data not shown). Together with threonine, serine tends to occupy those positions within ligands that were sensitive to alanine substitutions (compare Fig. 1 and Fig. 3).

Novel Putative Interactors of CIN85 SH3 Domains—Homology searches of protein databases with the TAI5-defined recognition consensus of CIN85 SH3 domains, PX/P/A/XXR, revealed a great number of putative CIN85 interactors, including all known proteins that were demonstrated to bind CIN85 and CD2AP/CMS proteins through their SH3 domains (not shown). A recurrent and self-consistent theme in functional studies on CIN85/CMS proteins suggests that they function as organizers interfacing different protein interaction sub-networks, such as the specialized protein adhesion machinery at the sites of cell-to-cell and cell-to-matrix contacts, the endocytic apparatus and the cytoskeleton (8). In addition, both a CIN85 mutant lacking the coiled-coil domain and a CIN85 SH3 domain triplet localized exclusively in the nucleus, suggesting the existence of associations with nuclear proteins that are mediated by CIN85 SH3 domains (9). Among the proteins that contained a minimal CIN85 recognition consensus PX/P/A/XXR, synaptopjanin 1, PAR2, ZO-2, and TAF170 were chosen as individual representatives of endocytosis, regulation of cytoskeleton dynamics, cell-to-cell adhesion, and basal transcription machinery, respectively, to test their putative interactions with CIN85 in biochemical experiments. Fig. 5A shows that all four chosen proteins were specifically precipitated from mouse brain lysates with CIN85 SH3 domains in GST pull-down experiments, suggesting that CIN85 can be found in complexes containing the indicated proteins in vivo. To verify a direct association with CIN85, synaptopjanin 1, PAR2, ZO-2, and TAF170 and their associated complexes were immunoprecipitated from mouse brain lysate and resolved by SDS-PAGE. The separated proteins were transferred then to a PVDF membrane and incubated with individual CIN85 SH3 domains conjugated to alkaline phosphatase. All three CIN85 SH3 domains bound synaptopjanin 1 on Far Western membranes, suggesting a direct association between synaptopjanin 1 and CIN85 SH3 domains (Fig. 5B). The PAR2 bound only the second CIN85 SH3 domain in Far Western assay, suggesting their direct association. The Far Western results for ZO-2 and TAF170 proteins were inconclusive. This could be explained by the poor performance of the
Fig. 2. Cross-reactivity of CIN85 SH3 domains. Eight peptide ligands selected by individual CIN85 SH3 domains from a random 16-mer peptide library were assayed in phage end point ELISA experiments for binding to their cognate CIN85 SH3 domain as well as to the other two SH3 domains of CIN85 as indicated. White bars correspond to a negative control. The y axis on the histograms indicates A values at 405 nm. The experiments were repeated three times in triplicate. Error bars correspond to the S.D. of the mean for each triplicate and indicate well-to-well variations of the ELISA signal.
respective antibodies in the immunoprecipitation step (data not shown) and a relatively low sensitivity of the chosen alkaline phosphatase detection method. Therefore, although ZO-2 and TAFII70 are present in complexes pulled down by CIN85 SH3 domains, direct interactions between these proteins and CIN85 remain to be demonstrated.

**DISCUSSION**

Taking advantage of a novel screening format, TAIS, the recognition properties of CIN85 SH3 domains were delineated. An analysis of the peptide ligands selected by the CIN85 SH3 domains allowed the characterization of a minimal recognition consensus SH3 PXXR abolished binding of the peptide ligands. Additional positions of the peptide ligands were sensitive to alanine substitution. These additional specificity determinants may be responsible for the unique ligand preferences displayed by individual domains (Fig. 2 and “Results”). Alanine substitution of the proline or arginine residues flanking the minimal CIN85 recognition consensus abolished binding of an SH3-1 ligand to its cognate domain as well, indicating the critical role of these residues for binding to all three CIN85 SH3 domains (not shown). The y axis indicates relative ELISA kinetics slopes expressed in arbitrary units. Mutagenesis experiments were repeated three times for triplicate samples. Error bars correspond to the S.D. of the mean for triplicate samples.

**Fig. 3.** Alanine-scanning mutagenesis of peptide ligands of SH3-2 and SH3-3 domains of CIN85. Alanine substitution of either proline or arginine flanking CIN85 SH3 recognition consensus PX/P/AXXR abolished binding of the peptide ligands to their cognate SH3 domains (see peptides 6 and 11 on both panels), confirming the recognition consensus of SETA SH3 domains defined by TAIS. Additional positions of the peptide ligands were sensitive to alanine substitution (positions 10 and 12 for the SH3-2 ligand and positions 13 and 14 for the SH3-3 ligand), indicating the existence of additional specificity determinants outside and/or within the PX/P/AXXR core. These additional specificity determinants may be responsible for the unique ligand preferences displayed by individual domains (Fig. 2 and “Results”). Alanine substitution of the proline or arginine residues flanking the minimal CIN85 recognition consensus abolished binding of an SH3-1 ligand to its cognate domain as well, indicating the critical role of these residues for binding to all three CIN85 SH3 domains (not shown). The y axis indicates relative ELISA kinetics slopes expressed in arbitrary units. Mutagenesis experiments were repeated three times for triplicate samples. Error bars correspond to the S.D. of the mean for triplicate samples.

**Fig. 4.** A point mutation in the PX/P/AXXR recognition core abolishes binding of CIN85 SH3 domains to a Cbl-b fragment. The 36-amino acid-long fragment of Cbl-b shown was previously reported to bind CIN85 SH3 domains. Exhaustive mutagenesis of all PXXR sites (canonical SH3 binding motifs) within this fragment, however, failed to abrogate the interaction (12). The CIN85 SH3 recognition consensus, PX/P/AXXR, defined by TAIS, predicted a unique CIN85 SH3 binding site on the Cbl-b fragment in question (underlined). A point mutation substituting arginine 911 of Cbl-b by alanine was sufficient to abolish binding of the 36-amino acid fragment of Cbl-b to all three SH3 domains of CIN85 in vitro, confirming the prediction. The y axis indicates A values at 405 nm. End point phage ELISA experiments were repeated three times in triplicates. Error bars correspond to the S.D. of the mean for triplicate samples.

**A. GST-pull-downs**

**B. Far-Western**

**Fig. 5.** Interaction of CIN85 SH3 domains with PAK2, ZO-2, TAFII70, and synaptotagmin 1. A. GST-pull-downs from mouse brain extracts were performed using SH3 domains of CIN85 immobilized on Sepharose beads as GST fusions. Protein complexes were resolved by SDS-PAGE, blotted to PVDF membranes, and probed with the indicated antibodies. GST alone was used as a negative control. B, synaptotagmin 1 (SY1) and PAK2 interact with CIN85 SH3 domains on a Far Western blot (WB). Protein complexes immunoprecipitated (IP) from mouse brain extract with anti-synaptotagmin 1 and anti-PAK2 antibodies were resolved by SDS-PAGE, blotted to a PVDF membrane, and probed with biotinylated SH3 domains conjugated to alkaline phosphatase as indicated above. The WW domain of the Yes-associated protein (YAP) conjugated to alkaline-phosphatase was used as a negative control (NC).
tagenesis of CIN85 SH3 peptide ligands and experiments addressing cross-reactivity between different domains suggested the existence of additional specificity determinants residing within and/or adjacent to the minimal CIN85 SH3 binding core (Figs. 2 and 3).

Using the newly defined CIN85 SH3 recognition consensus as a query in pattern searches allowed for the accurate mapping of putative CIN85 SH3 binding sites within the known CIN85 interactors such as c-Cbl, BLNK, Cbl-b, AIP1/Alix, SB1, and CD2 proteins. It is unlikely to be coincidental that the predicted unique binding site of the CIN85 SH3 domains within the CD2 cytoplasmic tail overlaps exactly with the binding site that was biochemically defined on CD2 for the first SH3 domain of CD2AP/CMS protein (6). Sharing overall domain organization, CIN85 and CD2AP/CMS proteins are 39% identical and 54% similar in amino acid sequence and hypothesized to form a novel adaptor protein family (8). The amino acid homology between CIN85 and CD2AP/CMS is concentrated within SH3 modules, suggesting similar recognition properties of their SH3 domains. As an example, the first SH3 domains of CIN85 and CD2AP display 68% identity and 85% similarity in amino acid sequence. For comparison, the first and the second domains of CIN85 share 64% identity and 82% similarity, whereas the average amino acid conservation within the SH3 domain family has been estimated to be 28% of identity.2 In addition, a striking functional similarity between CD2AP and CIN85 proteins was recently demonstrated (7).

Interestingly yet, the primary binding site of the first SH3 domain of CD2AP on the CD2 cytoplasmic tail was concluded to be “a Type II SH3 ligand (PPLPRPR)” in a previous report (6). This conclusion is in disharmony with the observation from another group that suggested PXXP-independent binding of the closely related CIN85 SH3 domains (12) and with the definition originally proposed by S. Schreiber and coworkers (31) for Class II SH3 ligands as PXXPXR. Although at present we cannot exclude the possibility that CD2AP SH3 domains indeed display recognition pattern(s) distinct from that of CIN85 SH3 domains, one explanation eliminating the disharmony would be the fact that the indicated binding site of CD2AP/CMS on the CD2 cytoplasmic tail does conform to the CIN85 SH3 minimal recognition consensus as defined in the present report: **PPLPRPR**.

It has been shown that SH3 domains bind peptide ligands in two opposite pseudo-symmetrical orientations. Depending on the orientation, two classes of SH3 ligands are distinguished, Class I, (R/K)XXPXXP, and Class II, PXXPXR(K/R) (23, 31). Some of the SH3 domains, such as Src SH3, bind their ligands in both orientations with similar affinities (32), whereas others prefer only one ligand orientation, as exemplified by Crk SH3 domain (27). CIN85 SH3 domains appear to bind their peptide ligands in one preferential orientation that resembles the Class II orientation (see Fig. 1). Dustin et al. (6) classified the interaction site of the first SH3 domain of CD2AP on the CD2 adhesion receptor, PPLPRPR, as a Type II SH3 ligand, without explicitly indicating whether the interaction in question was PXXP-dependent. This could easily lead to a potential confusion, because this interaction was later interpreted as PXXP-dependent (8), even though this fact had not been proven experimentally. To avoid similar confusion, we propose classification of CIN85 SH3 ligands as Class IIA, where A stands for atypical, i.e. PXXP motif-independent.

The length of the CIN85 SH3 recognition consensus core PX(P/A)XXR defined by TAILS is similar to the footprint size of previously reported binding sites of other SH3 domains such as PXLXXK for Crk SH3 PXXXPR for Src SH3 and PFPVPRP for phospholipase Cγ SH3 (27, 29). It is tempting to speculate that in the XP binding groove formalism (18, 22, 23), the second groove of CIN85 SH3 domains is distorted into a (P/A)X-accepting groove. Consistent with this hypothesis, the SH3 domain of PAK-interacting exchange factor, which is the closest relative of CIN85/CMS SH3 domains in amino acid sequence (53% identity and 75% similarity with CIN85 SH3-1), does not bind PXXP motifs but instead interacts with an atypical SH3 binding sequence in PAK1 and PAK2 kinases, PPFVAPRPEHTSKS (33, 34). It is worth noting that this atypical PAK-interacting exchange factor SH3 binding sequence of PAKs, which displays both cross-isofrom and cross-species conservation, is remarkably similar to the CIN85 SH3 ligands, PPPVAPRPEHTKS, thus paralleling the high amino acid conservation between the SH3 domains of PAK-interacting exchange factor and CIN85.

The affinities of the best peptide binders of CIN85 SH3 domains isolated in the present report were similar to the affinity of a characterized Src SH3 peptide ligand for its cognate domain in phage ELISA experiments and were estimated to be on the order of 10 μM (data not shown). To be informative, more accurate affinity measurements should await detailed biochemical and structural studies addressing the unusual recognition properties of CIN85 SH3 domains and the analysis of putative specificity determinants surrounding the characterized core consensus. In the absence of these studies, affinity measurements might not be of rich informative content. As an example, independent affinity measurements of the interaction between the same CD2-derived peptide and the first SH3 domain of CD2AP performed by the same method, surface plasmon resonance, resulted in widely discordant Kd values for the same interaction spanning almost 3 orders of magnitude, 0.15 μM (6) and 100 μM (35).

To further assess the predictive value of the newly defined CIN85 SH3 recognition consensus, four proteins containing CIN85 SH3 putative binding sites were tested for their potential interactions with CIN85 SH3 domains in GST-pull down and Far Western blotting experiments. Synaptojanin 1, PAK2, ZO-2, and TAF170 were chosen as representatives of the protein interaction sub-networks allegedly connected through the CIN85 adapter. The PKA family of serine/threonine kinases plays a critical regulatory role in cytoskeletal polarization at specialized sites of cell-to-cell, cell-to-matrix contacts and in the dynamics of local cytoskeletal remodeling that accompanies endo-, phago-, and macropinocytosis (36–38). ZO-2 belongs to the membrane-associated guanylate kinase (MAGUK) family of proteins (39). By providing a structural and functional scaffold through its multiple protein interaction modules, it is thought to orchestrate the organization of specialized protein complexes at the cytoplasmic face of tight junctions in epithelial and endothelial cells (40). More recently, the discovery of ZO-2 shuttling between cell-to-cell contacts and nucleus suggested the novel role of the protein as a signaling link between tight junctions and nuclear functions such as splicing and/or transcription (41, 42). Synaptojanin 1 is a lipid phosphatase with an essential role in endo-/exocytosis, cytoskeletal remodeling, and vesicular trafficking (28, 30).

The presence of PAK2, ZO-2, and synaptojanin 1 in protein complexes precipitated from mouse brain lysates with CIN85 SH3 domains (Fig. 5A) supports the emergent role of CIN85/CMS proteins as functional connectors that act at the interface between the endocytosis of activated receptors and cytoskeletal polarization and dynamics at cell-to-cell and cell-to-matrix contacts (8). It is reasonable to hypothesize that all the “perception” events at the cell periphery that are mediated through the

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2 Software package Pfam is available at www.sanger.ac.uk/Software/Pfam/browse/8.shtml.
activation of stimulus-specific receptors, such as growth factor receptors, integrins, adhesion, and activation receptors, are necessarily connected to the regulation of gene expression, allowing the cell to respond equally and dynamically to cues from its environment. In this regard the interaction of CIN85 SH3 domains and TAFII70 suggested by our experiments provides a putative link between the basal transcription machinery and cell periphery perception systems.

CIN85 SH3 domains display atypical recognition properties. The delineation of the minimal recognition consensus shared by all three CIN85 SH3 domains, PXPlAIXXX, clarifies seemingly contradictory results obtained in the previous studies addressing protein interactions mediated by SH3 domains of the CIN85/CMS protein family. It is likely that the SH3 domains of CIN85 constitute a specific sub-class of SH3 domains that bind atypical and related non-PXXP peptide sequences.

There are indications that SH3 domains of at least two other proteins, CD2AP/CMS and PAK-interacting exchange factor, recognize similar non-PXXP motifs. Even though all three CIN85 SH3 domains share the PXPlAIXXX recognition consensus and cross-react in our and other investigators’ hands, their specificities appear to be overlapping but not identical. Additional specificity determinants residing within or adjacent to the PXPlAIXXX recognition core must be characterized. The newly defined CIN85 SH3 recognition pattern provides a reasonable predictive power allowing a mapping of CIN85 binding sites within its known and novel putative interactors.

Synapoijin 1, PAK2, ZO-2, and TAFII70 were predicted to bind CIN85 SH3 domains in homology searches of protein databases with PXPlAIXXX motif as a query. All four proteins were selectively and quantitatively precipitated from mouse cerebral cortex preparation. We express our gratitude to Dr. S. Kajigaya (NHLBI/NIH) for comments and help in manuscript preparation. We thank Dr. V. Galvan for comments on manuscript.

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