Direct Binding of p130Cas to the Guanine Nucleotide Exchange Factor C3G*

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p130Cas (Cas; crk-associated substrate) belongs to a new family of docking molecules. It contains one Src homology (SH) 3 domain in its amino-terminal region followed by a region containing binding motifs for SH2 and SH3 domains. To gain further insight into Cas signaling we used the SH3 domain of Cas in a two-hybrid screen to search a human placenta library for binding partners. The screen confirmed a previous finding of its binding to the focal adhesion kinase (FAK) but also identified C3G, a guanine nucleotide exchange factor. We found direct interaction between Cas and C3G in vitro and in vivo. A series of analysis with C3G deletion mutants revealed a proline-rich Cas-binding site (Ala5-Pro1-Pro2-Lys3-Pro3-Leu4-Pro5) located NH2-terminal to the previously characterized Crk binding motifs in C3G. Mutagenesis studies showed that Pro1, Lys3, and Pro4 within the ligand-binding site are critical for high affinity interaction. These results, combined with sequence alignments of proline-rich binding elements from proteins known for Cas binding, define the consensus sequence APPXXPPX which is recognized by the CasSH3 domain. Cas shows structural characteristics of a docking molecule and may serve to bring C3G to specific compartments within the cell.

Cas1 was initially identified as a highly phosphorylated protein of 130-kDa in v-Src and v-Crk transformed rat 3Y1 fibroblasts (1). It belongs to a new family of structurally related proteins also including HEF1 (human enhancer of filamentation)Cas-L and Efs (embryonal Fyn-associated substrate)/Sin (2–4). Cas contains an NH2-terminal SH3 domain followed by a stretch of proline-rich sequences, a central substrate domain composed of a cluster of potential SH2-binding sites, and a COOH-terminal domain which contains consensus binding sites for the SH3 and SH2 domains of c-Src (5). Recently, it has been shown that the SH3 domain of Cas binds the tyrosine kinases FAK, RAFTK (related adhesion focal tyrosine kinase)/PYK2 and FRNK (FAK-related non-kinases) (6–8) and the two protein tyrosine phosphatases PTP1B and PTP-PEST (9, 10). Cas becomes tyrosine-phosphorylated in response to many different stimuli including B-cell receptor engagement (12), growth factors such as nerve growth factor (13), epidermal growth factor (14), and platelet-derived growth factor, neuropeptides, phorbol esters, and bioactive lipids (15), osmotic shock of 3T3L1 adipocytes (16) as well as integrin mediated cell-cell and cell-matrix adhesion (17–19). Recently, it has been demonstrated that Cas can act as a downstream mediator of FAK-promoted cell migration (20).

Elevated tyrosine phosphorylation appears to result in increased association of Cas with SH2 domain-containing signaling molecules to trigger downstream signaling pathways. The SH2 domains of phosphatidylinositol 3-kinase-p85, Grb2, Nck, phospholipase C-γ, and Crk have been found to bind phosphorylated Cas (18, 20). Two guanine nucleotide exchange factors, Sos and C3G, which bind to the NH2-terminal SH3 domain of Crk (CrkSH3(N)), have also been detected in Cas immunoprecipitation complexes (18, 21). The physiological relevance of these associations is not clearly understood, but may be involved in the activation of different downstream pathways. Sos has been shown to be an activator of the Ras/MAP kinase cascade (reviewed in Ref. 22) and C3G was shown to transmit signals to JNK1 in fibroblasts (23). Recent studies indicate that the substrate specificity of C3G is directed against the small G-proteins Rap1 and R-Ras (24, 25). In certain systems, Rap1 can act as Ras antagonist (reviewed in Refs. 26 and 27). However, it has also been shown that Rap1 can mediate sustained ERK activation in rat phaeochromocytoma cells induced by nerve growth factor (28).

In normal fibroblasts, Cas is evenly distributed in the cytoplasm and a small fraction localizes to focal adhesions (11) or in cellular fractions enriched with membranes of the endoplasmatic reticulum (10). Although the exact role for Cas has not been identified it seems to be an important molecule in multiple signaling pathways. Cas becomes tyrosine-phosphorylated in response to many different stimuli including B-cell receptor engagement (12), growth factors such as nerve growth factor (13), epidermal growth factor (14), and platelet-derived growth factor, neuropeptides, phorbol esters, and bioactive lipids (15), osmotic shock of 3T3L1 adipocytes (16) as well as integrin mediated cell-cell and cell-matrix adhesion (17–19). Recently, it has been demonstrated that Cas can act as a downstream mediator of FAK-promoted cell migration (20).

In this study we found that Cas associates directly, in vivo and in vitro, with C3G. This association was mediated through the interaction of the SH3 domain of Cas with a proline-rich motif in C3G and was independent of tyrosine phosphorylation. Mapping of this motif revealed that it is distinct from the well studied proline-rich motifs of C3G that bind to the CrkSH3(N) domain. The specificity of binding between the CasSH3 domain and C3G was analyzed by mutagenesis of the component residues and a consensus motif for the SH3 domain of Cas was subsequently generated.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—** Saccharomyces cerevisiae strains were used, HF7c (29) and SFY526 (30). Human 293T kidney epithelial (293T) cells and mouse NIH3T3 fibroblast cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum or calf serum, respectively. The human placenta MATCHMAKER cDNA library (HL400SAB) and the MATCHMAKER Two-Hybrid system (PT1265–1) were obtained from CLONTECH. Polyclonal antiserum to CrkII (C-18) and C3G (C-19) and the monoclonal antibody to glutathione S-transferase (GST) (B-14) were purchased from Santa Cruz Biotechnology.
Mouse monoclonal antibodies against phosphotyrosine (RC20), FAK, and Cas were purchased from Transduction Laboratories.

**Plasmid Construction**—The cDNA fragment encoding the rat CasSH3 domain (amino acid residues 99 to 161) was generated by polymerase chain reaction using customized primers. BamHI and EcoRI sites were added to the 5'- and 3'-ends and cloned into pGBT9 (CLONTECH). BamHI and EcoRI sites were added to the 5'- and 3'-ends and cloned into pGEX-2TK (Pharmacia) and pGEX-4T-3 (Pharmacia). The cDNA of CasSH3 in pGEX-4T-3 was cut with BamHI and NotI and subcloned in-frame into the mammalian expression vector pEG (31) that expresses proteins fused with GST. Human full-length C3G (hC3G) was previously cloned into the mammalian expression vector pcAGGS (32). GST-fusion protein constructs for v-CrkSH3 domain wild type, mutant W405L, and c-CrkSH3(C) have been described previously (33, 34). GST-fusion peptides derived from the C3G sequence (amino acid residues 216 to 226 and 265 to 276) were constructed with double-stranded oligonucleotides that possessed a 5' BamHI and a 3' EcoRI overhang and cloned in-frame into pGEX-2TK vector. For mapping the Cas-binding site in C3G, deletion constructs of C3G were generated by polymerase chain reaction with customized primers and were cloned into pACT2 (CLONTECH). All polymerase chain reaction products and all junctions were verified by DNA sequencing.

**Two-hybrid Library Screening**—The HF7c yeast strain, which carries HIS3 and lacZ genes (lacZ reporter) under the control of GAL4 17- and UASGal4 plasmids transformed with pGBT9-CasSH3, was expressed first with pGBT9-CasSH3 (pGBT9 carries the TRP1 gene as selectable marker) and subsequently with a human placenta cDNA library cloned in pGAD10. The LEU2 gene as selectable marker). Competent yeast cells were obtained using the YEASTMAKER yeast transformation system (CLONTECH), and cells were transformed with the indicated plasmids according to the manufacturer’s instructions. Double transformants were grown on yeast plates lacking leucine, tryptophan, and histidine. After 6 days, yeast colonies were individually assayed for β-galactosidase activity by filter assay. The filter assay was carried out as described (35). Briefly, transformants growing on a filter were lysed by freeze thawing in liquid nitrogen, and each filter was incubated in Z-buffer (16.1 g/liter Na2HPO4•7H2O, 5.5 g/liter NaH2PO4•H2O, 0.75 g/liter KCl, 0.246 g/liter MgSO4•7H2O, pH 7.0) containing 50 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide) at 30 °C (up to 24 h for screening experiments). Positive clones were selected, and target plasmids containing library cDNA inserts were isolated according to the CLONTECH manual and transformed into HB101 bacteria for amplification. Target cDNAs were subjected to DNA sequence analysis.

**Expression of GST Fusion Peptides and in Vitro Binding Assay**—Expression and affinity purification of GST fusion proteins were carried out as described elsewhere (36). Briefly, bacteria harboring the above described expression plasmids were cultured in medium containing 40 mg/ml ampicillin, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.1 mM, and bacteria were cultured for an additional 4 h. Cells were harvested and resuspended in HMK buffer (150 mM NaCl, 10 mM sodium phosphate, 10 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20) at 4 °C. The reaction was stopped by adding 1 ml of HMK stop buffer (1 mg/ml bovine serum albumin, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium phosphate, pH 8.0) and the pellet washed twice with cold 1× phosphate-buffered saline containing 1% Triton X-100, twice with cold phosphate-buffered saline, and once with 10 mM Tris-HCl, pH 8.0. Radiolabeled GST fusion protein was eluted at room temperature with 1 ml of elution buffer. After incubation in blocking solution, blots were incubated with 0.5 ml of [32P]radiolabeled and purified GST-CasSH3 fusion protein or GST alone. 100 μg of GST fusion protein bound to glutathione beads (100 μl) were incubated with 1 volume of HMK buffer (100 mM sodium chloride, 12 mM magnesium chloride, 20 mM Tris-HCl, pH 7.5) containing 100 units of cAMP-dependent heart muscle protein kinase (Sigma), 1 μM dithiothreitol, and 50 μCi of [γ-32P]ATP for 30 min at 4 °C. The reaction was stopped by adding 1 ml of HMK stop buffer (1 mg/ml bovine serum albumin, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium phosphate, pH 8.0) and the pellet washed twice with cold 1× phosphate-buffered saline containing 1% Triton X-100, twice with cold phosphate-buffered saline, and once with 10 mM Tris-HCl, pH 8.0. Radiolabeled GST fusion protein was eluted at room temperature with 1 ml of elution buffer. After incubation in blocking solution, blots were incubated with 0.5 ml of [32P]radiolabeled probe overnight in fresh blocking solution, washed extensively with TBS/Tween, and then exposed to autoradiography films (Kodak).

**RESULTS**

**Yeast Two-hybrid Screen Identifies the Guanine Nucleotide Exchange Factor C3G as a CasSH3 Interacting Protein**—CasSH3 domain (Fig. 1) was expressed as a fusion protein with the GAL4 DNA-binding domain in the reporter yeast strain HF7c and used to screen for interactions with a library of human placenta cDNA-encoded polypeptides fused to the transcription activation domain of GAL4. Out of 2 × 10^6 independent transformants, 36 cDNAs supported Cas-dependent transcription activation of two independent reporter genes (HIS3 and LacZ). Analysis of these clones by restriction enzyme digests and sequencing showed that four clones encoded the COOH-terminal noncatalytic part (amino acid residues 752–1025) of FAK (Fig. 2A). This polypeptide contained only one of the proline-rich sequences described previously as binding sites for the SH3 domain of Cas (6, 7).

Six additional Cas interacting cDNAs, representing two distinct overlapping clones C1 and C2, encoded sequences identical to the NH2-terminus of the human guanine nucleotide exchange factor C3G (Fig. 2A). The open reading frame of clone C1 is identical to amino acid residues 11 to 446 of C3G. Clone C2 contained a 124-nucleotide insertion at amino acid residue 648 which resulted in a truncated protein due to an in-frame stop codon. The central region of C3G contains four similar proline-rich XPXPLXXK sequences (Fig. 2A) which bind with high specificity the CrkSH3(N) domain (37). Since clone C2 encompassed all four proline-rich Crk-binding sites, two cDNA clones C1 and C2 were sequenced to see whether the CrkSH3(N) domain was capable of interacting with both C1 and C2 clones similarly to the CasSH3 domain in a yeast two-hybrid assay. Interestingly, of two clones only one (C2) supported CrkSH3(N)-dependent transcription activation (Fig. 2B, lane 2). These results suggested that either the binding sites for the CasSH3 and CrkSH3(N) domains in C3G are...
different, or that Cas interacts through the same proline-rich sequence of C3G as Crk but with higher affinity.

**CasSH3 Domain Binds C3G Directly In Vitro and In Vivo**—To analyze the interaction between the CasSH3 domain and C3G in mammalian cells, we examined their *in vitro* binding. We compared the binding of the CasSH3 and individual Crk SH3 domains to C3G. As shown by Knudsen et al. (37) the interaction of Crk with C3G is mediated by the NH2-terminal SH3 domain of Crk adapter proteins, which we used as positive control, while no binding has been observed by the COOH-terminal SH3 domain of CrkII (c-CrkSH3(C)), which we introduced as negative control. GST fusion proteins containing the different SH3 domains were captured on glutathione beads, and equivalent amounts were incubated with cell extracts from NIH3T3 fibroblasts. As shown in Fig. 3A, binding of endogenous C3G to the CasSH3 domain was observed to the same extent or even better when compared with the v-CrkSH3 domain. No binding was detectable with c-CrkSH3(C) and GST alone. Furthermore, no binding was observed with v-CrkSH3(W405L), containing a point mutation in the SH3 domain (34).

To determine whether the interaction of the SH3 domain of Cas with C3G is direct or is mediated by other proteins, a Far Western blot analysis was carried out. Bacterially expressed GST-CasSH3 domain fusion protein was purified and labeled with 32P on a specific cAMP-dependent protein kinase site located at the COOH-terminal end of GST. The labeled fusion protein was used to probe FAK, C3G, and Crk immunoprecipitates of NIH3T3 cell lysates, which were resolved on SDS-PAGE and transferred to Immobilon-P membranes. As shown in Fig. 3B (*upper panel*), the labeled CasSH3 domain bound predominantly to a single protein with a molecular mass of about 120 kDa (*first lane*) which corresponds to FAK and to a single protein with a molecular mass of 145 kDa (*third lane*) which corresponds to C3G, as confirmed by probing with specific antibodies. The direct interaction of the CasSH3 domain and FAK was a confirmation of a previous finding by Harte et al. (7) and used as positive control in our experiment. Weak binding of labeled CasSH3 domain to C3G but not to Crk could also be detected in Crk immunoprecipitates (Fig. 3B, *second lane*). C3G binds to the family of Crk adapter proteins and therefore it can be found in Crk immunoprecipitates. However, the amount of endogenous C3G coimmunoprecipitated by Crk was low. No binding of 32P-labeled GST was observed (data not shown). These results show that the SH3 domain of Cas is able to interact directly with C3G.

We were also able to detect the *in vivo* interaction of endogenous Cas and C3G in C3G immunoprecipitates of total cell lysates of human promyelocytic HL60 cells, NIH3T3 mouse fibroblasts, and primary chicken embryo fibroblasts (data not shown). However, by applying that method we could not conclude that this *in vivo* interaction is direct. Crk proteins could act as bridging molecules between Cas and C3G, since they are known to bind to both. Thus we chose to transiently overexpress only the SH3 domain of Cas together with C3G in human 293T kidney epithelial cells. When cotransfected the GST CasSH3 domain and C3G, we detected C3G in anti-GST im
munneprecipitates by anti-C3G Western blotting (Fig. 4, upper panel). Conversely, we detected CasSH3 in anti-C3G immunoprecipitates by anti-GST Western blotting (Fig. 4, second panel). These data suggest that the CasSH3 domain and C3G associate directly in vivo.

Identification of the Proline-rich Motif Binding to the SH3 Domain of Cas—The site of interaction of C3G with Cas was investigated by assessing the ability of various C3G constructs to support transcription activation in a Cas dependent manner using the yeast two-hybrid assay system (Fig. 5A). All C3G deletion constructs were cloned into the yeast expression vector pACT2 and expressed as HA-tagged GAL4 activation domain fusion peptides. That allowed us to assay for protein expression of the examined constructs (data not shown). First, we found that the 3'-end of clone C2, construct DL1, which includes three Crk-binding sites, is unable to activate transcription in CasSH3 background. These data immediately suggested that the sequence motif in C3G recognized by the SH3 domain of Cas is different from the Crk-binding sites. Two clones, C1 and C2, overlapped in a region of 330 amino acids, which includes one cluster of PXXP motifs located near the 3'-end of that region (amino acid residues 439 to 446). However, since this cluster of PXXP motifs is also present in construct DL1, it cannot be a binding site for the CasSH3 domain. Two additional clusters of PXXP motifs located in the middle of this overlapping region, amino acid residues 215 to 231 (NH2-terminal cluster) and 267 to 273 (COOH-terminal cluster), could also serve as potential CasSH3 domain-binding sites. All C3G constructs (DL2, DL3, DL4, and DL8) containing both clusters of those proline-rich sequences supported CasSH3-dependent transcription activation. Deletion of the NH2-terminal cluster (residues 215 to 231) of PXXP motifs in construct DL7 did not abolish the interaction between the SH3 domain of Cas and C3G. In contrast, the C3G construct DL5 which lacks the COOH-terminal cluster (residues 267 to 273) of PXXP motifs failed to interact with the CasSH3 domain in our two-hybrid assay.

The shortest C3G constructs which were still able to bind the SH3 domain of Cas (DL4 and DL7) contained this COOH-terminal PXXP motif, APPKPPLP (Fig. 5B). This sequence is different from the Crk binding motif, and indeed DL4 did not interact with CrkSH3 in the two-hybrid assay. The binding capacity of this sequence was further tested by the ability of the peptide GST-V265APPKPPLPGIR to bind endogenous Cas protein (Fig. 6A). As negative controls, GST fusion peptides carrying proline to alanine or proline to glutamine mutations of the central two prolines (P270A/P271A, P270E/P271E) disrupting all possible PXXP motifs, the core consensus motif for SH3 domain binding, were no longer able to interact with Cas. We also confirmed that endogenous Crk did not bind to the CasSH3 domain.
DL5, which contains the NH$_2$-terminal cluster (residues 215 to 231) of PXXP motifs did not support the transcription activation with CasSH3 in the two-hybrid assay. To confirm this observation, we also synthesized GST-T$^{236}$SPVPKPPSAP, which is similar to the above described motif, and examined its capacity to bind Cas protein. We found that this peptide does not bind either Cas or CrkSH3 domains (data not shown).

Fine Mapping of the CasSH3 Binding Motif in C3G—To analyze the importance of individual amino acid residues within the CasSH3 domain binding motif, a mutagenesis study of the V$^{266}$APPKPPPLPGIR peptide was performed. GST fusion peptides carrying individual point mutations of all proline residues to alanine and lysine to alanine (P267A, P268A, K269A, P270A, P271A, and P273A) or a double mutation (P270A/P271A) were constructed. As shown in Fig. 7, among the proline to alanine mutations only the mutation of residue 267 and P271A were the widely expressed nonreceptor tyrosine kinase FAK to downstream signaling pathways other than extracellularly regulated kinases as proposed by Schlaepfer et al. (45). The consequence of Cas binding to C3G could be the activation of the JNK pathway by integrin stimulation. Recently, it has been demonstrated that in 293T cells C3G activates JNK1 by a Ras-independent mechanism (46). We believe that the binding of Cas to its target molecules is a dynamic process which may be regulated by activation of integrins and other upstream pathways. The existence of a linear signal transduction pathway in the action of platelet-derived growth factor involving phosphatidylinositol 3-kinase and Rac that leads to the tyrosine phosphorylation of Cas have been demonstrated, and a similar pathway has been proposed for the action by low concentrations of epidermal growth factor (14, 15). However, further work is needed to clarify the physiological role of the CasC3G complex formation.

C3G contains in its central region four proline-rich binding motifs that can bind the small adapter protein Crk (37). In this report we clearly demonstrated that the Cas-C3G association is not mediated by those Crk-binding sites on C3G. Nonetheless, the SH3 domain of Cas binds to the NH$_2$-terminal region of C3G which harbors one proline-rich Cas-binding site distinct from the Crk-binding sites on C3G. The presence of several

**DISCUSSION**

Cas belongs to a new family of cytoplasmic docking molecules. It becomes tyrosine phosphorylated after cell adhesion and phosphorylated Cas can be detected in focal adhesion complexes (3, 7, 38). The structural organization of focal adhesions is characterized by a complex network of protein-protein interactions which link the actin cytoskeleton to extracellular matrix proteins (39).

To further identify molecules of the signaling transduction pathway involving Cas, we employed the yeast two-hybrid screen using the SH3 domain of rat Cas as bait and searched for interacting molecules encoded by a human placenta cDNA library. Among the interacting peptides identified by the screen were the widely expressed nonreceptor tyrosine kinase FAK that is critical in integrin-mediated signal transduction pathways (6, 40–42). FAK has already been described to associate with Cas in a two-hybrid screen when FAK residues 1–748 were used as bait (6), and its isolation in our two-hybrid screen demonstrated that the CasSH3 domain used as bait was folded properly and functional.

We also isolated C3G, a 145-kDa protein with guanine nucleotide exchange activity for Rap1 and R-Ras (24, 25, 43). The results reported herein show that besides the Cas-FAK interaction Cas can also form a stable complex with C3G in vitro and in vivo. The potential cellular role of Cas has still not been clearly resolved to date, with at least one report suggesting that Cas is involved in FAK-mediated cell movement (20). Interestingly, in 3T3 cells containing constitutively active c-Src and in COS-7 cells stimulated with fibronectin, deletion of the Src-binding domain of Cas resulted in total loss in the recruitment of Cas to focal adhesions (11). In contrast, in unstimulated cells the localization of Cas to focal adhesions was not affected by the deletion of the Src-binding domain. These data suggest that after integrin activation Cas and FAK form a ternary complex with activated Src, where Src acts as an adapter molecule that links Cas and FAK. Subsequently, the SH3 domain of Cas is released of its initial binding to FAK or FAK-like molecules. A truncated Src protein encompassing only the SH3 and SH2 domain of c-Src, expressed in Src cells, bound to both FAK and Cas and promoted their association in vitro (44). In that scenario, C3G could bind to the SH3 domain of Cas and link FAK to downstream signaling pathways other than extracellularly regulated kinases as proposed by Schlaepfer et al. (45). The consequence of Cas binding to C3G could be the activation of the JNK pathway by integrin stimulation. Recently, it has been demonstrated that in 293T cells C3G activates JNK1 by a Ras-independent mechanism (46). We believe that the binding of Cas to its target molecules is a dynamic process which may be regulated by activation of integrins and other upstream pathways. The existence of a linear signal transduction pathway in the action of platelet-derived growth factor involving phosphatidylinositol 3-kinase and Rac that leads to the tyrosine phosphorylation of Cas have been demonstrated, and a similar pathway has been proposed for the action by low concentrations of epidermal growth factor (14, 15). However, further work is needed to clarify the physiological role of the CasC3G complex formation.

**FIG. 7. Mutational analysis of the Cas binding sequence in C3G.** Sequences shown here were used as GST-C3G fusion peptides to precipitate endogenous Cas. NIH3T3 cell lysates (1 mg of total protein) were incubated with 10 μg of GST fusion peptides bound to glutathione S-agarose beads as indicated above, and the blots were probed with anti-Cas antiserum. Arrowheads point to the position where a single mutation to alanine almost entirely abolished binding.

**Fig. 6. Cas binds to proline-rich sequences upstream of Crk-binding sites in C3G.** Specific binding of endogenous Cas and c-Crk to GST-C3G fusion peptides. 1 mg of total protein of NIH3T3 cells were incubated with 10 μg of wild-type GST-C3G fusion peptide (amino acid residues 265–276, lanes 2 and 6), the double mutant GST-C3G fusion peptide M5 (P270/271A, lanes 3 and 7, and P270/271E, lanes 4 and 8), the wild-type GST-C3G fusion peptide (amino acid residues 282–291, lane 9), and the point mutant K289L GST-C3G fusion peptide (amino acid residues 282–291, lane 10) bound to glutathione S-agarose. The left panel was first probed with anti-Cas antiserum, stripped with stripping buffer (see “Experimental Procedures”), and re-probed with anti-Crk antiserum (middle panel). The right panel was probed with anti-Crk antiserum.
protein binding motifs in C3G raises the possibility that two or more motifs may function in a cooperative fashion. It is possible that Crk brings C3G near the CasSH3 domain after binding via its SH2 domain to tyrosine-phosphorylated Cas and a cooperative binding stabilizes it. Okada and Pessin (47) have demonstrated the dissociation of the C3G-Crk complex in Chinese hamster ovary cells following epidermal growth factor and insulin stimulation. Taken together, these findings suggest that Crk may initiate the binding of C3G to Cas before Crk gets released. However, in this report we clearly demonstrated that Cas can bind C3G directly.

SH3 domains bind proline-rich motifs with the core consensus sequence PXXP (48). Amino acid residues immediately adjacent to the proline residues seem to be important for specificity of different SH3 domains (49, 50). Cas has already been shown to interact via its SH3 domain with FAK, and it has been proposed that this interaction is mediated by the ligand consensus sequence X{P}X{P}X{P}X{P}X{P} for left-handed proline polypeptide II helix (6, 7), which requires a positively charged amino acid residue at the COOH terminus of the peptide ligand (51). On the other hand, SH3 domain ligands, including AFAP-110, CDC42 GAP, and Shc, missing at least one critical consensus residue have been recently reported (50). PTP1B and PTP-CDC42 GAP, and Shc, missing at least one critical consensus residue at the COOH terminus of the peptide ligand (51). On the other hand, SH3 domain ligands, including AFAP-110, CDC42 GAP, and Shc, missing at least one critical consensus residue have been recently reported (50). PTP1B and PTP-PEST (10, 52) have proline-rich sequences, PPRPKP and PPKPPR, respectively, similar to that APPKPSR motif in FAK. 

**Table I.** Alignments of sequences tested for binding the CasSH3 domain

| Protein | Motif | P_2 | P_1 | P_0 | P_1 | P_2 | P_3 | P_4 | P_5 | P_6 | Binding |
|---------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------|
| PTP-PEST | 1 | S | P | P | K | P | P | R | Yes |
| PTP1B  | 1 | P | P | P | P | R | P | R | Yes |
| FAK     | 1 | E | A | P | P | K | P | P | S | Yes |
| C3G     | 1 | T | S | V | P | K | P | P | S | No  |
| Consensus | X | X | P | p | + | P | p | X |

* Nomenclature for binding position is from Yu et al. (22).

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