The widely expressed cellular Crk protein has the domain structure SH2-SH3-SH3. We have previously demonstrated that the more N-terminal SH3 domain of Crk (CrkSH3(N)) specifically binds several cytoplasmic proteins. A cDNA encoding one of these proteins was isolated and found to have two different splice forms. The sequence is virtually identical to CSG, a guanine-nucleotide exchange factor. The center region of the 145-155-kDa protein contains four similar proline-rich sequences which are capable of binding individually to the SH3(N) domains of c-Crk and v-Crk. Comparison of these sequences in CSG to proline-rich sequences in other Crk-binding proteins suggests that positively charged amino acids following the prolines play an important role in the binding to the CrkSH3(N) domain. The endogenous C3G could be coprecipitated with Crk from cell lysates of cells expressing high levels of c-Crk or v-Crk. Comparison of these sequences in CSG to proline-rich sequences in other Crk-binding proteins suggests that positively charged amino acids following the prolines play an important role in the binding to the CrkSH3(N) domain. The unique binding specificity supports the idea that C3G plays an important role in Crk signaling pathways.

Src homology 2 and 3 (SH2 and SH3) domains are globular protein modules present in a large variety of functionally distinct proteins (1-3). They mediate binding events that control the activity and localization of many proteins involved in the transmission of signals from the cell surface to the nucleus. SH2 domains bind to phosphorylated tyrosine residues (4-6). The specificity of these phosphorylation-dependent interactions is determined by sequences adjacent to the phosphorylated tyrosines and has been extensively analyzed (7-9, 10 and references therein). In contrast to the abundant data on SH2 domain specificity, the binding specificity of SH3 domains is less well understood.

The presence of SH3 domains in proteins of lower eukaryotes such as Saccharomyces cerevisiae is indicative of the functional importance of SH3 domains throughout evolution. Despite significant sequence diversity, structural comparisons of many SH3 domains show similar folds (11). The first SH3 binding protein was isolated through its ability to bind to the Ab1SH3 domain, and the sequence responsible for this interaction was localized to a 10-amino acid proline-rich fragment (12, 13). It has been suggested that proline-rich SH3 binding motifs have a structure similar to that of a polyproline II helix (14). In such a helix each turn consists of three amino acids. Amino acids which are three positions apart are therefore oriented coplanar in space. Ultrastructural analysis of the interaction of a high affinity, proline-rich binding peptide with the phosphatidylinositol 3-kinase-pS5-a-SH3 domain demonstrated that two coplanar proline residues form contacts with two grooves on the surface of the SH3 domain (15). These grooves contain highly conserved aromatic amino acids and are spaced approximately at a distance of one turn of a polyproline II helix.

The Crk proteins belong to a family of proteins that consist almost entirely of SH2 and SH3 domains, with little intervening sequence. This family presently includes v-Crk (16), two forms of c-Crk proteins, c-Crk-I and c-Crk-II (17, 18), CRKL (19), Grb2/ASH (20-22) and its homologs Sem5 (23) and Drk (24, 25), Grb3-3 (26), and Nck (27). Expression of v-Crk or elevated expression of c-Crk-I leads to cell transformation and increased cellular phosphotyrosine levels (4, 16, 18, 28, 29). The biological role of c-Crk proteins is currently unknown. Since these proteins lack apparent catalytic domains, their function probably lies in their ability to bind specific proteins via their SH2 and SH3 domains. Proteins that interact with the CrkSH2 domain via phosphorylated tyrosine residues have been first identified in cells transformed by v-Crk or v-Src (4, 29, 30). The CrkSH2 domain was shown to preferentially bind phosphotyrosyl peptides that contain a Tyr(P)-X-X-Pro motif (7, 30). Such a high affinity binding motif is generated upon phosphorylation of c-Crk-II by c-Abl in the spacer region between the two CrkSH3 domains. Binding of the CrkSH2 to this phosphotyrosine residue may regulate c-Crk functions (31).

The CrkSH3(N) domain has previously been shown to interact with several proteins, including Sos, Ab1, Arg, and unidentified proteins between 145 and 155 kDa as well as an abundant protein of 185 kDa (31-33). We are currently identifying these SH3-binding proteins to determine their functional properties and to better understand the binding specificity of the Crk SH3 domains. In this study we report the cloning of one of these proteins, C3G, that was also identified recently by Tanaka et al. (34). While other CrkSH3- binding proteins like Sos5 and Ab1 bind to multiple SH3 domains (12, 13, 35), we...
found that C3G is very specific for Crk. Analysis of the CrkSh3(N) binding sequences within C3G identified proline-rich sequences that bind to the CrkSh3(N) and no other SH3 domain tested.

**MATERIALS AND METHODS**

**Expression of SH3-containing GST-fusion Proteins**—GST-GAPSH3, GST-CrkSH3, GST-AbiSH3, GST-PLCγSH3, GST-SpectrinSH3, GST-Nck, GST-StrSH3, and all GST-Crk constructs have been described (31, 38). The Grb2 N- and C-terminal SH3 domains were kindly provided by A. M. Pendergast (37). The phophatidylinositol 5-kinase p58α SH3 domain was a gift of L. Cantley (38). Unlabeled and 35S-labeled GST-fusion proteins were prepared as described (32).

**Library Screening for C3G Binding Proteins**—2 × 106 plaques of a HeLa expression library (Clontech) were screened with 32P-labeled GST-Crb SH3 SH3 for the first screen. Four positive plaques were identified. Two of them gave a strong signal and the corresponding plaques were plaque purified in subsequent screens using unlabeled GST-CrkSH3 SH3 as a probe. Bound GST-CrkSH3 SH3 was detected with an affinity-purified GST antisera and [32P]labeled Protein A (Amersham Corp.). The first screen with the 32P-labeled probe gave a very low background and the required result is the appearance of two isolated phages containing inserts of 1.2 kb with an identical restriction pattern. The inserts were excised from the phage with EcoRI, subcloned into pBluescript II SK (−/−) (Stratagene) and pGEX-1N (AMRAD) or pGEX-3X (Pharmacia) and their identity confirmed by sequencing. Two of these clones was used and one probe to obtain a 5.5-kb clone from a pC815-E1224 human embryonic fibroblast library (a gift of Toru Miki and Stuart Aaronson) (39) which encoded a 5′-truncated C3G cDNA that extended to the poly(A) tail. The original C3G clone of 1.2 kb was also used to probe a blot of HeLa poly(A) selected RNA. The cloning region of C3G was cloned by polymerase chain reaction. The clones were sequenced using a commercial sequencing kit (ABI).

**C3G GST-fusion Constructs**—pGEX constructs of the C3G proline-rich sequences were constructed by subcloning into pGEX vectors. The correct reading frame was confirmed by sequencing through the junctions. CB-2, CB-3, and CB-4 were generated from polymerase chain reaction products and sequenced entirely. Proteins generated from these constructs were expressed and purified essentially as previously described (32). The first screen with the 35S-labeled probe gave a very low background and the required result is the appearance of two isolated phages containing inserts of 1.2 kb with an identical restriction pattern. The inserts were excised from the phage with EcoRI, subcloned into pBluescript II SK (−/−) (Stratagene) and pGEX-1N (AMRAD) or pGEX-3X (Pharmacia) and their identity confirmed by sequencing. Two of these clones was used and one probe to obtain a 5.5-kb clone from a pC815-E1224 human embryonic fibroblast library (a gift of Toru Miki and Stuart Aaronson) (39) which encoded a 5′-truncated C3G cDNA that extended to the poly(A) tail. The original C3G clone of 1.2 kb was also used to probe a blot of HeLa poly(A) selected RNA. The cloning region of C3G was cloned by polymerase chain reaction. The clones were sequenced using a commercial sequencing kit (ABI).

**Antisera**—The C3G antisera (RF 45) was produced in rabbits immunized with GST-C3G-C2,4,4 amino acids 410-652, see Fig. 4). Antibody binding to C3G was confirmed by Western blotting and showed a doublet of 145/155 kDa. Gag (3C2) and Crk antisera have previously been described (41, 42).

**SH3 Blot Assay**—1 µg of GST or equimolar amount of SH3-fusion protein (GST-C3G, GST-CrkSH3, GST-AbiSH3, GST-PLCγSH3, GST-SpectrinSH3, GST-Nck, GST-StrSH3, and all GST-Crk constructs) was subjected to SDS-PAGE, electroblotted, and incubated with 2 µg/ml (70,000 dpm/ml) 32P-labeled GST-C3G CB region as described elsewhere (32). Filters were exposed overnight.

**Precipitation of C3G and Crk Proteins from Cell Lysates**—Crk Sh3 (rat fibroblasts expressing v-Crk (43)), c-CrWCEF (chick embryo fibroblasts overexpressing c-Crk (44)) and HeLa cells were harvested in RIPA (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM Na2EDTA) with protease inhibitors as described elsewhere (32). Particulate material was removed by centrifugation for 30 min at 10,000 g. Cytosolic HeLa proteins (S100) for precipitation of C3G were obtained as described elsewhere (32). Protein complexes were precipitated with GST-Sepharose beads (Pharmacia Biotech Inc.). For precipitation with the C3G antibody, 1 mg of total cell lysate of Crk SH3(N) or c-Crk SH3(N) precipitated with 10 µl of anti-C3G. Protein complexes were precipitated with protein G-Sepharose beads (Pharmacia Biotech Inc.). In all precipitation experiments, beads were washed three times with RIPA. Proteins were then separated by SDS-PAGE, electrophelotted and probed with the antibodies described in the figure legends.

**ELISA**—GST-CB1 to 4 proteins were biotinylated with sulfoNsacimidyl 6-biotinamidohexanolacetate (Pierce) according to the manufacturer's instructions. ELISA plates (Nunc) were coated with 100 ng/well; GST-Grb2, GST-CrkSH3 SH3, or g in 0.1 M NaHCO3, pH 9.6, overnight at room temperature (45). Wells were then blocked with 2% bovine serum albumin. After washing three times with blocking buffer (200 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween-20, 1 mM EDTA, 0.2% horse serum albumin, and 0.1% ovalbumin), biotinylated GST-CB fusion proteins were added at the concentrations indicated in Fig. 6. To measure bound GST-CB fusion proteins, wells were incubated with horseradish peroxidase-coupled streptavidin (Life Technologies, Inc.) A chromogenic substrate, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS, Pierce) was then added and the OD was measured at 405 nm after 5 and 15 min and the 5 min OD value subtracted from the 15 min OD value to yield ΔODmin. In each experiment, the ΔODmin at saturation of SH3 domain binding sites was considered 100%. ΔODmin values for all other points within the same experiment were expressed as a percentage of this 100% value. Binding to gat alone was subtracted at each data point.

**RESULTS**

**Cloning of a Protein that Binds to the CrkSh3 Domains**—The N-terminal CrkSh3 domain (CrkSh3(N)) binds specifically to a limited number of proteins from HeLa cell lysates (32). A HeLa expression library was screened to clone proteins encoding CrkSh3 binding proteins. A phage containing a 1.2-kb insert with an open reading frame was isolated. In a filter binding assay, the protein expressed from the phage showed strong binding to gst-CrkSh3 SH3, weak binding to gst-Grb2 and no binding to gat-Nck or gat alone. The sequence of the 1.2-kb phage insert is almost identical to the middle region (amino acids 280-653) of C3G, a CrkSH3-binding protein that was recently published by Tanaka et al. (34). However, the two sequences differ at nucleotide 681 which changes threonine 287 in the previously reported sequence to a proline in our sequence (Fig. 1A). Since this proline sequence was subsequently shown to be involved in the binding to the CrkSh3(N) domain, its presence was further confirmed by sequencing polymerase chain reaction amplified HeLa mRNAs. Probing of HeLa poly(A)+ RNA with the 1.2-kb clone detected a 6.5-kb band (Fig. 1B). In addition, two differentially spliced C3G mRNAs were identified by polymerase chain reaction. The shorter one has a 114-nucleotide deletion between nucleotides 151 and 265 (amino acids 50-88) of the coding sequence. Even though this difference was not detectable in Northern blots, multiple bands between 145 and 155 kDa appeared in Western blots that may represent the two splice variants. The C-terminal domain of C3G is homologous to the catalytic domain of the guanine-nucleotide exchange factor CDC25 (46). The amino acid sequence of the phage insert contains four stretches of proline-rich sequences (Fig. 1A and C). They were subsequently shown to bind the CrkSh3 domain and were therefore named Crk Binding sequence 1 to 4 (CB1-4). Alignment of the sequences showed remarkable similarity spanning nine amino acids (Fig. 1C). CB-2 and CB-4 have 8/9 amino acids identical with CB-1, whereas CB-3 has 6/9 identical amino acids with CB-1. (CB-1-4) are good candidates for SH3 domain binding sequences since they contain a Pro-X-X-Pro motif (14). One additional proline-rich sequence is localized N-terminal to the first CB motif (amino acids 267-276). It differs significantly from the other four CB sequences and from sequences in other known Crk binding proteins (Table I) and was not analyzed in detail.

**C3G Preferentially Binds to the CrkSh3(N) Domain**—Since the library screen involved a GST-fusion protein that contained both c-Crk SH3 domains, the domain in Crk sufficient for the binding to C3G was not yet clearly identified. The C3G CB-region containing all four CB-sequences was expressed as an 35S-labeled GST-fusion protein and tested for binding to full length Crk, the CrkSh2 domain and the individual Crk SH3 domains which were immobilized on a filter (Fig. 2A). The C3G CB-region bound only to the isolated CrkSh3(N) domain and full length c-Crk. To assess whether C3G CB-sequences can also bind to other SH3 domains, the 35S-labeled C3G CB-fragment was tested against a panel of SH3 domains (Fig. 2B).

3 B. S. Krouse, S. M. Feller, and H. Hanafusa et al., unpublished observation.
Only gest-fusion constructs that contained the CrkSH3(N) domain bound strongly to the 35S-labeled C3G CB-region. Grb2, the individual Grb2SH3 domains and the phosphatidylinositol 3-kinase-p85-a-SH3 domain only bound weakly. Binding to the Grb2 and phosphatidylinositol 3-kinase-p85-a SH3 domains was only observed in this filter binding assay but did not occur in precipitations from solution or in the ELISA (Figs. 3 and 6). This may result from the partial denaturation of the blotted SH3 domains.

Subsequently the binding the C3G CB region to cellular proteins other than Crk was analyzed. Blotted HeLa proteins, initially precipitated with unlabeled gest-CR-1, were probed with 35S-labeled gest-CB-1. Apart from a faint signal of about 38 kDa, no additional binding proteins were detected beyond non-specific binding to gest. This hand was also detected when 35S-labeled cell lysates were precipitated with gest-CB-1 and could be Crk or the Crk-related protein CRKL. It is not possible to distinguish between Crk and CRKL (19) in this assay, since the

| Consensus: | PP AL PK K R |
|---|---|

**Table 1**

| Protein  | CrkSH3 binding sequence |
|----------|------------------------|
| C3G/CB-1 | SPPPALPKKRRQ           |
| C3G/CB-2 | DTPPALPPKRRR           |
| C3G/CB-3 | EKPPPLPKKNN            |
| C3G/CB-4 | APPPALPPKQRQ           |
| Abd/CB-1 | LQAPELPTKRT            |
| Abd/CB-2 | AVSPLPPKRRR            |
| Arg/CB-1*| PRPLILPSKRT            |
| Arg/CB-2*| SGPSPALPKQRD           |
| mSos1/P-1| PPPPPVPPRRR            |
| mSos2/P-1*| LPPPLPPKRKF            |
| Consensus| XPPXLPXXRXR            |
proteins containinjg
procedures
proteins have almost identical molecular weights. The unambiguous identification of this CB-1-binding protein awaits further analysis with antisera specific for Crk and CRKL. The specificity of C3G-SH3 domain interactions in solution was further analyzed by incubating proteins of the cytosolic fraction of HeLa cells (S100) with a panel of SH3-gst-fusion proteins. Protein complexes were precipitated with glutathione-beads, washed with RIPA and probed with an antisera raised against C3G. C3G was detectable as a broad band between 145 and 155 kDa in precipitates with gst-fusion proteins of full length c-Crk, the CrkSHXN domain and v-Crk (Fig. 3). All these constructs contain the CrkSH3(N) domain. No binding was observed to any other SH3 domain including Grb2, the individual Grb2 SH3 domains and the phosphatidylinositol 3-kinase-pf35-n-SH3 domain. When the antisera was preincubated with immunogen in a control experiment the 145/155 kDa band was the only signal that was no longer visualized. The proteins precipitated with Grb2 (Fig. 3) were not competed by the antigen. These data demonstrate that the unique binding specificity of the C3G-CB region toward the Crk SH3(N) domain is retained in the full length C3G protein.

**Binding of the Individual Proline-rich C3G-CB Sequences to v-Crk and c-Crk**—The four proline-rich motifs in C3G are remarkably similar, but the differences in the amino acid sequences could influence the binding to the Crk SH3 domain. To investigate the binding of each individual proline-rich sequence, gst-fusion proteins containing only a single proline-rich sequence were expressed (Fig. 4). Since the v-Crk SH3 domain slightly differs from the c-Crk SH3 domain in its sequence, the binding of both c-Crk and v-Crk proteins was tested. Cell lysates of v-Crk expressing rat fibroblasts (v-Crk/3Y1) or c-Crk overexpressing chick embryo fibroblasts (c-Crk/CEF) were incubated with gst-CB-1 to -4 and analyzed by Western blot for the binding to Crk proteins. Both c-Crk and v-Crk bound well to all CB constructs tested (Fig. 5). The binding was stable in RIPA, suggesting strong binding of each proline-rich CB-sequence to the Crk proteins. These experiments show that there are four potential Crk binding sites in the C3G protein.

**Analysis of CrkSH3-C3G Binding by ELISA**—To quantify the affinity of the C3G-CrkSH3 interaction we utilized an ELISA. The binding of each proline-rich sequence was analyzed. Gst-CB fusion proteins were biotinylated and probed for binding to immobilized gst-CrkSH3SH3, gst-Grb2, or gst alone. Binding to the Crk SH3 domain was saturable in all cases (Fig. 6). However, Grb2 showed little binding and the binding did not saturate even at the highest concentrations of gst-CB fusion proteins used in these experiments. Binding to gst alone was minimal and was subtracted from the binding to gst-CrkSH3SH3 and gst-Grb2. The high binding affinity between gst-CrkSH3SH3 and the four proline-rich sequences is reflected in half-saturation points of 10.4 nM (gst-CB-1), 27 nM (gst-CB-2), 11.3 nM (gst-CB-3), and 2.85 nM (gst-CB-4). The ELISA binding data are consistent with the solution phase binding results shown in Fig. 3 and suggest that the affinity of Grb2 to C3G is not high enough for stable binding.

**Coprecipitation of Crk and C3G from Cell Lysates**—Since the endogenous cellular Crk-II does not form detectable complexes with cytoplasmic proteins in different unstimulated cells (32), we used cells that overexpress Crk proteins to examine the formation of complexes between C3G and Crk. The endogenous C3G was precipitated with the C3G antiserum from c-Crk/CEF or v-Crk/3Y1 cell lysates and the immune complexes probed for Crk proteins (Fig. 7, A and B). v-Crk is a fusion protein containing viral Gag sequences and was detected with a monoclonal antibody directed against Gag. Crk proteins were only detected in precipitates with the C3G antiserum but not with the preimmune serum. In summary, our data strongly suggest that the interaction between C3G and Crk is highly specific and therefore likely of biological relevance.

**DISCUSSION**

We have cloned a Crk binding protein, C3G, that contains a domain homologous to guanine-nucleotide exchange factors for Ras GTPases and binds with remarkable specificity to the SH3(N) domain of Crk. The stable association between Crk and C3G in cell lysates suggests a biologically important interaction of these two proteins in a yet undefined c-Crk signaling pathway, as well as a possible role for C3G in v-Crk transformation. While this manuscript was in preparation, a virtually identical protein sequence was reported by Tanaka et al. (34).

However, our results differ significantly from the previous report in the elucidation of C3G sequences that mediate binding to the Crk SH3(N) domain and the binding specificity of
Fig. 4. CrkSH3 binding sites in C3G and C3G fragments that were used for bacterial expression. Schematic diagram of C3G showing the positions of the proline-rich CrkSH3 binding sequences (black boxes) and the constructs used in this study. Numbers indicate the amino acid boundaries of the C3G fragments.

Fig. 5. Binding of individual proline-rich sequences of C3G to v-Crk and c-Crk. A, total cell lysates (300 µg) of v-Crk/3Y1 cells were incubated with 20 µg of gst-fusion proteins as indicated (see also Fig. 4). Complexes were precipitated with glutathione-beads. The precipitates were washed with RIPA, separated by SDS-PAGE (11%) and blotted. The blot was probed with a monoclonal antibody raised against the Gag portion of the v-Crk protein. B, c-Crk protein was precipitated from total cell lysates (500 µg) of c-Crk/CEF with gst-fusion proteins as indicated. The precipitated proteins were separated and immobilized as in A and probed with the Crk antiseraum. 50 µg of total cell lysate was Western blotted to show the migration of the c-Crk protein.

C3G for SH3 domains. In our study, four homologous, proline-rich sequences, CB-1 to -4, were identified. Each sequence can bind individually and with similar affinity to the SH3 domains of c-Crk and v-Crk. In contrast, Tanaka et al. (34) found one strong and one weak Crk binding sequence, which correspond to our CB-4 and CB-3 sequences, respectively (Fig. 1C). The reason for the weak binding of the construct corresponding to part of the CB-3 sequence is probably the truncation of this sequence after the first lysine residue following the proline-rich stretch of amino acids. A similar truncation in the CB-1 sequence resulted in a dramatic decrease of the binding affinity. Our data indicate that basic amino acids following the prolines in certain positions strongly increase the affinity of proline-rich sequences to the CrkSH3(N) domain. Alignment of the

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Fig. 6. Binding of proline-rich sequences to Crk and Grb2 in an ELISA. Wells were coated with 100 ng/well gst-CrkSH3SH3 or gst-Grb2. Biotinylated gst-CB-1 (squares), gst-CB-2 (diamonds), gst-CB-3 (circles), gst-CB-4 (triangles) were added at concentrations indicated. Binding of biotinylated gst-CB proteins was detected by a colorimetric assay as described under "Materials and Methods." Each data point reflects the mean of three independent experiments. The standard error of the mean was usually less than 10%.

Fig. 7. Coprecipitation of Crk and C3G. A, total cell lysates of c-Crk/CEF's (1 mg) were precipitated with anti-C3G or control sera as indicated above each lane and blots were probed for c-Crk with a Crk antiseraum. B, total cell lysates of v-Crk/3Y1 (1 mg) were precipitated with anti-C3G or control antibodies as indicated above each lane and probed for v-Crk.

CrkSH3(N) binding sequences identified in C3G and the Abl kinase (33) with proline-rich sequences of the CrkSH3(N) binding proteins Sos and Arg (32), also points to the importance of
basic amino acids in CrkSH3(N) binding sequences. The consensus sequence for high affinity binding to the CrkSH3(N) domain obtained from this alignment (Table I) includes two basic amino acids following the prolines and differs therefore significantly from the consensus sequence previously proposed (34).

Factors that determine the specificity of the various SH3 domains for certain proline-rich sequences are currently not well understood. Only one example of a highly specific SH3 interaction has been previously reported. It occurs between a proline-rich sequence of p47phox and the p67phox SH3 domain (47, 48). Other proline-rich sequences which have been tested often interact with multiple SH3 domains. The results vary to some extent, depending on the experimental system. Interactions of different SH3 domains with short proline-rich peptides or protein fragments have been analyzed in vitro, using purified proteins (13, 35). However, binding of the corresponding full length protein is not always detectable in vitro. For example, the GTPase dynamin binds in vitro to the SH3 domain of the p85-α subunit of PI3-kinase (35), but no interaction of these proteins was detected when p85-α and dynamin were overexpressed together in cells (49). This lack of correlation between in vitro and in vivo binding results has also been observed for the Ab1SH3 binding proteins 3B1-1 and 3B2-1 and some partial clones obtained by screening of expression libraries with Crk. The binding of the C3G proline-rich sequences to SH3 domains of Grb2 and phosphatidylinositol 3-kinase-p85-α which are immobilized on a membrane (Fig. 2B) is likely a similar artifact of the assay system and was not observed in solution phase binding assays. In agreement with the specific, high affinity interaction between Crk and C3G, indicated by the coprecipitation of these proteins from cell lysates, apparent Kᵋ values (half-saturation points) in the nanomolar range were obtained for the binding the CrkSH3SH3 protein in the ELISA, while Grb2 showed very poor, nonsaturating binding. The ELISA dissociation constants are about 1000-fold lower than the values obtained with small proline-rich peptides in solution binding studies (15). This difference results most likely from the surface adsorption of one of the binding partners. We propose that C3G is not important for intracellular signaling through Grb2.

The consequence of Crk binding to C3G could be the activation of Ras or a Ras-related protein. C3G contains a C-terminal domain with homology to the Ras guanine-nucleotide releasing factor (FRF) CDC25 of S. cerevisiae (34, 50). It belongs therefore into a growing family of mammalian GRFs with CDC25 homology. This family includes currently a brain specific Ras-GRF (CDC25') and the widely expressed Sos proteins (50, 51). In addition to their catalytic exchange factor domain, CDC25' and the Sos proteins contain functionally equivalent, non homologous sequences that control their association with growth factor receptors (52, 53). Sos has four proline-rich stretches of amino acids C-terminal of the catalytic domain which mediate receptor binding via the Grb2 adapter protein, and CDC25' has sequences at the N terminus which are crucial in ligand-induced activation of the exchange factor activity (52). While the biological relevance of having two C3G splice variants (Fig. 1A) is currently not understood, divergent N-terminal sequences seem to be common in the family of Ras GRFs. Four different RNAs, which are likely a consequence of differential splicing, were detected for CDC25' (52). They encode a full-length protein and three proteins with truncated N termini. Only the longest protein can function in a ligand-dependent manner. It may therefore be interesting to functionally compare the two forms of C3G.

The specific complex formation between SH3 domains and cellular proteins can have at least three functional conse-quences. Recent reports indicate that the binding of SH3-containing proteins to enzymes can regulate their catalytic activity. Binding of Grb2 or the SrcSH3 domain to dynamin (35), as well as the binding of the SrcSH3 domain to phosphatidylinositol 3-kinase-p85-α (53), increases the enzymatic activities of dynamin and PI3-kinase. Besides the regulation of enzymatic activities (35, 53, 54), SH3 domains can mediate the substrate recognition of enzymes (31, 33) and the targeting of proteins to specific subcellular locations (55). Future studies will aim to determine whether the binding of C3G to Crk influences C3G activity or whether the binding to Crk merely relocalizes the C3G protein. The best studied SH3 domain-dependent interaction of functionally similar proteins is the binding of the adapter protein Grb2 to the mammalian guanine-nucleotide exchange factor Sos. Upon exposure of cells to some mitogenic stimuli, Grb2 is recruited from the cytoplasm to the membrane where it forms a link between tyrosine kinases and the Ras pathway. Several activated receptor tyrosine kinases generate high affinity binding sites for the Grb2SH2 domain (24, 56–59). The Grb2 SH3 domains in turn bind to proline-rich sequences at the C terminus of Sos (21, 60), thereby bringing Sos in proximity to Ras. The expression of v-Crk or c-Crk-I in PC12 cells potentiates neurite outgrowth. Based on these results, it has been postulated that Crk can activate the Ras pathway (61, 62). This activation could result from the binding of Sos and C3G to the CrkSH3(N) domain. While the function of Sos as a Ras exchange factor has been established (57), the biological activity of C3G in higher eukaryotes is not yet characterized. However, C3G is able to complement a temperature sensitive CDC25 mutant yeast strain (34), suggesting that C3G acts as an exchange factor for a member of the Ras or Raf family of GTPases. The yeast complementation assay cannot distinguish these activities. A more precise functional analysis of C3G signaling pathways should greatly advance our understanding of the biological functions of c-Crk as well as mechanisms of v-Crk transformation.

Acknowledgments—We thank Rosemary Williams for excellent technical assistance. We are grateful to Toru Miki and Stuart A. Aaronson for supplying the pgp2515-M426 cDNA library, to Ann Marie Pendegast for the Grb2SH3 constructs, and to Lewis Cantley for glutathione fusion construct of the phosphatidylinositol 3-kinase-p85-α-SH3 domain. We also thank Ray Birge and Paul van Bergen en Henegouwen for comments on the manuscript. We acknowledge the communication of the C3G sequence by Michiyuki Matsuda prior to publication.

REFERENCES

1. Koch, C. A., Anderson, D., Moran, M. F., Killus, C., and Pawson, T. (1991) Science 252, 668–674
2. Mussacchio, A., Gilson, T., Lehto, V.-P., and Saraste, M. (1992) FEBS Lett. 307, 55–61
3. Mayer, B., and Baltimore, D. (1993) Trends Cell Biol. 3, 8–13
4. Matsuda, M., Mayer, B., and Hanafusa, H. (1991) Mol. Cell. Biol. 11, 1607–1613
5. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Rosh, M. D., Ross, G. B., Silverman, S., and Xyremko, J. (1992) Nature 358, 464-467
6. Cantley, L. C., Auger, K. R., Carpenter, C., Dackworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) Cell 64, 281–302
7. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gilb, G., Pawson, T., Haer, W. G., King, F., Roberts, T., Ratafotkz, S., Neel, B. G., Birge, R. B., Pajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 78, 267–277
8. Songyang, Z., Shoelson, S. E., McGuire, J., Olivier, P., Pawson, T., Bustelo, R. X., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Rattoksky, S., Feldman, R. A., and Cantley, L. C. (1994) Mol. Cell. Biol. 14, 2777–2785
9. Lee, C.-H., Kominos, D., Jacques, S., Margolis, B., Schlesinger, J., Shoelson, S. E., and Kuriyan, J. (1994) Structure 2, 429–430
10. Birge, R. B., and Hanafusa, H. (1993) Science 262, 1522–1524
11. Kuriyan, J., and Cowburn, D. (1993) Curr Op. Struct. Biol. 3, 838–837
12. Shoelson, S. E., Mayer, B. J., Thiel, G., and Baltimore, D. (1992) Science 257, 803–806
13. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Science 259, 1107–1111
14. Lim, W. A., and Richards, F. M. (1994) Struct. Biol. 1, 221–225
Yu, H., Chen, J. K., Feng, S., Dalgarro, D. C., Brauer, A. W., and Schreiber, S. L. (1994) Cell 76, 933-945
Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988) Nature 332, 272-275
Reichman, C. T., Mayer, B. J., Keshav, S., and Hanafusa, H. (1992) Cell Growth & Differ. 3, 451-460
Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1992) Mol. Cell. Biol. 12, 3462-3469
Hoeve, J., Morsius, C., Hoekstra, N., and Groffen, J. (1993) Oncogene 8, 2469-2474
Matsuoka, K., Shibata, Y., Yamakawa, A., and Takenawa, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9015-9019
Lowenstein, E. J., Daly, R. J., Bantzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) Cell 70, 431-442
Suen, K. R., Bustelo, X. R., and Barbacid, M. (1993) Mol. Cell. Biol. 13, 5000-5012
Clark, S., Stern, M. J., and Horvitz, H. R. (1992) Nature 356, 340-344
Olivier, J. P., Raabe, T., Henkemeier, M., Dickson, B., Bhamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993) Cell 73, 179-191
Simon, M. A., Dodson, G. S., and Rubin, G. M. (1993) Cell 73, 169-177
Path, I., Schweigtoffer, F., Roy, I., Mulon, M.-C., Boizian, J., Duchesne, M., and Tocque, E. (1994) Science 264, 971-974
Lehman, J. M., Riezman, G., and Johnson, J. P. (1990) Nucleic Acids Res. 18, 1048
Mayer, B. J., and Hanafusa, H. (1990) J. Virol. 64, 3081-3089
Matsuda, M., Mayer, B. J., Fukui, Y., and Hanafusa, H. (1990) Science 248, 1537-1539
Birge, R. B., Fajardo, J. E., Reichman, C., Shoelson, S. E., Songyang, Z., Cantley, L., and Hanafusa, H. (1993) Mol. Cell. Biol. 13, 4458-4456
Feller, S. M., Krudsen, B., and Hanafusa, H. (1994) EMBO J. 13, 2341-2351
Feller, S. M., Krudsen, B., Wong, T. W., and Hanafusa, H. (1995) Methods Enzymol., in press
Ben-R., Ye, Z.-S., and Baltimore, D. (1994) Genes & Dev. 8, 783-795
Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matsuda, K., Takenaka, T., Kurata, T., Nagashima, K., and Matsuda, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3443-3447
Gout, I., Dhand, R., Hiles, I., Giddings, B. W., Buday, L., Sizeland, A. M., and Brooks, M. W. (1993) Cell 76, 11167-11171
Chou, M. (1993) The rck Oncogene: A Link between Tyrosine and Serine/Threonine Kinases. Ph.D. Thesis, The Rockefeller University
Penninger, J. K., Quilliam, L. A., Cripe, L. D., Baezling, C. H., Dai, Z. I., Li, N., Bantzer, A., Rabun, K. M., Der, C. J., Schlessinger, J., and Gishizky, M. L. (1993) Cell 75, 175-185
Kapeller, B., Prasad, K. V. S., Janssen, O., Hou, W., Schaffhausen, B. S., Rudd, C. E., and Cantley, L. C. (1994) J. Biol. Chem. 269, 1927-1933
Miki, T., Matsui, T., Heidaran, M. A., and Aaronson, S. A. (1989) Gene (Amst.) 85, 197-146
Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31-40
Potz, W. M., Olsen, M., Boettiger, D., and Vogt, V. M. (1987) J. Gen. Virol. 68, 3177-3182
Mayer, B. J., and Hanafusa, H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2638-2642
Matsuda, M., Marshall, C. P., and Hanafusa, H. (1990) J. Biol. Chem. 265, 12000-12004
Reichman, C. (1993) Cloning, Characterization and Oncogenic Activation of a rck Proto-oncogene. Ph.D. Thesis, The Rockefeller University
Kudrinski, B. S., Silverstein, R. L., Leung, L. K., Harpel, P. C., and Nachman, R. L. (1986) J. Biol. Chem. 261, 10765-10771
Jones, S., Vignais, M.-L., and Brocho, J. R. (1991) Mol. Cell. Biol. 11, 2641-2646
Finan, P., Shimizu, Y., Gout, I., Hau, K., Truong, O., Butcher, C., Bennett, P., Waterfield, M. D., and Kellie, S. (1994) J. Biol. Chem. 269, 13752-13755
Sumimoto, H., Kage, Y., Nuno, H., Sasaki, H., Nose, T., Fukuzaki, Y., Choo, M., Minakami, S., and Takeshige, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5345-5349
Seedorf, K., Koertk, G., Lammer, R., Bashkin, P., Roger, D., Burgess, W. H., van der Bliek, A. M., Schlessinger, J., and Ullrich, A. (1994) J. Biol. Chem. 269, 16009-16014
Shou, C., Farnsworth, C. L., Neel, B. G., and Feig, L. A. (1992) Nature 358, 351-354
Bowtell, D., Fu, D. P., Simon, P., and Senior, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6511-6515
Cas, G., Papageorge, A. G., Vass, W. C., Zhang, K., and Lowy, D. R. (1993) Mol. Cell. Biol. 13, 7718-7724
Pieten, C. M., Herz, W. M., and Cambier, J. C. (1994) Science 263, 1609-1612
Mayer, B., and Baltimore, D. (1994) Mol. Cell. Biol. 14, 2883-2894
Bar-Sagi, D., Rotin, D., Bantzer, A., Mandiyan, V., and Schlessinger, J. (1993) Cell 74, 83-91
Buday, L., and Downward, J. (1993) Cell 73, 611-620
Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993) Nature 363, 88-92
Beltersperger, K., Konig, L. M., Cherniack, A. D., Klarlund, J. K., Chawla, A., Banejee, U., and Czech, M. P. (1993) Science 269, 1950-1952
Tobe, K., Matsuoka, K., Tanimoto, H., Ueki, K., Kaburagi, Y., Assi, S., Naguchi, T., Matsuda, M., Tanaka, S., Hattori, S., Fukui, Y., Akanuma Y., Yazaki, Y., Takenawa, T., and Kadowaki, T. (1993) J. Biol. Chem. 268, 11167-11171
Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45-51
Bennetseal, B. L., Birge, R. B., Fajardo, J. E., Glassman, R., Mahadeo, D., Krasemaeer, R., and Hanafusa, H. (1994) Mol. Cell. Biol. 14, 1964-1971
Tanaka, S., Hattori, S., Kurata, T., Nakamura, S., Fukui, Y., Nakamura, S., and Matsuda, M. (1993) Mol. Cell. Biol. 13, 4409-4415