Interaction between the Amino-terminal SH3 Domain of CRK and Its Natural Target Proteins*

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CRK is a human homolog of chicken v-Crk, which is an adaptor protein. The SH2 domain of CRK binds to several tyrosine-phosphorylated proteins, including the epidermal growth factor receptor, p130Cas, Shc, and paxillin. The SH3 domain, in turn, binds to cytosolic proteins of 135–145, 160, 180, and 220 kDa. We screened expression libraries by Far Western blotting, using CRK SH3 as a probe, and identified partial cDNA sequences of four distinct proteins, including C3G, DOCK180, EPS15, and clone ST12. The consensus sequence of the CRK SH3 binding sites as deduced from their amino acid sequences was Pro^1-Pro^2-X^3-Leu^4-Pro^5-X^6-Lys^7. The interaction of the CRK SH3 domain with the DOCK180 peptide was examined with an optical biosensor, based on the principles of surface plasmon resonance. A low dissociation constant of the order of 10^{-7} resulted from a high association rate constant (k_{assoc} = 3 \times 10^5) and low dissociation rate constant (k_{diss} = 3 \times 10^{-3}). All CRK-binding proteins except clone ST12 also bound to another adaptor protein, Grb2. Mutational analysis revealed that glycine at position +1 of ST12 inhibited the binding to Grb2 while retaining the high affinity binding to CRK SH3. The result suggests that the amino acid at position +1 also contributes to the high affinity binding of the peptides to the SH3 domain of Grb2, but not to that of CRK.

v-Crk was identified originally as an oncogene product of the CT10 retrovirus (1). Studies on v-Crk have yielded important information based on signal transduction research. First, comparison of the amino acid sequence of v-Crk with those of molecules involved in signal transduction identified a new domain, designated as SH3 (1, 2). Second, analysis of the proteins bound to v-Crk led to the finding that the SH2 domain of v-Crk binds to several proteins and that the binding is dependent on tyrosine phosphorylation (3). Finally, the isolation of a homolog of v-Crk, together with the isolation of Grb2, revealed the presence of proteins that consist mostly of the SH2 and SH3 domains, which are now known as adaptor proteins (4–7).

The cellular homolog of v-Crk has been isolated from chicken, human, and mouse (4, 5, 8). Alternative splicing of the human CRK gene yields two forms of translation products, designated as 28-kDa CRK-I and 40/42-kDa CRK-II (5). The latter contains an additional SH3 domain. Microinjection of CRK induces neuronal differentiation of PC12 cells, and overexpression of v-Crk accelerates the neuronal differentiation of PC12 cells induced by nerve growth factor and epidermal growth factor, which trigger the activation of cognate tyrosine kinase receptors (9–11). This CRK-dependent differentiation is inhibited by a dominant-negative Ras protein (9, 12). These results have assigned CRK as lying between receptor-type tyrosine kinases and the Ras family proteins in the signal transduction pathway.

It has been well established that the function of adaptor proteins is to bring the SH3-binding proteins into the close vicinity of the proteins bound to SH2 upon activation of tyrosine kinases (13, 14). Thus, many efforts are being devoted to the identification of the proteins bound to the SH2 and SH3 domains. Proteins known to bind to the SH2 domain of CRK include paxillin, p130Cas, Cbl, Shc, and epidermal growth factor receptor (3, 12, 15–17). We and another group have shown that the SH3 domain of CRK binds to proteins of 135–145, 160, 180, and 220 kDa, in vivo (9, 18). With the use of expression cloning, two of these, 135–145- and 180-kDa proteins, have been identified as C3G (9, 19) and DOCK180 (20), respectively. Except for C3G, which has been demonstrated to be a guanine nucleotide exchange protein for Rap1 (21), their biochemical functions are not known.

Pioneering work by Baltimore and his colleagues revealed that the function of SH3 is to bind to the peptide that forms a polyproline type II helix (22, 23). Further studies with synthetic peptides and phage libraries have deduced consensus sequences essential for binding to the SH3 domains (24–26). Moreover, crystallographic and NMR studies have established the general features of SH3 structure and the interaction of SH3 with proline-rich peptides (27–30). In these studies, however, the affinities of the SH3 domains for the proline-rich peptides were usually low; their K_d values ranged from 5 to 100 \mu M (24, 31–33). This is in contrast to the affinity of the SH2 domains for their targets; their K_d values are in the nm range (34). This might suggest that the combinations of SH3s and their target peptides used in the previous studies are not physiologic, or that there are other binding motifs that reinforce the SH3-mediated binding (28).

We have used modified Far Western blotting to identify proteins bound to the SH3 domain of the human CRK protein.
The specificity and affinity of the human CRK SH3 appeared comparable to those of antibodies, which are usually below the μM range. Moreover, we have found that at least 5% of DOCK180, a CRK SH3-binding protein, associates with CRK in vivo, suggesting high affinity between CRK and DOCK180. These findings led us to study the kinetics of the binding of CRK SH3 to its physiologic targets.

**Materials and Methods**

Far Western Blotting—Molecular cloning of the human CRK gene and the expression of its amino-terminal SH3 domain as a GST fusion protein were described previously. From λgt11 cDNA expression libraries, recombinant clones expressing SH3-binding proteins were identified by Far Western blotting with GST-CRK SH3 and anti-GST monoclonal antibody. Nonspecific binding of the filters was blocked by 2% skim milk in PBS containing 0.05% Tween 20 (PBS-Tween) for 12 h at 4 °C. The filters were incubated with 0.1 μg/ml GST fusion proteins in PBS-Tween for 2 h at 25 °C. After washing in PBS-Tween, bound peptides were detected by successive incubation with anti-GST monoclonal antibody, alkaline phosphatase-conjugated anti-mouse immunoglobulin antibody, and chromogen. We isolated four distinct cDNAs. Three of them, C3G (36), DOCK180 (20), and clone ST12 were novel. One clone, ST21, was found to encode a partial sequence of EPS15 (37).

Plasmids and Peptides—The expression and purification of Grb2 and of the SH3 domains of CRK, PLC-γ, and Fyn as GST fusion proteins have been described (5, 36, 39). The SH3 binding regions of DOCK180, C3G, and clone ST12 were also expressed as GST fusion proteins. The DOCK180 peptide used in this study corresponds to amino acid 1815 to the carboxyl terminus (amino acid 1865) of DOCK180, which contains one of two CRK binding regions. The amino acid sequence of the ST12 peptide fused to GST is SLPGPLTPVAEGQEIGMNTETSGTSAR.

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**Interaction of CRK SH3 with Its Natural Target Proteins**

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**RESULTS**

Detection of the Binding of CRK SH3 to a Proline-Rich Region of DOCK180—For the study of the kinetic parameters between CRK SH3 and DOCK180 with the optical biosensor, one of two proline-rich regions of DOCK180 (amino acids 1815–1865) was expressed as a GST fusion protein and immobilized on the sensor chip matrix. Fig. 1 shows the sensorgrams of binding of CRK SH3 to the immobilized proline-rich region of DOCK180. The analysis consisted of two phases. In the association phase, the purified CRK SH3 domain was passed over the sensor chip immobilized on the sensor chip matrix. Panel A shows the sensorgrams of binding of CRK SH3 to the immobilized proline-rich region of DOCK180. The analysis consisted of two phases. In the association phase, the purified CRK SH3 domain was passed over the sensor chip immobilized on the sensor chip matrix. Panel A shows the sensorgrams of binding of CRK SH3 to the immobilized proline-rich region of DOCK180. The analysis consisted of two phases. In the association phase, the purified CRK SH3 domain was passed over the sensor chip immobilized on the sensor chip matrix. Panel A shows the sensorgrams of binding of CRK SH3 to the immobilized proline-rich region of DOCK180. The analysis consisted of two phases. In the association phase, the purified CRK SH3 domain was passed over the sensor chip immobilized on the sensor chip matrix. Panel A shows the sensorgrams of binding of CRK SH3 to the immobilized proline-rich region of DOCK180. The analysis consisted of two phases. In the association phase, the purified CRK SH3 domain was passed over the sensor chip immobilized on the sensor chip matrix.

The binding of CRK SH3 to the proline-rich region of DOCK180 immobilized on the sensor chip was monitored by an optical surface resonance biosensor. Arrows indicate the beginning and end of the injection of CRK SH3 into the sensor chip. Panel B, slopes of dRU/dt versus RU plots of the interactions shown in panel A. Panel C, slopes in panel B replotted against the concentrations of the injected protein. The association and dissociation rate constants can be obtained from Equation 1. The affinity constant for CRK SH3 binding to DOCK180 was calculated from the equation $K_a = \frac{k_{\text{ass}}}{k_{\text{diss}}}$.

**Fig. 1. Determination of association and dissociation constants of CRK SH3 for DOCK180.** Panel A, the binding of CRK SH3 to the proline-rich region of DOCK180 immobilized on the sensor chip was monitored by an optical surface resonance biosensor. Arrows indicate the beginning and end of the injection of CRK SH3 into the sensor chip. Panel B, slopes of dRU/dt versus RU plots of the interactions shown in panel A. Panel C, slopes in panel B replotted against the concentrations of the injected protein. The association and dissociation rate constants can be obtained from Equation 1. The affinity constant for CRK SH3 binding to DOCK180 was calculated from the equation $K_a = \frac{k_{\text{ass}}}{k_{\text{diss}}}$.
Interaction of CRK SH3 with Its Natural Target Proteins

the RU decreased, indicating a decrease in the amount of CRK SH3 bound to the sensor chip matrix. When no ligand was immobilized on the sensor chip, there was no detectable binding of CRK SH3. We could not detect the binding of GST alone to the immobilized GST-DOCK180 peptide, either.

Determination of Kinetic Parameters—For estimation of the association and dissociation rate constants, we analyzed the binding of CRK SH3 to DOCK180 at various concentrations (Fig. 1A). Data for a dRU/dt versus RU plot were calculated from the sensogram (Fig. 1B). This linear plot allowed the estimation of the association rate constant ($k_{assoc}$) as well as the dissociation rate constant ($k_{diss}$) as described previously (40, 41). The analysis of the binding phase of CRK SH3 to the proline-rich sequence of DOCK gave a fairly fast $k_{assoc}$ of 2.8 × 10^7 M^{-1} s^{-1}, and the y intercept of the curve showed a slow $k_{diss}$ of 3.1 × 10^{-3} s^{-1} (Fig. 1C). The dissociation constant $K_d$ was calculated to be 1.2 × 10^{-7} in this experiment.

The $k_{diss}$ value was also determined directly from the dissociation phase of bound protein according to Equation 2 (Fig. 1D). The first 20 s of the dissociation phase was distorted by a bulk effect; and after 5 min, more than 50% of the ligand was still on the sensor chip. We collected data from 30 to 70 s after the removal of the ligand (Fig. 1D). The $k_{diss}$ value, 1.7 × 10^{-3}, calculated in this way agreed well with the $k_{diss}$ value determined from Equation 1 as shown above.

Specificity of the Binding of CRK SH3—We next examined the association of the proline-rich region of DOCK180 with various SH3-containing proteins (Fig. 2). Grb2 showed a binding profile similar to that of the SH3 domain of CRK, with a $K_d$ of 3.7 × 10^{-7}. The SH3 domains of Fyn and PLC-γ2 showed slow binding to DOCK180. Because of the limited binding, we could not analyze the dissociation phase of Fyn and PLC-γ2. In CRK SH3-D150K, Asp^{150} of CRK SH3, which is required for the binding of lysine in the CRK binding sequences, is substituted for lysine (42). This mutation prevents CRK from inducing neuronal differentiation in PC12 cells (9). As expected, CRK SH3-D150K did not bind to DOCK180. The flat curves for RU in the association phase are due to the trace amount of salt present in the sample. When we used the amino-terminal or carboxyl-terminal SH3 domain of Grb2, neither bound to DOCK180. Other SH3 domains for which we could not detect binding to DOCK180 included those of the vav protein and the p85 subunit of phosphatidylinositol 3-kinase.

Isolation of a Clone That Distinguishes CRK from Grb2—We obtained four cDNA clones by screening λgt11 expression libraries. They are C3G, DOCK180, EPS15 (37, 43), and clone ST12. All but one clone, ST12, also bound to Grb2 in Far Western blot analysis (Fig. 3). The mechanism by which Grb2 discriminates between clone ST12 and the other CRK-binding proteins was studied further. The proline-rich region of ST12 (50 amino acids in length) was expressed as GST fusion proteins and used for binding analysis with BIAcore (Table I). The GST-ST12 bound to the CRK SH3 and to the full-length CRK protein with affinities comparable to those for DOCK180. However, in contrast to DOCK180, GST-ST12 did not bind to Grb2. We performed at least two completely independent experiments for the determination of kinetic parameters, starting from protein preparation and coupling to the sensor chip. This might have resulted in a rather wide deviation of $K_d$ values.

Consensus Sequence of CRK SH3 Binding Regions and Mutational Analysis—Table II shows the alignment of the amino acid sequences of the CRK binding sites of the four CRK-binding proteins described in this paper. The consensus sequence deduced from this alignment is PXXPLPKX. For depicting each amino acid, we adopted the notation used previously (44). We examined five recombinant proteins (Fig. 4A) for the binding to the SH3 domain of CRK and Grb2 by Far Western blotting (Fig. 4B). In C3G-32, position 3 (lysine), which has been reported to be critical for the binding of CRK to C3G (45), was substituted for arginine. C3G-32 bound to both CRK SH3 and Grb2, similar to the wild-type C3G-B9 and C3G-31. In previous work (45), the substitution of this Lys to Arg caused a 10-fold reduction in the affinity, which might be insufficient to disrupt the binding of this peptide to CRK SH3 in the filter binding assay.

We compared the binding sequence of ST12 with that of C3G, we found that the consensus sequence was perfectly
Interaction of CRK SH3 with Its Natural Target Proteins

Table I

| Protein | C3G  | DOCK180 | ST12 | EPS15 |
|---------|------|---------|------|-------|
|         | bm1  | bm2     | bm3  |       |
|         | P    | P       | P    | P     |
|         | P    | P       | P    | P     |
|         | A    | A       | A    | A     |
|         | L    | L       | L    | L     |
|         | P    | P       | P    | P     |
|         | E    | E       | E    | E     |
|         | K    | K       | K    | K     |
|         | NO   | +       | +    | +     |

Consensus: P P X L P X K

Note: bm, binding motif.

Fig. 4. Mutational analysis of CRK binding sequences. Panel A, these mutated proteins were expressed in Escherichia coli as GST fusion proteins and purified on a glutathione column. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and either stained with Coomassie Blue or analyzed by Far Western blotting with CRK SH3 or Grb2 used as probes. We used 100 ng for Coomassie Blue staining and 5 ng for the Far Western blot analysis. Panel B, amino acids substituted in the listed mutants.

Conserved. The difference is in position +1 (Gly) and position −2 (Ser). We substituted position +1 (Ala) for glycine in C3G-35, position +1 (Ala) for glycine, and position −2 (Pro) for serine in C3G-36. Both C3G-35 and C3G-36 bound to the SH3 domain of CRK but not to Grb2. The result indicated that substitution of position +1 (Ala) for Gly abolished the binding of the C3G-derived peptide to Grb2.

Discussion

In this report, we have analyzed the binding of CRK SH3 to its physiologic target proteins by using an optical biosensor, the operation of which is based on the principles of surface plasmon resonance. The Kd values for binding of CRK SH3 to DOCK180 and C3G obtained by this method are of the order of 10−7, which is remarkably lower than those of several SH3 domains reported in preceding papers, which ranged from 10−5 to 10−3 (30, 46). The relatively low Kd value resulted from moderately slow dissociation and rapid association rate constants. To avoid the effect of the GST protein fused to the amino terminus of SH3, we also used SH3 domains fused to maltose-binding protein and obtained similar results.

The binding of GST alone to GST fusion peptides immobilized on the sensor chip was not detectable, probably because of the alkaline treatment of the sensor chip during the washing step.

Knudsen et al. (19, 45) studied the interaction of CRK SH3 with the four proline-rich motifs of C3G, termed CB-1 to CB-4, by using two different methods. In the first paper, they used peptide competition assay and reported that the Kd values ranged from 2.85 to 27 nM, claiming high affinity binding between CRK and each of the four proline-rich motifs of C3G (19). Fluorescence measurements based on interactions of the peptides with the aromatic residues were described in a second paper (45). The Kd values ranged from 1.89 ± 0.06 to 35.8 ± 1.2 μM with this method, significantly higher than those reported in the first paper. The Kd value for the binding of CRK SH3 to the DOCK180 peptide obtained in this paper with BIAcore method is of the order of 10−7. Many previous investigations measuring the Kd values of peptides with various SH3 domains adopted the fluorescence measurement method and claimed low affinity binding of SH3 domains (24, 31). Taking into consideration the fact that many of the SH3 domain-containing proteins and their target proteins can be communoprecipitated, the interaction between the SH3-containing proteins and their target proteins should be tight (14). Therefore, it is possible that the use of fluorescence spectroscopy for the measurement of the Kd value of the SH3 domain may yield higher values than do other methods. This may be either because both the SH3 domains and the ligands were in solution in the fluorescence measurement method or because the lengths of the peptides used in this method were relatively short. Recently, Sastry et al. (47) analyzed the binding between the SH3 domains of Grb2 and Sos by BIAcore. The reported Kd value was 1.68 nM, significantly lower than those reported previously.

The consensus sequence obtained from four cDNA clones, PPXLPPK, is essentially the same as reported previously, except that the position +3 (Pro) could be other amino acids in Abl, Arg, and Eps15R which have been identified as CRK SH3-binding proteins by others (43, 48, 49). This consensus sequence agrees quite well with the crystallography data on CRK published recently by Wu et al. (42), which demonstrated that positions +3, +2, 0, −1, and −3 are in contact with CRK SH3. Amino acids at positions +4, +1, and −2 extend away from the surface of SH3. The fact that position +1 (Gly) in done ST12 inhibits binding to Grb2 indicates that position +1 contributes to the binding to Grb2, but not to CRK SH3. In accordance with this result, Terasawa et al. (50) reported that the side chain carboxamide group of Asn51 of the amino-terminal SH3 of Grb2 will form hydrogen bonds to the main chain carboxyl oxygens of position +1 (50). Asn51 of Grb2 corresponds to Pro188 of Crk, which will not form hydrogen bond to the main chain carboxyl oxygens of position +1 (42). The result suggests that the hydrogen bond between Asn51 and the main chain carboxyl oxygens of position +1 is required for the high affinity binding to Grb2 and that Gly, the smallest amino acid, might destabilize the hydrogen bond.

The observation that neither the amino-terminal nor the carboxyl-terminal SH3 domain of Grb2 binds to the CRK-bind-

3 M. Matsuda, unpublished result.
ing proteins also suggests that the binding modes of CRK SH3 and Grb2 are different from each other. Binding of dynamin to Grb2 also requires both of the SH3 domains; therefore, this is not a sole example of the binding modes of Grb2 to its target proteins (51). The three-dimensional structure of Grb2 has recently been determined (52). As suggested previously (33), the amino-terminal SH3, carboxyl-terminal SH3, and SH2 domain are each independent in their binding. However, the two SH3 domains are in contact with each other; therefore, it still possible that these two domains cooperate in the recognition of target molecules.

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