The insulin-like growth factor receptor (IGF-IR) is a transmembrane tyrosine kinase-containing glycoprotein closely related to the insulin receptor (IR) and insulin receptor-related receptor (IRR). The IGF-IR and IR share similar heterotetrameric structures consisting of two extracellular α-subunits containing the ligand-binding domains and two transmembrane β-subunits with the ligand-sensitive tyrosine kinase activity (1, 2). Following activation of receptor tyrosine kinase, a number of substrates and signaling pathways are activated. IRS-1 and IRS-2 are rapidly phosphorylated on tyrosine residues (3–5), as are the cytosolic proteins p46Shc and p52Shc (6, 7). IRS-1, IRS-2, and Shc link the activated receptor to downstream signaling pathways. Phosphorylated tyrosine residues on IRS-1 and IRS-2 are binding sites for several SH2 domain-containing proteins including the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI 3′-kinase) (8), the tyrosine-specific phosphatase PTP1D (Syp) (9), and the small adaptor protein Grb-2 (10). Both IRS-1 and Shc interact with the juxtamembrane domain of either the IR or IGF-IR via the NPEY motif surrounding residue tyrosine 950 (Tyr950). Activated Shc proteins apparently bind only Grb-2 (11). Grb-2, whether bound to IRS-1 or Shc, binds SOS via its SH3 domain and activates the Ras/Raf/mitogen-activated protein kinase pathway (for recent reviews, see Refs. 3, 5, and 12). Recent studies have revealed that in addition to IRS-1, IRS-2, and Shc, substrates such as Syp, p85, and G-protein associated protein interact directly with the IGF-I receptor in vitro through their SH2 domains (13). G-protein associated protein binds to residue Tyr950 in the juxtamembrane domain, while Syp and p85 interact with residue Tyr1316 in the carboxyl-terminal domain (13).

The Crk family consists of proteins which include Crkl, CrkII, v-Crk, and CrkL, all containing SH2 and SH3 domains with very short intervening sequences (14, 15). Despite the lack of a kinase domain these proteins are phosphorylated in vitro and in vivo on tyrosine residues (16) and apparently play an important role in growth factor-stimulated signal transduction (17–19). The widely expressed CrkII protein contains an N-terminal SH2 domain followed by two SH3 domains (14, 20). Crkl is an alternatively spliced version of CrkII which lacks the C-terminal SH3 domain (14) and thus closely resembles the viral oncogenic form of Crk, v-Crk (21). CrkL is a recently identified member of the family with one SH2 and two SH3 domains (15). The SH2 and both of the SH3 domains are essential for signal transduction (22, 23).

We have recently demonstrated that the product of the c-CrkII protein proto-oncogene is phosphorylated on tyrosine residues by the activated IGF-I receptor (24). Crk-II directly binds the nucleotide exchange proteins C3G and mSOS, thereby activating the Ras/Raf/mitogen-activated protein kinase and/or Jun kinase pathways (25). In this study we present data demonstrating that CrkL, as well as CrkII, is an endogenous substrate of the IGF-IR and both CrkL and CrkII may specifically associate, using different mechanisms, with a recently described member of the IRS family of proteins, IRS-4. In addition we demonstrate that the biological responses of cells overexpressing CrkL or CrkII are very different.

**MATERIALS AND METHODS**

**Cell Culture, CrkL Overexpression, and IGF-I Stimulation**—The cDNA encoding full-length CrkL was excised from pSG5-CrkL (26) and
subcloned into the pCXX2 expression vector into blunt-ended EcoRI restriction site, substituting the CrkII cDNA (19). Plasmids containing CrkII cDNA in the correct and in the opposite orientations were selected and used to stably transfect human embryonic kidney cells (293) and NIH-3T3 mouse fibroblasts. Resulting cell lines, as well as each parental cell line, were cultured in Dulbecco’s modified essential medium (DMEM) (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Upstate Biotechnology, Inc., Lake Placid, NY). Prior to IGF-I stimulation, subconfluent cell cultures were serum starved for 18–20 h in DMEM supplemented with 0.1% insulin-free bovine serum albumin (Intergen, Purchase, NY) and 20 mM HEPES (pH 7.5). The cells were then stimulated without further serum or with 50 nM IGF-I (Cell Signaling Sumit, NJ) for various times as indicated in the figure legends. NIH-3T3 cells overexpressing IGF-I and insulin receptors have been previously described (27).

Immunoprecipitations and GST Fusion Protein Binding Study—Serum-starved or IGF-I-stimulated cells were lysed in Nonidet P-40 buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM CaCl2, 1 mM MgCl2, 10% glycerol, and 2.5 mM sodium molybdate, 0.3 mM sodium fluoride) or RIPA buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 2.5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 0.3 mM sodium molybdate, and 10 mM sodium fluoride); both buffers were supplemented with protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Proteins were immunoprecipitated overnight from 1 g of total proteins (1 mg/ml in Nonidet P-40 or RIPA lysis buffer) with 2 μg of anti-Crk- or -CrkII polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), 5 μg of anti-IRS-1/plekstrin homology (PH), or IRS-2 polyantibodies (Upstate Biotechnology), anti-IRS-1 C terminus polyclonal antibody at 4 °C, 40 μl of a 50% (w/v) suspension of protein A-Sepharose beads were added and the incubation was continued for an additional 4 h. The beads were washed three times with 1 ml of ice-cold washing buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 0.3 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride). Precipitated samples were boiled for 5 min in 2 × Laemmli loading buffer, fractionated by 7.5% SDS-PAGE, and transferred to nitrocellulose membranes.

The GST-CrkL expression construct (a gift of Dr. J. Groffen (Children’s Hospital, Los Angeles, CA) was described previously (28). GST-CrkL expressing vectors encoding the SH2 (aa 2–111), N-terminal SH3 (aa 125–199), and C-terminal SH3 (aa 199–295) domains of human CrkL were obtained by subcloning the corresponding polymerase chain reaction fragments into a pGEX-5x-3 expression vector (Pharmacia Biotechnology, Piscataway, NJ). GST-CrkII expression construct was the full-length GST-CrkII construct as a gift of Dr. Beatrice Knudsen (Rockefeller University, New York). GST-CrkII expression vectors containing the SH2 (aa 9–119) and SH2-SH3 (aa 9–208) domains of CrkII were similarly prepared. The expression plasmids GST-SH2RV encoding a mutated form of the SH2 domain of CrkII (substitution of Arg-38 by Pro), GST-SH2/R39V-SH3 (substitution of Arg-39 of CrkL by Val), and GST-SH2-SH3(WW-LL) (substitution of Arg-38 by Val) were created by specific mutagenesis and a recombinant technique similar to one described by Higuchi (28).

All constructs were sequenced to ensure in-frame ligation, sequence identity, and the presence of the engineered mutation. The expression construct encoding SH2 domains of the p85 subunit of PI 3’-kinase was a gift of Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI). All plasmids were transfected into Escherichia coli DH5α (Life Technologies, Inc., Gaithersburg, MD) and GST fusion proteins were expressed and purified as recommended (GST Gene Fusion System, Third Edition, Pharmacia Biotech). The purity, integrity, and concentrations of the fusion proteins were assessed by SDS-PAGE and Coomassie Blue staining. All proteins appeared as a single band of the expected molecular weight.

For GST fusion protein binding studies, 500 μg of total protein prepared from serum-starved or IGF-I-stimulated 293 cells in 500 μl of RIPA buffer was mixed with GST alone (3 μg) or equimolar amounts of GST fusion proteins (6–8 μg), and 40 μl of glutathione-Sepharose 4B beads (50% w/v suspension in RIPA buffer) and incubated overnight at 4 °C. Beads were then washed three times with 1 ml of ice-cold washing buffer. Proteins were fractionated and transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated in an nNOS phospho-antibody (4G10, diluted 1:2000), anti-IRS-1 antibody (C-Terminal; 1 μg/ml), anti-IRS-2 (1 μg/ml) (all from Upstate Biotechnology, Inc.), or anti-IRS-4 (4 μg/ml) (29).

Cell Cycle Analysis—Cells were collected, washed with ice-cold phosphate-buffered saline (pH 7.8), fixed in 70% ethanol, 30% saline and kept for 1–3 days at −20 °C. For DNA content analysis cells were rinsed with phosphate-buffered saline and incubated in phosphate-buffered saline containing 50 μg/ml RNase and 0.1% Triton X-100 for 30 min at room temperature followed by the addition of 20 μg/ml propidium iodine for an additional 20 min. The DNA staining was analyzed by Becton Dickinson FACScan flow cytometer. The percentage of cells in the various phases of the cell cycle was calculated using ModFit LT software (Verity Software House, Topsham, ME).

RESULTS

**CrkL Is an Endogenous Substrate of IGF-I Receptor**—We have recently shown that stimulation of 293 and NIH-3T3 cells with IGF-I resulted in rapid tyrosine phosphorylation of CrkII in a time- and dose-dependent manner (19, 24), suggesting that CrkII was an endogenous substrate for the IGF-I receptor in intact cells. Extending these studies, we analyzed the effect of IGF-I on CrkII phosphorylation and compared the roles of CrkL and CrkII in IGF-I receptor-mediated signal transduction. Treatment of NIH-3T3 cells overexpressing the IGF-I receptor with IGF-I stimulated tyrosine phosphorylation of CrkII protein in a time-dependent manner (Fig. 1). Analysis of the CrkII protein from the same cell lysates showed a similar pattern of phosphorylation, indicating that both CrkII and CrkL are endogenous substrates of the IGF-I receptor. The sizes of Crk proteins are usually referred to as 40–42 kDa for CrkII and 36–39 kDa for CrkL. Analysis of the cell lines of mouse (NIH-3T3) or human (293 cells) origin as well as in intact rat tissues (30) indicates that the size of CrkL is consistently larger (40–42 kDa) when compared with CrkII protein (36–38 kDa). The immunoblot shown in Fig. 1 demonstrates that both Crk

![Image](335x553 to 527x729)
proteins associate with tyrosine-phosphorylated pp120 in the unstimulated state and this interaction decreased following IGF-I treatment. The nature of this pp120 protein is as yet unknown (30–32). In addition, CrkL, but not CrkII, was able to associate with 70-kDa paxillin (data not shown and Ref. 30) and 160–180-kDa protein (presumably members of the IRS family of proteins) in an IGF-I-dependent manner.

**Biological Responses in NIH-3T3 cells Overexpressing Crk Proteins**—To study the role of the Crk proteins in IGF-I receptor-mediated signal transduction, CrkL was overexpressed in NIH-3T3 cells and analyzed in parallel with NIH-3T3 cells overexpressing CrkII. To standardize the conditions for CrkL overexpression with those for CrkII, the cDNA encoding human CrkII was substituted with the human CrkL cDNA in the same vector (see “Materials and Methods”) and the resulting expression plasmid was used to transfect NIH-3T3 cells. Control cells were generated by transfection of parental NIH-3T3 cells with the expression vector, containing the CrkL cDNA in the antisense orientation. Cell cycle analyses were performed on cells overexpressing CrkL, CrkII, and control cells. When cells were plated at the equivalent densities in medium supplemented with 10% FBS and incubated in this medium for 24 h, cell cycle analyses revealed that all cell lines tested had a high percentage of cells in S phase reflecting the high mitotic activity of these cells (Table I). Serum starvation resulted in cell cycle arrest in control and CrkII overexpressing cells as early as 24 h, while only partial inhibition of growth was seen in CrkL cells. Stimulation of serum-starved cells with IGF-I led to strong mitogenic responses of control and CrkL overexpressing cells while a very weak response was seen in CrkII cells. These results indicate that overexpression of CrkII inhibited IGF-I-dependent proliferation of NIH-3T3 cells while CrkL overexpressing cells partially lost their growth dependence on growth factors. These data are consistent with the hypothesis that CrkL confers an oncogenic transformation of NIH-3T3 cells. To verify this hypothesis we analyzed the effect of high cell density on further growth of the CrkL and CrkII overexpressing cell lines (Fig. 2A). As demonstrated, CrkL cells, in contrast to CrkII or control cell lines, showed only partial growth arrest in the highly confluent state. Finally, cells overexpressing CrkL formed 2 to 3 times more colonies in soft agar assays (Fig. 2B), compared with control or CrkII cells.

**Mechanism of Tyrosine Phosphorylation of Crk Proteins in Response to IGF Stimulation**—To study the mechanism of CrkL and CrkII phosphorylation, CrkL was stably expressed in 293 cells and the cell line overexpressing CrkL (clone L-17) was analyzed in parallel with 293 parental and CrkII overexpressing cells (clone II-6/7). The results obtained for these clones were verified by analysis of additional clones and cell lines transfected with empty vector. Treatment of these cell lines with IGF-I stimulated tyrosine phosphorylation of CrkL, as well as CrkII, in a time-dependent manner (Fig. 3, middle panels). In agreement with data presented by Senechal et al.

### Table I

| Time | Cell cycle phases | OP2 | OP5 | 3T3-7 | 3T3-9 | 3T3-L-22 | 3T3-L-24 |
|------|------------------|-----|-----|-------|-------|----------|----------|
|      |                  |     |     |       |       |          |          |
| ~24 h |                  | 52  | 51  | 49    | 44    | 34       | 49       |
|       |                  | 37  | 30  | 31    | 31    | 46       | 36       |
|       |                  | 11  | 19  | 20    | 25    | 20       | 15       |
| 0 h   |                  | 86  | 77  | 86    | 81    | 52       | 65       |
|       |                  | 7   | 12  | 6     | 4     | 28       | 22       |
|       |                  | 7   | 11  | 8     | 15    | 20       | 13       |
| 24 h  |                  | 85  | 65  | 85    | 75    | 84       | 80       |
|       |                  | 9   | 29  | 9     | 19    | 8        | 12       |
|       |                  | 6   | 6   | 7     | 13    | 13       | 14       |
|       |                  | 8   | 14  | 8     | 14    | 7        | 9        |
|       |                  | 7   | 14  | 9     | 10    | 6        | 9        |

**Fig. 2.A.** overexpression of CrkL in NIH-3T3 cells partially abolishes density-dependent cell cycle arrest. $3 \times 10^5$ cells were plated in 6-well plates in DMEM supplemented with 10% FBS and cell cycle analysis was performed at the various time periods indicated. 0 h time point represents cells at about 70% confluency, analyzed 24 h after plating. The medium containing DMEM + 10% FBS was changed daily. Cell lines used were the same as described in legend for Table I. The experiment was repeated twice with similar results.  • op2; ● op5; ▲ 3T3-7; ○ 3T3-9; □ 3T3-L-22; ■ 3T3-L-24. B, overexpression of CrkL in NIH-3T3 cells increases the number of anchorage-independent colonies in soft agar assay. Columns 1 and 2 represent OP-2 and OP-5 (control cells), columns 3 and 4 represent 3T3-7 and 3T3-9 (CrkII overexpressing cells), and columns 5 and 6 represent 3T3-L-22 and 3T3-L-24 (CrkL overexpressing cells), respectively. The results are representative of two independent experiments ($n = 4$).
and lanes 1–3) of 293 cells. CrkL and CrkII following IGF-I stimulation are presented on the upper panels. These blots were stripped and reblotted with an anti-CrkL or anti-CrkII antibody (middle panels); exposure times for CrkII and CrkL were 90 and 10 s, respectively. These results imply that CrkL and CrkII may differentially regulate the activation of cellular tyrosine kinases and/or phosphatases. In contrast to CrkII which appeared as only one tyrosine-phosphorylated band (Fig. 3, middle and lower panels), CrkL migrated as three bands with two upper bands phosphorylated on tyrosine residues and responsive to IGF-I stimulation. These data may indicate that in contrast to CrkII, which is tyrosine phosphorylated only on Tyr221, CrkL may possess at least two sites of tyrosine phosphorylation. One of these tyrosines is Tyr207, the analog of Tyr221 of CrkII (34). The localization and possible significance of the second phosphotyrosine of CrkL has yet to be determined. Alternatively, the slowest migrating CrkL protein may reflect serine/threonine phosphorylation in addition to tyrosine phosphorylation. While this possibility cannot be excluded it is of interest that only a single band of phosphorylated CrkII is seen even when serine and tyrosine residues are phosphorylated following epidermal growth factor stimulation (35).

Upon IGF-I stimulation of 293 cells both CrkL and CrkII co-precipitated a tyrosine-phosphorylated 180-kDa protein (Fig. 3, upper panel). Overexpression of either CrkL or CrkII in 293 cells increased the association with 180-kDa phosphoproteins and inhibited the interaction of its counterpart with this protein; thus, supporting the idea that both CrkL and CrkII may bind and compete for the same binding site(s) on pp180. CrkL co-precipitated much more pp180, compared with CrkII (Fig. 4B) and interaction of CrkII with this protein can be detected only in Nonidet P-40 but not in RIPA lysis buffer, possibly indicating that CrkL forms more stable complexes with pp180. This protein possesses some characteristics of IRS-4, including similar electrophoretic mobility and IGF-I- and insulin-dependent tyrosine phosphorylation (data not shown). However, despite the use of different commercially available antibodies we failed to identify this pp180 protein as IRS-4 (A).

**Specific Interaction of Crk Proteins with IRS-4**—To characterize the nature of Crk-associated pp180 we verified the presence of functional IRS proteins in 293 cells. For this purpose IRS-1, IRS-2, and IRS-4 were specifically immunoprecipitated and analyzed with anti-phosphotyrosine antibody. In agreement with Laval et al. (29), our analyses revealed (Fig. 4A) that IRS-4 was the major IRS protein in these cell lines. In addition, IGF-I-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 could be easily detectable. Overexpression of Crk proteins did not change the phosphorylation pattern of IRS-4. In contrast, in cells overexpressing CrkII, IGF-I-stimulated tyrosine phos-
phorylation of IRS-1 was inhibited while phosphorylation of IRS-2 was significantly increased, compared with parental or CrkL-17 cells. Finally, using anti-IRS-4 antibody Crk-associated 180-kDa protein was characterized as IRS-4 (Fig. 4B).

To further characterize the interaction of the Crk proteins with IRS-4, GST fusion proteins encoding different domains of CrkL and CrkII were used to precipitate pp180 from total lysates of 293 parental cells stimulated with IGF-I (Fig. 5). As expected, GST full-length CrkL precipitated IRS-4 (Fig. 5, B–D). The association of GST-CrkL with this protein was greater with the use of a combination of SH2 and N-terminal SH3 domains as compared with each domain separately. To further characterize this interaction, SH2 and SH3 domains were mutated and GST-SH2(R39V)-SH3 and GST-SH2-SH3(WW-LL) fusion proteins were used in vitro binding assays (Fig. 5B). These experiments showed that in accordance with the inducible nature of interaction of CrkL with IRS-4, the SH2 domain played major role in this association when the N-terminal SH3 domain and, possibly, the interdomain region augmented the interaction. In agreement with data obtained using immunoprecipitations, the GST fusion protein containing full-length CrkII was able to precipitate pp180, although less effectively than the GST-CrkL (Fig. 5, A and D). In contrast to CrkL, the interaction between GST-CrkII and the IRS-4 depended entirely on the presence of the SH2 domain and addition of SH3 domains negatively regulated this association.

Despite the presence of IRS-1 in 293 cells, no IRS-1 could be detected in GST-CrkL or GST-CrkII precipitates but IRS-2 could easily be recognized, supporting the idea that Crk proteins specifically bind IRS-4 in 293 cells. To verify this hypothesis a protein-depletion approach was used (Fig. 6A). Total cell lysates from parental 293 cells stimulated with IGF-I were pretreated with GST or GST-CrkL (Fig. 6A, lanes 1–4). Analysis of proteins remaining in the lysates with anti-IRS-1-PH antibody, GST-CrkL or GST-p85 showed that a significant amount of tyrosine-phosphorylated 180-kDa proteins could be detected in lysates pretreated with GST alone (Fig. 6A, lanes 5–7). Pretreatment with GST-CrkL (lanes 8–10) depleted the CrkL-bound protein while GST-p85 was able to precipitate significant amounts of phosphorylated 180-kDa protein which was characterized as IRS-1 (data not shown). These data indicated that at least two different pp180 proteins were present in 293 cells and only one, different from IRS-1, specifically associated with CrkL. Decreased amounts of IRS-1 in GST-CrkL pretreated cell lysates (compare lanes 5 and 8) may be explained by our observations that anti-IRS-1-PH antibody may precipitate both IRS-1 and IRS-4 (data not shown and Footnote 2).

To examine the possibility of direct interactions of CrkL and CrkII with the receptor, the ability of GST fusion proteins encoding full-length or different domains of the Crk proteins to bind to IGF-I or insulin receptors directly from total cell lysates was tested. Results of these studies demonstrated (Fig. 6B), that no direct interaction between GST-CrkL and IGF-I or insulin receptors could be detected. In contrast, the SH2 domain of CrkII alone demonstrated a strong and specific interaction with the IGF-I receptor, although no interaction could be found with full-length CrkII. These data, together with the observations that immunoprecipitations were unable to detect association of CrkII or CrkL with the IGF-I receptor in intact cells (data not shown), lead us to conclude that direct interaction with the IGF-I receptor probably was not the main mechanism whereby CrkL and CrkII undergo phosphorylation. Nevertheless, this experiment demonstrated that SH2 domains of CrkL and CrkII may have different binding specificity at least to some phosphoproteins.

It has been shown that both CrkII (18) and CrkL (34) may bind the guanine nucleotide exchange proteins SOS and C3G, thus recruiting them to the plasma membrane and activating Ras and Rap 1, an antagonist of Ras, respectively. Proposed models (36) have considered CrkII as the main adaptor protein for C3G (37), thus implicating CrkII as a possible anti-oncogene (38). The final decision of which of the two pathways, Ras or Rap 1, is activated is dependent on the relative amounts of the CrkII and Grb2 adaptors, as well as C3G and SOS.
complexity to this system has been described by the finding that in v-Crk-transformed cells C3G specifically activated Jun kinase (JNK) and stimulated anchorage-independent growth of v-Crk-transformed cells in a JNK-dependent manner. This pathway likely progresses through Crk, C3G, and an unknown Ras-like G protein to activate JNK (25, 39). Although the data presented in this paper have concentrated on upstream mechanisms leading to Crk protein phosphorylation, we compared the activity of JNK in 293 parental and Crk overexpressing cell lines. Overexpression of CrkL, but not CrkII, significantly stimulated the activity of endogenous JNK (Fig. 6C), demonstrating once more that the related adaptor proteins CrkL and CrkII may play different roles in molecular signaling.

**DISCUSSION**

We have shown that the interplay of adaptor proteins CrkL and CrkII might regulate the signal transduction of the IGF-I receptor in 293 and NIH-3T3 cells. While both proteins are substrates involved in IGF-I receptor signaling, they apparently demonstrate important different properties and different biological responses. Evidence supporting this hypothesis includes (a) the inhibition of IGF-I-dependent cell cycle progression by overexpression of CrkII, (b) the oncogenic potential of CrkL versus the absence of this potential in CrkII overexpressing cell lines, (c) the differential interaction with the newly characterized IRS-4 molecule, and (d) the differential regulation of phosphorylation status of selective proteins in CrkII and CrkL overexpressing cell lines.

To date, BCR-ABL (40), Steel factor (41), Type I interferon receptor (42), and some cytokines (43) are the stimuli known to induce CrkL phosphorylation. Similarly, CrkII is known to be phosphorylated in response to a variety of stimuli (44), indicating a broad range of specificity of this protein. This may suggest that multiple mechanisms are involved in the phosphorylation of Crk proteins. Importantly, we have found that CrkL undergoes tyrosine phosphorylation in the rat uterus in response to injections of IGF-I (30). Thus in vivo, CrkL appears to be an endogenous substrate of the IGF-I receptor.

The biological role of the cellular Crk proteins is not known. p130Cas and paxillin, two major Crk-binding proteins, are phosphorylated upon integrin stimulation, suggesting that Crk proteins are involved in signaling from focal adhesion complexes (45–47). CrkII was shown to be involved in signaling from epidermal growth factor, nerve growth factor, and T-cell receptors (31, 35, 48). CrkL may participate in the SF/Kit pathway, which has been linked to several hematologic and nonhematologic neoplastic disorders, or be involved in Type I interferon receptor signaling, mediating antiproliferative, antiviral, and immunomodulatory activities. Results in this paper and others (19, 24) strongly implicate both CrkL and CrkII in IGF-I receptor-mediated signal transduction. Analysis of cell cycle progression of NIH-3T3 cells overexpressing CrkL or CrkII in response to IGF-I stimulation revealed no significant influence of CrkL on IGF-I stimulated proliferation. This was due to the already high level of mitotic activity produced by CrkL overexpression. On the other hand, CrkII exhibited an inhibitory function. Furthermore, overexpression of CrkL resulted in the oncogenic transformation of NIH-3T3 cells as reflected by the increased number of anchorage-independent colonies in soft agar assays, as well as partial serum- and cell density-independent cell cycle progression. These data are consistent with the data of Senechal et al. (33), implicating CrkL as an oncogene. The inhibitory effect seen with CrkII overexpressing cells raises doubts in the interpretation of our previous results using IGF-I-induced thymidine incorporation assay in the same cells. (19). Thus FACS analysis is apparently a more direct and reliable measurement of cell cycle progression.

Previously it had been shown that overexpression of CrkL in 293T, Rat-1, and NIH-3T3 cells (33, 34) resulted in increased.

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**Fig. 6.** A, specific interaction of CrkL and CrkII with IRS-4 following IGF-I stimulation. 500 ng of cell lysates prepared from 293 parental cells stimulated with 50 nM IGF-I for 5 min were treated twice with 100 ng of GST alone (lanes 1 and 2) or GST-CrkL (lanes 3 and 4) and the remaining supernatants were additionally treated with 6 μg of GST-CrkL (CrkL) or GST-p85 (p85) or immunoprecipitated with 8 μg of anti-IRS-1-PH antibody (IRS-1). Bound proteins were analyzed with the anti-phosphotyrosine antibody. 1 and II stand for the first and second pretreatment steps, respectively. B, analysis of the in vitro interactions of CrkL and CrkII with IGF-I and insulin receptors. NIH-3T3 cells overexpressing insulin receptors (IR12) (lanes 1–6) or IGF-I receptors (WT b3) (lanes 7–12) were stimulated with 50 nM ligand for 3 min. 300 ng of total cell lysates in RIPA buffer were treated with 6 μg of GST fusion proteins encoding CrkII and CrkL as indicated. 20 μg of total cell lysates of IR12 and WT b3 were loaded in lanes 13 and 14. Proteins were analyzed with the anti-phosphotyrosine antibody. The arrows indicate the positions of autophosphorylated insulin (IR) and IGF-I (IGF-IR) receptor β subunits. C, overexpression of CrkL in 293 cells stimulates endogenous JNK kinase activity. The experiment was performed as described under "Materials and Methods." Each cell line was plated in duplicate. C stands for control reactions where no Jun kinase was added. Each experiment was repeated at least twice.
basal tyrosine phosphorylation of CrkL. Confirming these findings we additionally show that overexpression of CrkII stimulated basal tyrosine phosphorylation of both CrkII and CrkL. In addition to CrkL phosphorylation, CrkII may regulate IGF-I-stimulated tyrosine phosphorylation of the IRS family of docking proteins. Overexpression of CrkII in 293 cells noticeably inhibited IGF-I-dependent tyrosine phosphorylation of IRS-I but enhanced tyrosine phosphorylation of IRS-2. Although both IRS-1 and IRS-2 may interact with IGF-I receptor NPXY motif (Tyr950) through their phosphotyrosine binding domains, only IRS-2 possesses an additional domain which exhibits strong interaction with the activated IGF-I receptor domain outside of the juxtamembrane region (49, 50). Recently, we demonstrated in vitro that the SH2 domain of CrkII interacted with the phosphorylated tyrosines of the juxtamembrane region of IGF-I receptor and especially with tyrosine 950 (51). Based on these data we speculate that CrkII but not CrkL may compete with IRS-I but not with IRS-2 for binding of tyrosine 950 of the IGF-I receptor.

Our data suggest that Crk proteins become tyrosine phosphorylated in response to IGF-I stimulation via a specific association with the recently described IRS-4 (29). This interaction is dependent upon IGF-I stimulation and may be detected in intact cells as well as in vitro. In intact cells CrkL and CrkII compete with each other for IRS-4 binding, thus indicating that both Crk proteins may share similar binding sites on IRS-4. Analysis of the in vitro interaction revealed that the combination of the SH2 and N-terminal SH3 domains of CrkL formed a stable complex with IRS-4 most effectively. Furthermore, we demonstrate that the SH2 domain of CrkL plays the most important role in this interaction while the N-SH3 and inter-domain region play stabilizing roles in the CrkL-IRS-4 interaction. In additional experiments we show that partially purified IGF-I receptor effectively phosphorylated GST-CrkL fusion proteins on tyrosines following activation of the receptor (data not shown), while no direct interaction between the IGF-I receptor and GST-CrkII could be detected. These data may suggest that other intermediate proteins, juxtaposing CrkL to the IGF-I receptor, may be involved in CrkL phosphorylation. Interestingly, a recent study has shown that direct binding of bacterially expressed v-Abl to GST-CrkL was not necessary to phosphorylate this adaptor protein in vitro (34). GST-CrkL fusion proteins effectively bound IRS-4 in an IGF-I-dependent manner, although lower amounts of IRS-4 could be found to associate with CrkII using immunoprecipitation techniques. This interaction was completely dependent on the presence of the SH2 domain with the addition of the SH3 domains negatively affecting this interaction. We speculate that such differences in the mechanisms of binding of the Crk proteins to IRS-4 may reflect different dynamic structures of CrkL and CrkII. These structures may be responsible for different mechanisms of association of Crk proteins with effector molecules and, as a result, lead to different biological roles for these adaptor proteins. The significantly decreased association of C3G, SOS, and c-Abl with immunoprecipitated CrkII, as compared with CrkL, in Ba/F3 cells has recently been described (52) and supports our hypothesis.

If phosphorylation of CrkL is solely dependent on tyrosine phosphorylation of IRS-4, then overexpression of CrkII should effectively block CrkL phosphorylation by competing for binding sites on IRS-4. Surprisingly, IGF-I-inducible tyrosine phosphorylation of CrkL was not affected by CrkII overexpression. Furthermore, stable CrkL-IRS-4 complex could be detected even after 90 min of stimulation of 293 parental and CrkL overexpressing cell lines (data not shown). However, overexpression of CrkII not only decreases the amount of IRS-4 co-phosphorylated with CrkL, but this interaction could not be detected in RIPA lysis buffer (data not shown), possibly indicating different stability of the CrkL-IRS-4 association in this particular case. It has been shown that upon phosphorylation, CrkII forms an intramolecular complex through the association of its SH2 domain with the phosphorylated Tyr221, possibly abolishing the interaction of CrkII with SH2 and SH3 domain-associating proteins (10, 37). Although the ability of CrkL to form a similar structure has not been evaluated, we were not able to detect CrkII nor CrkL in anti-phosphotyrosine immunoprecipitates (data not shown), supporting the idea that CrkL was able to form the intramolecular complex. To explain these data we hypothesize that CrkL is able to bind tyrosine-phosphorylated IRS-4 on two different regions. The “distal” region of IRS-4 may form stable complexes with CrkL, but CrkL in this position cannot effectively be phosphorylated by the IGF-I receptor; overexpressed CrkII may compete with CrkL for this binding site. In addition, CrkL may bind, although with lower affinity, a more “proximal” region on IRS-4, thereby becoming phosphorylated and then dissociating from the IGF-I receptor-IRS-4 complex due to formation of an intramolecular complex. A similar mechanism may be used, although less effectively, by CrkII. However, the significantly lower level of tyrosine phosphorylation of CrkII compare with CrkL after IGF-I stimulation of 293 cells, the inability of CrkL to inhibit this phosphorylation, the direct interaction of CrkII with the IGF-I receptor in vitro (51), and the possible competition of CrkII with IRS-I for binding to the receptor may indicate that the direct association of CrkII with the IGF-I receptor may contribute, at least in part, to IGF-I-dependent phosphorylation of CrkII in 293 cells. In fact, in order for GST-CrkII fusion proteins to be efficiently phosphorylated by purified IGF-I receptors in the in vitro kinase assay, the SH2 domain of CrkII must be present (51). Localization of Crk proteins-binding sites on IRS-4 are necessary to verify these hypotheses.

Alternatively, CrkII and CrkL may use other adaptor proteins or IGF-I-activated pathway(s) involving unknown tyrosine kinases to be phosphorylated on tyrosines in the IGF-I-dependent manner. We have begun to study potential adaptor proteins which may mediate such signaling pathways. Shc and p85 are potential intermediate proteins linking Crk to the tyrosine kinase-containing receptors (18, 41). In preliminary experiments we could find no interaction of the Crk proteins with Shc. In contrast, the p85 subunit of PI 3′-kinase could be co-precipitated with CrkL and CrkII, but the amount of p85 correlated with the co-precipitated IRS-4 (data not shown). Further experiments are necessary to characterize this interaction. The possible involvement of the adaptor protein Cbl is currently under investigation.

In conclusion, we have presented data that allow us to begin to delineate the different biological roles of the closely related adaptor proteins CrkL and CrkII. These differences have been found at the level of IGF-I-stimulated tyrosine phosphorylation of these proteins, as well as the ability of CrkII and CrkL to differentially regulate tyrosine phosphorylation of a number of proteins upon overexpression. Specifically we have described the association of the Crk proteins with the newly characterized IRS-4 protein and have proposed different mechanisms of CrkL and CrkII tyrosine phosphorylation in response to IGF-I stimulation. In addition we speculate that different dynamic structures for the highly homologous CrkL and CrkII may be responsible for different mechanisms of association of these proteins with effector molecules, thus activating different signaling pathways and resulting in different biological role of these proteins.
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REFERENCES

1. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, L. M., Dull, T. J., Gray, A., Cossent, L., Liao, Y.-C., Tsukahara, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) Nature 313, 117–142.

2. Ullrich, A., Gray, A., Ram, A., Yang-Feng, T., Tsukahara, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., and Fujita-Yamaguchi, Y. (1986) EMBO J. 5, 2593–2597.

3. Cheatham, B., and Kahn, C. R. (1995) Endocr. Rev. 16, 117–142.

4. Patti, M.-E., Sun, X.-J., Bruening, J. C., Araki, E., Lipes, M. A., White, M. F., and Kahn, C. R. (1995) J. Biol. Chem. 270, 24670–24673.

5. Waters, S. B., and Pessin, J. E. (1996) Trends Cell Biol. 6, 1–4.

6. Pronk, G. J., Miglade, J., Pellici, G., Pawson, T., and Bos, J. L. (1993) J. Biol. Chem. 268, 5747–5753.

7. Isakoff, S. J., Yu, Y.-P., Su, Y.-C., Blakie, P., Yajnik, V., Rose, E., Weidner, K. M., Sachs, M., Margolis, B., and Skolnik, E. Y. (1996) J. Biol. Chem. 271, 3959–3962.

8. Bruning, J., Winnay, J., Cheatham, B., and Kahn, C. (1997) Mol. Cell. Biol. 17, 1513–1521.

9. Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G.-S. (1993) J. Biol. Chem. 268, 11479–11481.

10. Skolnik, E. Y., Lee, C.-H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., and Schlessinger, J. (1993) EMBO J. 12, 1929–1936.

11. Skolnik, E. Y., Batzer, A., Li, N., Lee, C.-H., Loweinstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) Science 260, 1953–1955.

12. Keller, S. R., and Lienhard, G. E. (1994) Trends Cell Biol. 4, 115–119.

13. Seely, B. L., Reichart, D. R., Staubs, P. A., Zhu, B. H., Hsu, D., Maegawa, H., Milaraki, K. L., Saltiel, A. R., and Olefsky, J. M. (1995) J. Biol. Chem. 270, 19151–19157.

14. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1995) Mol. Cell. Biol. 15, 3482–3489.

15. Matsuda, M., Tanaka, S., Hattori, S., Kurata, T., and Shibuya, M. (1996) Mol. Cell. Biol. 16, 3419–3423.

16. de Jong, R., Haataja, L., Voncken, J., Araki, E., Lipes, M. A., White, M. F., and Kahn, C. R. (1995) J. Biol. Chem. 270, 24670–24673.

17. Parrizas, M., Gazi, A., Levitzki, A., Wertheimer, E., and LeRoith, D. (1997) Endocrinology 138, 142–143.

18. Higuchi, R. (1889) in PCR Technology: Principles and Applications for DNA Amplification (Erlich, H. A., ed) pp. 61–70, Stockton Press, New York.

19. Lavan, B. E., Fantin, V. R., Chang, E. T., Lane, W. S., Keller, S. R., and Lienhard, G. E. (1997) J. Biol. Chem. 272, 24103–24107.

20. Butler, A. A., Blakesley, V. A., Koval, A., de Jong, R., Groffen, J., and LeRoith, D. (1997) J. Biol. Chem. 272, 27660–27664.

21. Sawadkosi, S., Ravichandran, K. S., Lee, K. K., Chang, J.-H., and Burakoff, S. J. (1995) J. Biol. Chem. 270, 26583–26586.

22. Nakamura, S., and Hattori, S. (1994) Oncogene 13, 4409–4415.

23. Kizaka-Kondoh, S., Matsuda, M., and Okayama, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12177–12182.

24. Beiter-Johnson, D., and LeRoith, D. (1995) J. Biol. Chem. 270, 5187–5190.

25. Tanaka, S., Ouchi, T., and Hanafusa, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2356–2361.

26. ten Hoeve, J., Kaartinen, V., Fioretti, T., Haataja, L., Voncken, J., Heisterkamp, N., and Groffen, J. (1994) Cancer Res. 54, 2563–2567.

27. Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Piskie, E., Prasad, K. V. S., and Griffin, J. D. (1997) J. Biol. Chem. 272, 10248–10253.

28. Ahmad, S., Alsayed, Y. M., Druker, B. J., and Platanias, L. C. (1997) J. Biol. Chem. 272, 29999–29994.

29. Chin, H., Saito, T., Arai, A., Yamamoto, K., Kiyama, R., Miyaoka, N., and Miura, O. (1997) Biochem. Biophys. Res. Commun. 239, 412–417.

30. Matuda, S., and Kurata, T. (1996) Cell Signalling 8, 335–340.

31. Salgia, R., Uemura, N., Okuda, K., Li, J., Piskie, E., Sattler, M., de Jong, R., and Griffin, J. D. (1995) J. Biol. Chem. 270, 24633–24639.

32. Hamaasaki, K., Shimada, M., Morino, N., Furuya, H., Nakamoto, T., Aizawa, S., Iwamatsu, A., Hirai, H., and Nojima, Y. (1986) Biochem. Biophys. Res. Commun. 142, 338–343.

33. Sawada, K., and Teer, D. (1985) Cancer Res. 45, 923–929.

34. Uemura, N., Salgia, R., Li, J., Piskie, E., Sattler, M., and Griffin, J. D. (1997) Leukemia 11, 376–385.