The Crk proto-oncogene product is an SH2 and SH3 domain-containing adaptor protein which we have previously shown to become rapidly tyrosine phosphorylated in response to stimulation with insulin-like growth factor I (IGF-I) in NIH-3T3 cells. In order to further characterize the role of Crk in the IGF-I signaling pathway, NIH-3T3 and 293 cells were stably transfectcd with an expression vector containing the Crk cDNA. The various resultant 3T3-Crk clones expressed Crk at approximately 2-15-fold higher levels than parental 3T3 cells. In 3T3-Crk cells, Crk immunoreactivity was detected in insulin receptor substrate-1 (IRS-1) immunoprecipitates. Stimulation with IGF-I resulted in a dissociation of Crk protein from IRS-1. In contrast, the association of the related adaptor protein Grb2 with IRS-1 was enhanced by IGF-I stimulation. Similar results were obtained in stably transfected 293-Crk cells, which express both IRS-1 and the IRS-1-related signaling protein 4PS. In these cells, IRS-1 and 4PS both associated with Crk, and this association was also decreased by IGF-I treatment, whereas the association of Grb2 with IRS-1 and 4PS was enhanced by IGF-I. Overexpression of Crk also enhanced IGF-I-induced mitogenesis of NIH-3T3 cells, as measured by [3H]thymidine incorporation. The levels of IGF-I-induced mitogenesis were proportional to the level of Crk expression. These results suggest that Crk is a positive effector of IGF-I signaling, and may mediate its effects via interaction with IRS-1 and/or 4PS.

The c-Crk protein is a cellular homolog of the viral oncogene v-Crk product, an SH2 and SH3 domain-containing protein, encoded by the avian sarcoma virus CT10 (1). Crk proteins share homology with the adaptor proteins Grb2 and Nck (2, 3), and are known to associate with two guanine nucleotide releasing proteins, C3G and mSos (4, 5). These studies suggested that Crk may participate in growth factor-induced activation of Ras. In addition, we have recently shown that stimulation of IGF-I receptors results in rapid tyrosine phosphorylation of Crk (6).

Thus, it was of interest to further characterize mechanisms by which Crk could be involved in IGF-I receptor signaling pathways.

IRS-1 is a major phosphotyrosine substrate of the IGF-I and insulin receptors (7-9). IRS-1 acts as a docking protein, associating through multiple phosphorylated tyrosine residues with various SH2 domain-containing proteins, including the p85 subunit of phosphatidylinositol 3-kinase (10-13), Grb2 (14-16), Syp (17-19), and Nck (20). 4PS (also termed IRS-2) is an IRS-1-related molecule that functions similarly in coupling the IL-4 receptor to intracellular signaling pathways (21) and can also partially mediate insulin signaling in IRS-1-deficient animals (22, 23). Given that stimulation with IGF-I results in tyrosine phosphorylation of Crk (6) and the interaction of IRS-1 with the Crk-related molecules Grb2 and Nck, it was of interest to determine whether IRS-1 and 4PS also associate with Crk. In the present study we find that c-Crk associates with IRS-1 and 4PS and enhances IGF-I-induced mitogenesis.

MATERIALS AND METHODS

Cell Culture—Both human embryonic kidney carcinoma (293) cells and NIH-3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Upstate Biotechnology Inc., Lake Placid, NY). Prior to growth factor stimulation, subconfluent cultures of cells in 60- or 100-mm dishes were switched to serum-free DMEM supplemented with 0.1% insulin-free bovine serum albumin (Intergen, Purchase, NY) and 20 mM HEPES (pH 7.5) for 18 h. Cells were treated with IGF-I (Genentech, San Francisco, CA) diluted in serum-free DMEM at 37°C for various time points and concentrations, as indicated.

Stable Transfections—Cells were stably transfected with either pCXN2-CRK II, a Crk expression vector driven by the cytomegalovirus promoter and carrying neo' (kindly provided by M. Matsuda, Tokyo, Japan), or the pCXN2 vector from which the Crk cDNA had been excised. The Crk cDNA was excised from pCXN2-CRK II by cleavage with EcoRI and religation. Cells were transfected with 10 μg of either pCXN2-CRK II or the pCXN2 vector using the Lipofectamine reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's recommended conditions. Selection in 0.5 mg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) began 48 h after transfection. After 2 to 3 weeks of selection in G418, individual colonies were isolated using 0.8-mm glass cloning cylinders (PGC Sciences, Gaithersburg, MD).

Characterization of Resulting Clones—Clones were screened for Crk overexpression by immunoblotting for Crk as described below. Twenty-nine 3T3-Crk clones were obtained with Crk levels ranging from 2–15-fold overexpression, as compared to parental NIH 3T3 cells or 3T3-Neo cells (those transfected with the pCXN2 vector). Twelve 293-Crk clones were obtained with Crk levels ranging from 3–12-fold overexpression, as compared to parental 293 cells or 293-Neo cells, transfected with the pCXN2 vector. IGF-I receptor numbers in 3T3-Neo and 3T3-Crk cells were determined by Scatchard analysis using 125I-IGF-I as a tracer, as described previously (24).

Immunoprecipitations—After treatment with growth factors, cells were washed twice with ice-cold phosphate-buffered saline and har-
vested in a lysis buffer containing 50 mM HEPES (pH 7.4), 2 mM sodium orthovanadate, 100 mM NaCl, 4 mM sodium pyrophosphate, 200 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1% Triton X-100. Lysates were incubated for 1 h at 4 °C, then centrifuged at 10,000 × g for 30 min at 4 °C to remove Triton-insoluble material. Protein content of the lysates was determined by the Bio-Rad method. 600 μg of protein from each dish was immunoprecipitated overnight at 4 °C with rabbit polyclonal anti-sonic (1:100) specific for either IRS-1 or 4PS (21, 25), followed by adsorption to 50 μl of 30% Protein A-Sepharose beads (Pharmacia Biotech Inc.) for 5 h at 4 °C. Immunoprecipitates were washed 3 times with ice-cold immunoprecipitation buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 0.5% Nonidet P-40. The entire immunoprecipitated samples were then boiled for 2 min in sample buffer containing 50 mM Tris (pH 6.7), 2% SDS, 2% β-mercaptoethanol, and bromphenol blue as a marker. Samples were then run on 9% SDS-PAGE gels and transferred to nitrocellulose membranes using standard electrophoresis and electroblotting procedures. Prestained molecular weight markers were obtained from Sigma (#6DS-7B).

Immunoblotting—Nitrocellulose membranes were blocked with either 3% insulin-free bovine serum albumin (for phosphorylation blotting) or 3% nonfat dry milk in a PBST buffer containing 10 mM sodium phosphate, 0.1% Tween 20. Blots were then immunolabeled overnight at 4 °C for phosphorysorine (RC20H, 1:2500, Transduction Labs, Lexington, KY), Crk (1:1000, Transduction Labs, Lexington, KY), Grb2 (1:1000, Transduction Labs, Lexington, KY), IRS-1 (1:500) or 4PS (1:500) (21). Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's conditions. Some blots were stripped and re-probed with another antibody. Blots were stripped by incubation for 1 h at 50 °C in a solution containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 0.7% β-mercaptoethanol. Blots were then washed for 1 h in several changes of PBST at room temperature, and probed with ECL to confirm that antibodies had been completely removed. Blots were then re-blocked and immunolabeled as described above.

Thymidine Incorporation Assays—Subconfluent cell monolayers in 12-well plates were grown to quiescence in serum-free DMEM with 0.1% bovine serum albumin for 24 h. Cells were then incubated for 16 h in DMEM containing 0.1% bovine serum albumin plus various concentrations of IGF-I. Each cell line was plated in triplicate for each concentration of IGF-I. The media was replaced with fresh serum-free DMEM containing 1 μCi/well of [3H]thymidine, and the cells were incubated for an additional hour. The cells were rinsed twice with ice-cold phosphate-buffered saline, twice with ice-cold 5% trichloroacetic acid, and twice with ice-cold 95% ethanol. The cells were then lysed in 0.3 ml of 1 N NaOH, neutralized with 0.3 ml of 1 N HCl, and solubilized samples were counted by liquid scintillation.

RESULTS

NIH-3T3 cells were stably transfected with a Crk cDNA, as described. 3T3-Crk clones were selected in G418 and screened for Crk expression by immunoblot analysis. Out of 29 clones obtained, several clones were selected to use for further studies. As shown in Fig. 1A, two clones, 3T3-Crk7 and 3T3-Crk9, expressed 10- and 3-fold higher levels of Crk, respectively, than 3T3 parental or 3T3-Neo cells, as determined by densitometric scanning. These clones were chosen in order to evaluate the effects of relatively high levels of Crk expression (3T3-Crk7) and moderate levels of Crk expression (3T3-Crk9) on IGF-I action. Crk was also stably transfected into the 293 human embryonic kidney carcinoma cell line which, like NIH-3T3 cells, expresses endogenous IGF-I receptors. Interestingly, these cells also express relatively high levels of the IRS-1-like signaling protein 4PS, and IGF-I activates both IRS-1 and 4PS in this cell line (see below). Twelve 293-Crk clones were obtained after G418 selection, and several were selected for further study, including 293-Crk4 and 293-Crk6. These cell lines expressed 10-fold and 12-fold higher levels of Crk immunoreactive protein, respectively, as compared to 293 parental cells (Fig. 1B).

Serum-deprived 3T3-Crk7 cells were incubated for 5 min in the presence or absence of 100 nM IGF-I. Cleared whole cell lysates were immunoprecipitated with anti-IRS-1 serum and proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described. Using prestained molecular weight markers as a guide, blots were cut into three strips containing tyrosine-phosphorylated IRS-1 (180kDa), Crk (40kDa), or Grb2 (23kDa). As shown in Fig. 2A, association of IRS-1 with Crk was also detected in two independent clones that expressed lower levels of Crk, 3T3-Crk17 and 3T3-Crk 9. These clones express Crk at 8-fold and 3-fold higher levels, respectively, than in parental cells (data not shown). IGF-I also decreased the amount of Crk associated with IRS-1 in these cells (Fig. 2B).

We found a similar Crk association with IRS-1 in 293-Crk6 cells. In this cell type, IGF-I induced tyrosine phosphorylation of IRS-1, and IRS-1 immunoprecipitates also contained Crk and Grb2 proteins (Fig. 3). Again, whereas Crk association with IRS-1 was markedly decreased by IGF-I, Grb2 association was enhanced. In addition to expressing IRS-1, 293 cells also express high levels of the IRS-1-related signaling protein, 4PS as determined by immunoblot analysis utilizing an anti-4PS serum that does not recognize IRS-1.2 IGF-I also induced tyrosine phosphorylation of 4PS (Fig. 3). Crk and Grb2 were detected in 4PS immunoprecipitates, and similarly, Crk association with 4PS was decreased by IGF-I stimulation, whereas

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2 D. Betnner-Johnson, V. A. Blakesley, Z. Shen-Orr, M. J. Jimenez, B. Stannard, L-M. Wang, J. Pierce, and D. LeRoith, unpublished observations.
Grb2 association was increased. Identical results were obtained with the 293-Crk4 clone (data not shown).

We considered the possibility that overexpression of Crk could alter the level of IRS-1 or IGF-I receptor expression. In Fig. 4 it can be seen that IRS-1 levels were not different in 3T3-Neo and 3T3-Crk cells, as measured by immunoblotting. We also found IGF-I receptor numbers to be equivalent in these cells, as measured by radioligand binding. Receptor numbers per cell were: 3T3-Neo4, 54,000; 3T3-Neo11, 67,000; 3T3-Crk7, 61,000; and 3T3-Crk9, 52,000.

In order to evaluate the functional effects of Crk overexpression on IGF-I signaling, the mitogenic effects of IGF-I were compared in two 3T3-Crk clones and two 3T3-Neo clones using a \([^{3}H]\) thymidine incorporation assay. As shown in Fig. 5, 3T3-Crk7 and 3T3-Crk9 were significantly more responsive to IGF-I than were 3T3-Neo4 and 3T3-Neo11. While the 3T3-Crk7 exhibited the highest levels of IGF-I-induced thymidine incorporation, 3T3-Crk9 displayed intermediate levels, and the two 3T3-Neo cell lines had the lowest levels of thymidine incorporation. These findings are consistent with the levels of Crk expressed by these cell types, namely, 3T3-Crk7 > 3T3-Crk9 > 3T3-Crk-Neo (see Fig. 1).

DISCUSSION

Very little is known about the role played by Crk in growth factor receptor signal transduction. These studies show that in two Crk overexpressing cell types, Crk interacts both with IRS-1 and with the IRS-1-related signaling molecule, 4PS. IRS-1 and 4PS function as multisite “docking proteins” which link insulin and IGF-I receptors as well as IL-4 receptors (9, 21, 25, 26) with various post-receptor signaling systems. The typical mechanism of such associations is via interaction of phosphotyrosine residues within the IRS-1 molecule with SH2 do-
mechanism of interaction of these molecules, and to delineate the intracellular pathways by which Crk is involved in IGF-I induced mitogenesis.

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The Proto-oncogene Product c-Crk Associates with Insulin Receptor Substrate-1 and 4PS: MODULATION BY INSULIN GROWTH FACTOR-I (IGF) AND ENHANCED IGF-I SIGNALING

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