In Vivo Regulation of CrkII and CrkL Proto-oncogenes in the Uterus by Insulin-like Growth Factor-I

Differential Effects on Tyrosine Phosphorylation and Association with Paxillin*

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Changes in CrkII and CrkL phosphorylation are associated with insulin-like growth factor receptor activation in cultured cells. We examined whether similar changes also occur following administration of recombinant human insulin-like growth factor-I to the intact animal. In female rats starved overnight, CrkL phosphorylation was significantly increased 12 min after insulin-like growth factor-I administration. Tyrosine phosphorylation of CrkII was not detectable in either control or treated animals. Paxillin, a 65–70-kDa phosphoprotein containing high affinity binding sites common for the Src homology 2 (SH2) domains of CrkII and CrkL, was observed in both CrkII and CrkL immunoprecipitates. Insulin-like growth factor-I treatment stimulated the association of CrkII with paxillin. In contrast, the same treatment resulted in the dissociation of the CrkL-paxillin complex. Similar effects of insulin-like growth factor-I treatment on the association of CrkL with tyrosine phosphorylated paxillin were observed in fibroblasts overexpressing CrkL. This study demonstrates that the activation of the insulin-like growth factor-I receptor induces changes in the tyrosine phosphorylation and protein-protein interactions of the Crk proteins in vivo. The different responses of CrkII and CrkL to insulin-like growth factor-I receptor activation suggest distinct roles for these two adapter proteins in signal transduction.

The insulin-like growth factors (IGFs)-I and IGF-II are related structurally and, to some extent, functionally to insulin. The expression of the IGFs and their receptors is essentially ubiquitous during fetal and postnatal development, acting as autocrine/paracrine factors to regulate cellular mitogenesis and differentiation that are asssociated with tissue and organ development (1, 2). The IGFs have been demonstrated in vitro to act as progression factors in several cell types, stimulating progression from G1 to S phase of the cell cycle. The cellular responses to both IGF-I and -II are believed to occur primarily in response to the activation of the type I IGF receptor (IGF-IR). The IGF-IR is structurally similar to the insulin receptor, consisting of \( \alpha \) subunits, while the \( \beta \) subunit contains the tyrosine kinase domain and various motifs responsible for the interaction with intracellular signaling proteins such as Shc, insulin receptor substrate (IRS)-1, and IRS-2. In addition to effects on mitogenesis and differentiation, the IGF-IR has been implicated in the regulation of cell motility, apoptosis, and tumorigenesis (1–3).

Several second messenger systems have been implicated in signaling the changes in cell behavior associated with IGF-IR activation. The phosphorylation of IRS-1 is an immediate consequence of IGF-IR activation (2, 4). Phosphorylated IRS-1 then acts as a docking protein for multiple signaling proteins including Grb2, the p85 subunit of phosphoinositol-3'-kinase, the phosphatase PTP1D (Syp), Nck, and CrkII (4, 5). Adapter proteins such as Grb2, Nck, and Crk function to mediate protein-protein interactions and are believed to have an important role in regulating the activity of intracellular second messenger systems (6).

v-Crk and its cellular homologs CrkII (7), and the more recently discovered Crk-like or CrkL protein (8), are composed of Src homology (SH) domains and lack intrinsic enzymatic activity. SH2 and SH3 domains are modular structures found in many proteins and have been implicated in protein-protein interactions (6). Both Crk proteins are comprised of a single N-terminal SH2 domain and two SH3 domains (SH3-N, SH3-C), with a third member of the Crk family, CrkL, lacking the SH3-C due to the alternate splicing of CrkII mRNA (7). The inducible binding of the Crk SH2 domains to phosphotyrosine motifs contained in the target proteins is believed to localize effector proteins bound constitutively to the Crk SH3 domains. Thus, the SH2 domain of CrkII has recently been suggested to link the focal adhesion proteins p130Cas and paxillin to the Jun kinase (JNK) pathway (9). The guanine nucleotide exchange protein C3G is bound constitutively to the SH3 domains in CrkII and is believed to activate JNK, which has been shown to be essential for the oncogenic functions of v-Crk (9). Both Crk proteins have also been shown to mediate the association of C3G with tyrosine phosphorylated Cbl (10, 11). However, the Crk SH3 domains also bind to other signaling proteins including the nonreceptor tyrosine kinase Abl (12) and DOCK180 (13), suggesting that several pathways may be regulated by Crk. For example, CrkL reportedly links paxillin to BCR-Abl, an oncogenic form Abi (14).

CrkII is tyrosolphosphorylated following the activation of the nerve growth factor receptor (trkA) (15), the B-cell antigen receptor (BCR) (16), and the IGF-IR (5, 17). Two nonreceptor kinases, c-Arg and c-AbI, have been shown to tyrosine phos-
phosphorylate Crk in vitro, while in fibroblasts derived from c-Ab1 knockout mice (AbI-/-), Crk phosphorylation is inhibited (12). The tyrosine phosphorylation of tyrosine 221 or 222, depending on the species, located between the SH3-N and SH3-C of CrkII results in the formation of an intramolecular binding site for the SH2 domain (18). The formation of this intramolecular bond is believed to prevent the N-terminal SH2 and SH3 domains from associating with other proteins (12, 18). Thus tyrosine phosphorylation represents a possible mechanism to control the protein binding activity of CrkII. Since a similar tyrosine-containing motif is also present in CrkL, the phosphorylation of this tyrosine (tyrosine 207) may regulate CrkL binding activity by a similar mechanism.

While the function and regulation of the Crk proteins have been extensively examined in cell culture and in vitro systems, few studies have examined Crk in the intact animal. Accordingly, we examined whether the phosphorylation and protein-protein association of endogenous CrkII and CrkL change in response to IGF-IR activation in an IGF-responsive developing tissue, the uterus. The IGF-IR is known to have a critical role in the response to IGF-IR activation in an IGF-responsive developing tissue, and IRS-1 (20) may regulate CrkL binding activity by a similar mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibodies to CrkII and Paxillin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies to phosphotyrosine (PY20 and 4G10) were purchased from Transduction Laboratories and Upstate Biotechnology, Inc. (Lake Placid, NY) respectively. Polyclonal antibodies to CrkII, CrkL, and IRS-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked sheep-anti-mouse and donkey-anti-rabbit IgGs and the ECL system were purchased from Amersham Corp. (Arlington Heights, IL). The recombinant human (rh-) IGF-I used for these studies was kindly provided by Ciba Geigy (Summit, NJ). Female Sprague-Dawley rats (50–80 d) were purchased from Taconic (Germantown, NY). All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the NIDDK, Bethesda, MD. Dulbecco’s modified Eagle’s medium (DMEM), glutamine, and penicillin/streptomycin/fungizone were from Transduction Laboratories and Upstate Biotechnology, Inc. (Lake Placid, NY). Bovine serum was from Upstate Biotechnology, Inc. (Arlington Heights, IL). Bovine serum albumin (BSA) and 20 mM HEPES (pH 7.5) for 18 h prior to stimulation with 10 nM rhIGF-I.

Stable transfectants of NIH3T3 cells overexpressing CrkII were generated using pCOWN2-CrkII essentially as described for CrkII (5). CrkII cDNA was excised from pS85-CrkII (21) and subeloned into pCOWN. The resulting plasmid was used for lipofection (Life Technologies, Inc., Grand Island, NY) followed by selection using Geneticin (418, Life Technologies, Inc.).

**Immunoprecipitation and Western Blot Analysis**—Immunoprecipitations were performed using 800–1600 μg of protein. For IRS-1 immunoprecipitations, lysates were diluted 1:4 in buffer B (50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 0.67 mM Na3OVO4, 0.4 mM sodium molybdate, 10 mM NaF, 0.133% SDS, 0.5% deoxycholate, 1% Triton X-100, and 5% glycerol). For all other immunoprecipitations, the samples were diluted in buffer C (50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 0.2 mM PMSF, 1% Triton X-100). Samples were precleared by incubation with 50 μl of protein A-Sepharose (10% w/v in 50 mM Tris-Cl, pH 7.5) for 1–2 h at 4 °C. After an overnight incubation at 4 °C with antibody, samples were incubated with protein A-Sepharose for 2–4 h at 4 °C, washed with ice-cold buffer C (20 mM HEPES, 150 mM NaCl, 10 mM EDTA, 0.1 mM EGTA, 0.2 mM PMSF, 0.2 mM Na3OVO4, 1% Triton X-100, and 0.5% Nonidet P-40). Proteins were resolved by electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose filters for 4 h at 0.23 mA. Filters were blocked with 5% BSA in PBS plus 0.1% Tween 20 (PBST) for 1 h at room temperature. Primary antibodies in PBST plus 1% BSA were allowed to bind to proteins overnight at 4 °C. After washing in PBST, the filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, followed by extensive washes in PBST plus 0.1% Triton X-100. Proteins were detected using chemiluminescence (ECL, Amersham Corp., Arlington Heights, IL).

**Data Analysis—Calculations and statistical analysis (t-test using Bonferroni’s correction for multiple comparisons) were performed using Excel Version 6.0 (Microsoft Corp.).**

**RESULTS**

**IGF-I Increases IRS-1 Tyrosine Phosphorylation**—Prior to determining whether IGF-IR activation induced changes in Crk phosphorylation, we examined as a positive control the effect of IGF-I treatment on the tyrosine phosphorylation of IRS-1. Fig. 1 is a representative Western blot showing the effect of IGF-I treatment on the tyrosine phosphorylation of IRS-1 in the rat uterus. Treatment of female rats starved overnight was associated with an approximately 2.0-fold increase in the tyrosine phosphorylation of IRS-1, which was statistically significant at 3 min (p < 0.05) (Fig. 1A and B). Immunoblotting with an anti-IRS-1 antibody confirmed that approximately equal amounts of IRS-1 protein were being precipitated, with the phosphorylation data shown in Fig. 1B adjusted to account for any differences in the amount of IRS-1 protein in the precipitates.

**Tyrosine Phosphorylation of CrkL Is Increased by IGF-I Treatment**—CrkL immunoprecipitates from lysates of starved female rats treated with rhIGF-I or diluent using a polycyclical antibody revealed a broad band that migrated slower than CrkII. Immunoblotting of CrkL immunoprecipitates with anti-phosphotyrosine antibodies showed that only the slower migrating isoform of CrkL was phosphorylated on tyrosine residues in both the basal and IGF-I-stimulated state (Fig. 2A). Treatment with rhIGF-I was associated with an increase in the tyrosine phosphorylation of the upper CrkL band compared with basal levels (Fig. 2A). The amount of tyrosine phosphorylation in CrkL immunoprecipitates as determined by densitometry exhibited an approximately 2-fold increase in response to rhIGF-I treatment 3, 6, and 12 min after injection (Fig. 2B, and data not shown).

**CrkII Exhibits Undetectable Levels of Tyrosine Phosphorylation**—To examine CrkII tyrosine phosphorylation, CrkII immunoprecipitates were blotted with anti-phosphotyrosine antibodies or anti-CrkII antibodies. As with CrkL, a broad band was observed in immunoprecipitates using a polyclonal CrkII antibody, which on some gels resolved into a doublet. Tyrosine phosphorylation data shown in Fig. 2B.
phosphorylation of CrkII was not detected, even upon prolonged exposure of immunoblots (30 min, data not shown). In comparison, tyrosine phosphorylation was easily observed in CrkL immunoprecipitates after a 30-s exposure. Treatment with rhIGF-I did not result in a change in CrkII phosphorylation in the rat uterus (data not shown).

IGF-I Differentially Regulates the Association of CrkII and CrkL with Tyrosine Phosphorylated Paxillin in the Uterus—Having shown that IGF-I differentially regulates Crk phosphorylation, we next examined whether IGF-I also affected the association of CrkII and CrkL with other signaling proteins. We observed that a diffuse tyrosylphosphorylated protein in the region of 60–80 kDa was present in both CrkII and CrkL immunoprecipitates. Paxillin has an apparent molecular weight similar to the tyrosylphosphorylated protein we observed and has been shown to contain high affinity binding sites for the Crk SH2 domain (12, 22). The presence of paxillin in CrkII and CrkL immunoprecipitates was confirmed by immunoblotting (Figs. 3 and 4). Treatment of starved female rats with rhIGF-I differentially regulated the association of tyrosylphosphorylated paxillin with CrkL and CrkII. The amount of paxillin in CrkL immunoprecipitates was reduced following rhIGF-I treatment (Fig. 3). The apparent dissociation of the paxillin-CrkL complex was observed to occur as early as 3 min after IGF-I treatment (data not shown). In contrast, treatment with rhIGF-I stimulated a marked increase in the association of paxillin with CrkII (Fig. 4). The increase in the association of paxillin with CrkII occurred as early as 3 min (data not shown).

The Effect of IGF-I Treatment of Fibroblasts Overexpressing CrkL—Since there have been no reports concerning the regulation of the association of either Crk protein with paxillin by IGF-I in vitro, we next examined whether the changes in the association of Crk with paxillin observed in vivo also occur in cultured cells. The data from cell culture studies were similar to that observed in vivo. Fig. 5 shows the effect of IGF-I treatment on the association of CrkL with paxillin in NIH3T3 fibroblasts overexpressing CrkL (D20 and D22). IGF-I treatment resulted in a marked reduction in the association of tyrosine-phosphorylated paxillin (Fig. 5).

**DISCUSSION**

CrkII and CrkL are involved in many processes requiring changes in the activity of intracellular signaling proteins, rang-
IGF-I treatment increased the association of CrkII with paxillin in the rat uterus. A recent report has shown that v-Crk signals via C3G and a Ras-like protein to the JNK pathway, with paxillin and p130Cas postulated to act as docking sites important for the colocalization of the substrates necessary for the activation of this pathway (9). Our data indicate that this pathway may also play an important role, and be physiologically relevant, in defining the actions of IGF-I on the growth and development of the uterus. While in vitro data indicate that the JNK pathway is activated by v-Crk, it is also possible that CrkII is affecting other pathways. For example, CrkII has also been shown to be important for Ras-induced ERK activation by the nonreceptor tyrosine kinase Abl while having no effect on epidermal growth factor receptor signaling to Ras (27).

CrkL phosphorylation in the uterus increased markedly following IGF-I stimulation. In contrast, CrkII tyrosine phosphorylation was not detected and was not affected by IGF-I stimulation. The c-Abl and c-Arg tyrosine kinases have been reported to interact with and phosphorylate both CrkL and CrkII in vitro, while Crk phosphorylation is markedly reduced in cells not expressing Abl (12). The constitutive interaction of CrkII and CrkL with Abl occurs through the binding of the Crk SH3 domains to a proline-rich motif in c-Abl. One possible explanation for the differential phosphorylation of CrkII and CrkL observed in the uterus is that the subcellular localization of CrkII, CrkL, and c-Abl are discrete in a way that does not favor phosphorylation of CrkII. Presumably, the Abl kinase is able to interact with and tyrosine phosphorylate CrkL in response to the stimulation of the IGF-IR. Another possibility is that a novel tyrosine kinase, specific for CrkL, is activated following IGF-I stimulation and is responsible for the phosphorylation of CrkL. Given the previous reports showing that CrkL
and CrkII associate with similar proteins, and that Crk is not phosphorylated in Ab1+ fibroblasts (12, 21, 28), the former hypothesis appears to be more likely.

CrkII bound to paxillin under conditions where there was dissociation of CrkL, and in fact, CrkII association with paxillin increased markedly following IGF-I stimulation. This result suggests that the CrkLI SH2 domain was no longer able to bind to tyrosine residues on paxillin following IGF-I stimulation. The phosphorylation of CrkL on tyrosine 207 and CrkII on tyrosine 221 results in the formation of an intramolecular SH2 binding site, inhibiting the intermolecular interactions of the Crk proteins (12, 18). If this model is correct, the increased tyrosine phosphorylation of CrkL associated with IGF-I stimulation in the present study may account for the dissociation of CrkL from paxillin. Since we were unable to detect either CrkL or CrkII in paxillin immunoprecipitates (data not shown), we cannot determine which of the CrkII or CrkL isoforms bind to paxillin or, indeed, if phosphorylated CrkL forms a complex with paxillin.

The difference in the effect of IGF-IR stimulation on the binding of CrkII and CrkL to paxillin is particularly intriguing given that the CrkII and CrkL SH2 domains have been reported to bind to the same two (PYXXP motifs (Tyr-31 and Tyr-118) of the paxillin protein (14, 26). This result may imply that CrkL associates with paxillin in the unstimulated state and is displaced by CrkII following IGF-IR activation of a CrkL tyrosine kinase. CrkL appeared to be more abundant in the uterus; therefore, when CrkL tyrosine phosphorylation is low it may act to inhibit the downstream effects of CrkII by saturating the Crk SH2 binding sites present in paxillin. Importantly, this result also implies that in the natural state CrkL and CrkII may be activating distinct signaling pathways, perhaps with mutually exclusive functions. In any event, the results from this study imply that the protein binding activity of CrkII and CrkL may be regulated independently following IGF-IR activation.

The effect on IGF-IR activation on the association of CrkL and CrkII with paxillin may indicate a role for the focal adhesion complex in IGF-IR signal transduction. Paxillin, as a component of the focal adhesion complex (26, 29), may serve as a physiological response to IGF-IR activation (2, 4). In the uterus, estrogen response to IGF-IR activation (2, 4). In the uterus, estrogen is associated with an increase in the activity of the IGF-IR in the intact animal. These observations suggest that the formation and dissociation of multiprotein complexes containing Crk proteins are a physiological response to IGF-IR activation and may be involved in the stimulation of uterine growth by IGF-I. The different responses of CrkII and CrkL in terms of phosphorylation and association with paxillin indicate that these two proteins may possess distinct roles in the IGF-IR signal transduction process. Together, the results from this study provide important insights into the signal transduction process of a mitogenic receptor in the growing animal and provide the basis for future studies.

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