The Functional Role of CrkII in Actin Cytoskeleton Organization and Mitogenesis*  

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Crk is a member of a family of adapter proteins predominantly composed of Src homology 2 and 3 domains, whose role in signaling pathways is presently unclear. Using an in situ electroporation system which permits the introduction of glutathione S-transferase (GST) fusion proteins into cells, we found that c-CrkII bound to p130cas, but not to paxillin in serum-starved rat-1 fibroblasts overexpressing the human insulin receptor (HIRc cells) in vivo. 17 nM insulin stimulation dissociated the binding of c-CrkII to p130cas, whereas 13 nM insulin-like growth factor-I, 16 nM epidermal growth factor (EGF), and 10% serum each showed little or no effect. We found that stress fiber formation is consistent with a change in the p130cas-c-CrkII interactions before and after growth factor stimulation. Microinjection of either GST-Crk-SH2 or -Crk-(N)SH3 domains, or anti-Crk antibody each inhibited stress fiber formation before and after insulin-like growth factor-I, EGF, and serum stimulation. Insulin stimulation by itself caused stress fiber breakdown and there was no additive effect of microinjection. Microinjection of anti-p130cas antibody also blocked stress fiber formation in quiescent cells. Microinjection of the Crk-inhibitory reagents also inhibited DNA synthesis after insulin-like growth factor-I, EGF, and serum stimulation, but not after insulin. These data suggest that the complex containing p130cas-c-CrkII may play a crucial role in actin cytoskeleton organization and in anchorage-dependent DNA synthesis.

Crk, a member of a family of adapter proteins, was originally reported from an avian retrovirus encoding the oncogene product v-Crk (1). The mammalian cellular homologues of v-Crk have been subsequently identified as c-CrkI and c-CrkII, which are alternatively spliced forms of a single gene (2, 3). In addition, another closely related gene product, c-Crk-L has been isolated (4).

A potential role for Crk in the regulation of the mammalian actin cytoskeleton has been suggested. In v-Crk-expressing cells, the level of tyrosine phosphorylation of a limited number of specific cellular proteins is increased, despite the lack of any tyrosine kinase catalytic domain in v-Crk itself (5). Among these tyrosine-phosphorylated proteins, components of focal adhesions, p130cas and paxillin, known to associate with actin stress fiber formation (6, 7), have been shown to associate with the SH2 domain of v-Crk (5, 6, 8). v-Crk was shown to activate the Rho GTPase signaling pathway, thought to regulate actin cytoskeleton organization, and served as a scaffolding protein during the assembly of focal adhesions (7). DOCK180, which was cloned as an SH3 domain-binding partner of c-Crk, alters cell morphology upon translocation to the cell membrane (9). Thus, c-Crk has been implicated in actin cytoskeleton signaling pathways, but the detailed mechanisms of its actions are still unclear.

In this study, to examine the mechanism of c-Crk function in growth factor-induced actin cytoskeleton organization and mitogenic pathways, we have examined Crk interactions with several signal transduction molecules in rat-1 fibroblasts overexpressing human insulin receptor (HIRc cells). The functional involvement of c-Crk in these cellular functions was directly assessed by single cell microinjection analysis in the presence and absence of several growth factors in HIRc cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Rat-1 fibroblasts expressing 1 × 106 human insulin receptors per cell (HIRc cells) were cultured as described previously (10). These cells were found to express c-CrkII but not c-CrkI by Western blot analysis (data not shown). Porcine insulin and IGF-I were kindly provided by Lilly, and EGF was purchased from Life Technologies, Inc. (Grand Island, NY). Bromodeoxyuridine (BrdUrd), a monoclonal anti-Crk antibody, a mouse monoclonal anti-phosphotyrosine (anti-pY) antibody, a mouse monoclonal anti-p130cas antibody (against carboxyl terminus), a mouse monoclonal anti-Sos antibody, and a mouse monoclonal anti-paxillin antibody were from Transduction laboratories (Lexington, KY). A rabbit polyclonal anti-CrkII antibody, a rabbit polyclonal anti-c-abl antibody, a rabbit polyclonal anti-C3G antibody, a rabbit polyclonal anti-p130cas antibody (against carboxyl terminus) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A rabbit polyclonal anti-GST antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse IgG and fluorescent isothiocyanate or rhodamine-conjugated anti-mouse and anti-rat IgG antibodies were from Jackson Laboratories (West Grove, NY). Electrophoresis reagents were from Bio-Rad. TRITC-phalloidin and all other reagents were purchased from Sigma.

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1 The abbreviations used are: SH2, Src homology domain 2; IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; PIP2, phosphatidylinositol 3,4-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate.
Glutathione S-Transferase (GST)-Crk-SH2 and GST-Crk-(N)SH3 Domain Fusion Proteins Preparation—The GST fusion proteins containing SH2 domain or (N)SH3 domain of c-Crk were a generous gift of R. B. Birge and H. Hanafusa (8, 11). The fusion proteins were expressed in *Escherichia coli* and purified by chromatography on glutathione-Sepharose 4B beads. The purified proteins were concentrated and dialyzed against 10 mM Tris-HCI, pH 7.4, and 100 mM KCl for microinjection or to phosphate-buffered saline (PBS) for electroporation.

Western Blotting Studies—Cell monolayers were starved for 36 h in serum-free Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% BSA. The cells were then treated with 5 nM insulin, 15 nM IGF-I, 16 nM EGF, or 10% fetal bovine serum for the indicated times at 37 °C. Cells were lysed in a buffer containing 20 mM Hepes, 3 mM MgCl2, 2 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X-100, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, and 1.5 μM pepstatin (pH 7.4). The cell lysates were centrifuged to remove insoluble materials. The supernatants were used for immunoprecipitation with the indicated antibodies or precipitation with glutathione-Sepharose 4B for 3 h at 4 °C. The precipitates were separated by SDS-polyacrylamide gel electrophoresis and transferred to Protran (Schleicher & Schuell) using a Bio-Rad Transblot apparatus. The membranes were blocked in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, 4% BSA (pH 7.5) for 2 h at room temperature. The membranes were then probed with specific antibodies for 2 h at room temperature. After washing the membranes in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 7.5), blots were incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection using the ECL reagent according to the manufacture’s instructions (Amersham Corp.).

Cellular Electroporation—HIRC cells were plated on glass slides coated with conductive indium tin oxide and grown within windows (7 × 15 mm) framed by nonconducting adhesive. Cells approaching confluence were starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium containing 0.1% BSA. Electroporation was performed with an Epizap pulse generator and electrode manufactured by Ask Science (12). Plated cells were subjected to six consecutive electrical pulses, each at 25 V to a capacitance of 16 microfarads. During pulsations, the cells were submerged in PBS containing 7 mM of the indicated GST fusion protein. The cells were washed once with serum-free Dulbecco’s modified Eagle’s medium containing 0.1% BSA and incubated in the same medium for 12 h at 37 °C. The cells were lysed as described above. The cell lysates from 10 slides (400 mg of protein) were used for immunoprecipitation with anti-CrkII antibody and for precipitation with glutathione-Sepharose 4B (Amersham Corp.).

Microinjection—Cells were grown on glass coverslips and rendered quiescent by starvation for 36 h in serum-free Dulbecco’s modified Eagle’s medium containing 0.1% BSA. Anti-Crk antibody and GST fusion proteins were solubilized in microinjection buffer consisting of 5 mM sodium phosphate and 100 mM KCl (pH 7.4), and then microinjected using glass capillary needles. Immunofluorescent staining as described below was performed on injected cells indicated that about 80% of the cells were successfully microinjected. In all injection experiments, results represent the mean of at least three identical experiments in which at least 250 cells were injected.

Actin Localization—One hour after microinjection, cells were stimulated with 17 nM insulin, 13 nM IGF-I, 16 nM EGF, or 10% fetal bovine serum for 5 min and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were permeabilized in 0.2% Triton X-100 for 5 min, washed in PBS, and incubated at room temperature for 45 min with TRITC-phalloidin (0.5 μg/ml) in PBS. Fluorescent isothiocyanate-labeled donkey anti-mouse IgG antibody (1:100) was added to the incubation mixture for coverslips containing injected cells.

*BrdUrd* Incorporation—One hour after microinjection, cells were incubated with *BrdUrd* plus vehicle, 17 nM insulin, 13 nM IGF-I, 16 nM EGF, or 10% fetal bovine serum for 16 h at 37 °C. The cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized and blocked with a solution containing 5% BSA and 0.5% Nonidet-P-40 in PBS. The cells were incubated with rat polyclonal anti-BrdUrd antibody in a buffer containing 10 mM MCP, and DNase I for 1 h at room temperature. The cells were then stained with rhodamine-labeled donkey anti-rat IgG antibody, fluorescein isothiocyanate-labeled donkey anti-mouse IgG antibody, and Hoechst (Sigma), which stains all nuclei, for 1 h at room temperature.

After staining, coverslips were washed successively in PBS and deionized *H2O* for 5 min and mounted in PBS containing 15% polyvinyl alcohol, 33% glycerol, and sodium azide. After the coverslips were mounted, the cells were analyzed with a Axioskop fluorescence microscope (Zeiss, Germany). For actin localization, individual cells displaying parallel actin fibers that colocalized with the nucleus were then scored as positive for stress fibers. Cells that showed actin staining at the periphery were scored as positive for membrane ruffles. The percentage of total counted cells displaying each phenotype is represented by the stress fiber and ruffling indexes, respectively.

**RESULTS**

c-CrkII Association with Tyrosine-phosphorylated p130Cas—We first evaluated tyrosine-phosphorylated proteins which are co-immunoprecipitated with c-Crk in the basal state in HIRC cells. Immunoprecipitation by anti-CrkII antibody followed by Western blotting with anti-pY antibody showed a tyrosine-phosphorylated 130-kDa band (pp130 in Fig. 1). This band was also immunoprecipitated by another anti-Crk antibody which recognizes a distinct epitope (Fig. 1). The pp130 was identified as p130Cas by subsequent anti-p130Cas blotting of the anti-Crk antibodies immunoprecipitants (Fig. 1, middle panel). c-CrkII was faintly tyrosine phosphorylated (pp40) in the basal state (Fig. 1). c-CrkII was not detected by Western blotting with anti-Crk antibody in HIRC cell lysates (data not shown). A quantitative analysis of p130Cas-c-CrkII binding using Western blotting and densitometry showed that an estimated 43% of total c-CrkII binds to p130Cas, and 21% of total p130Cas binds to c-CrkII in quiescent and serum-starved HIRC cells.

*I vivo* Assessment of CrkII Associated Molecules by Electroporation of GST Fusion Proteins—To determine which signaling molecules bind to which domain(s) of c-CrkII in vivo, we electroporated GST fusion proteins containing either the Crk SH2 domain or the Crk-(N)SH3 domain. Cell lysates were then immunoprecipitated with anti-CrkII antibody (Fig. 2, lanes 1–3) and the supernatants from this procedure were precipitated with glutathione-Sepharose 4B (Fig. 2, lanes 4–6). The final supernatants are seen in Fig. 2, lanes 7–9. The CrkII antibody precipitates >95% of endogenous CrkII (Fig. 2, panel A), and since this antibody is directed against the COOH-terminal CrkII SH3, as predicted, it does not precipitate either the GST-Crk-SH2 or GST-Crk-(N)SH3 (Fig. 2, panel B, lanes 1–3). The GST fusion proteins were efficiently electroporated...
into the cells and were quantitatively precipitated by glutathione-Sepharose 4B, as seen in Fig. 2, panel B, lanes 4–6. Control studies using immunostaining of cells and Western blotting with GST antibody showed that >90% of the cells incorporate GST fusion proteins at an average level of 10 pmol of GST protein/mg of total cellular protein under our electroporation conditions (data not shown). As seen in Fig. 2, panels C and D, when the electroporated cells are precipitated with CrkII antibody, which does not recognize the GST fusion proteins, incorporating GST-Crk-SH2 into the cells completely disrupts the association between endogenous CrkII and a tyrosine-phosphorylated p130cas (lane 2), whereas GST-Crk-(N)SH3 is without effect (lane 3). In addition, precipitation of the GST fusion proteins from the CrkII immunodepleted supernatants revealed that p130cas was now associated with GST-Crk-SH2, but not GST-Crk-(N)SH3 (Fig. 2, C and D, lanes 4–6). Cells electroporated with PBS alone showed the same results as the cells electroporated with GST alone (data not shown). These results indicate that endogenous CrkII binds to p130cas via its SH2 domain and that GST-Crk-SH2 is able to displace endogenous CrkII from p130cas in vivo.

Crk-SH2 domain preferentially binds to (p)YXXP motifs (13). Paxillin possesses (p)YXXP motifs as does p130cas (14), and paxillin is tyrosine phosphorylated in these cells in the basal state (data not shown and Ref. 15). v-Crk has been shown to bind to paxillin in v-Crk-transformed cells (8), and GST-Crk-SH2 binds to paxillin (pp60) in vivo (Fig. 2, C and E, lane 5), however, anti-CrkII antibody failed to co-immunoprecipitate any detectable paxillin (Fig. 2, C and E, lanes 1–3). In vitro GST fusion protein precipitation studies showed that 1 μg of GST-Crk-SH2 precipitated both p130cas and paxillin but 0.1 μg of GST-Crk-SH2 did not, while anti-CrkII antibody immunoprecipitated only p130cas but not paxillin (data not shown). Another anti-CrkII antibody also did not immunoprecipitate the pp68 band (Fig. 1). These data suggest that the full-length c-CrkII protein binds only to tyrosine-phosphorylated p130cas, and not to tyrosine-phosphorylated paxillin, although GST-Crk-SH2 can bind to both docking proteins similarly, even in vivo. In the basal state, c-cbl, which also has been reported to bind to the Crk-SH2 domain (16, 17), did not bind to c-CrkII or to the GST fusion proteins (data not shown). The electroporated GST-Crk-(N)SH3 partially blocked the association between c-CrkII and the guanine nucleotide exchange factor C3G, or between c-CrkII and c-abl in vivo (Fig. 2, F and G). Sos, another exchange factor, was not detected in anti-CrkII antibody immunoprecipitants, whereas a small amount of Sos was precipitated with the GST-Crk-(N)SH3 in vivo (Fig. 2H).

Effect of Growth Factor Stimulation on Tyrosine Phosphorylation of CrkII or on CrkII Association with Signaling Molecules—As shown in Fig. 1, there is a low level of tyrosine phosphorylation of c-CrkII in the basal state and it has been shown that Tyr221 can undergo phosphorylation in response to growth factors (18, 19). To assess this and the effect of growth factor stimulation on c-CrkII interactions, we stimulated HIRc cells with 17 nM insulin, 13 nM IGF-I, 16 nM EGF, or 10% serum followed by immunoprecipitation with anti-CrkII antibody and Western blotting of the precipitates with anti-pY antibody, anti-CrkII antibody, anti-p130cas antibody, anti-paxillin antibody, or anti-c-cbl antibody. All three growth factors caused a time-dependent increase in c-CrkII tyrosine phosphorylation, whereas serum was without effect (Fig. 3A).

As regards the interactions of c-CrkII, as shown in Fig. 3C, c-CrkII strongly associates with a tyrosine-phosphorylated 130-kDa band in the basal state, and this association is almost completely disrupted after 5 min of insulin stimulation. p130cas bloting confirmed that this tyrosine-phosphorylated 130-kDa molecule is p130cas (Fig. 3D). A slight effect of IGF-I on dissociation of c-CrkII/p130cas was also detected, and EGF caused a transient (1 min only) effect which was coincided with the transient appearance of an 120-kDa tyrosine-phosphorylated band. This EGF-induced tyrosine-phosphorylated band was identified as c-cbl by anti-c-cbl antibody blotting (Fig. 3E). Serum stimulation had no significant effect (Fig. 3, C-E). There were no other detectable tyrosine-phosphorylated bands in the anti-CrkII antibody immunoprecipitants before or after growth factor stimulation (data not shown).

We then evaluated the tyrosine phosphorylation state of c-CrkII binding molecules by stimulation with growth factors, followed by precipitation with anti-p130cas and anti-c-cbl antibodies and Western blotting with anti-pY antibody. p130cas...
was strongly tyrosine phosphorylated in the basal state, and insulin led to marked dephosphorylation of this protein, which was almost complete by 5 min, while IGF-I had a more modest effect. EGF led to a transient dephosphorylation of p130\textsuperscript{cas}, which peaked at 1 min, and returned to basal level by 5 and 20 min (Fig. 4, right panels). Taken together, our results indicate that c-CrkII binds to p130\textsuperscript{cas} in the basal state, and this c-CrkII-p130\textsuperscript{cas} complex can be disrupted by the tyrosine dephosphorylation of p130\textsuperscript{cas} induced by insulin, weakly by IGF-I, and transiently by EGF stimulation.

With respect to c-cbl, insulin, IGF-I, and serum stimulation had no effect on c-cbl tyrosine phosphorylation. EGF had a measurable, but transient effect which peaked at 1 min (Fig. 4, left panels), and this event may induce the formation of the c-CrkII-c-cbl complex seen in Fig. 3, C and E.

**Effects of Microinjection of GST Fusion Proteins and Antibodies on Cytoskeleton Organization and DNA Synthesis**—As seen in Fig. 2, C and D, electroporation of GST-Crk-SH2 into the cells almost completely inhibited the endogenous CrkII SH2 domain binding to p130\textsuperscript{cas}, whereas GST-Crk-(N)SH3 was less competitive for the endogenous CrkII-(N)SH3 domain binding to C3G and to c-abl (Fig. 2, F and G). To see if these interactions were important for actin cytoskeletal signaling and for mitogenic signaling, we carried out single cell microinjection studies in which GST-Crk-SH2, GST-Crk-(N)SH3, and anti-Crk antibody were introduced into HIRc cells followed by stimulation with insulin, IGF-I, EGF, or serum. Following stimulation, actin cytoskeletal organization and BrdUrd incorporation into newly synthesized DNA were monitored by immunofluorescence staining.
As can be seen in Fig. 5, A and B, 62% of serum-starved HIRc cells have stress fibers. The stress fiber response to growth factor stimulation in preimmune mouse IgG-injected cells (Fig. 5B, white bars) was quite comparable to the changes in the association between c-CrkII and p130<sub>cas</sub> by growth factor stimulation (Fig. 3). Microinjection of the anti-Crk reagents induced partial stress fiber breakdown in the basal state, and even after IGF-I, EGF, or serum stimulation (Fig. 5, A and B), while control injections of the same concentration of GST, GST-Grb2-SH2, or GST-Grb2-(N)SH3 domain had no effect (Fig. 5C). Insulin stimulation by itself caused marked stress fiber breakdown, and there was no additive effect, or inhibition, resulting from microinjection of these reagents. Microinjection of anti-p130<sub>cas</sub> antibody also decreased actin stress fiber formation, indicating that the p130<sub>cas</sub>-c-Crk-II pathway is important in maintenance of the cytoskeletal structure (Fig. 5C).

We next evaluated the effect of microinjection of these anti-Crk reagents on membrane ruffling of HIRc cells. Insulin and IGF-I led to a membrane ruffling response in 81 and 65% of the cells, respectively (Fig. 6, A and B), whereas EGF and serum stimulation were without effect (data not shown). All of the injected anti-Crk reagents inhibited membrane ruffling induced by insulin and IGF-I (Fig. 6B). Taken together with the data in Fig. 5, it seems likely that c-CrkII plays a role in the signaling pathway leading to stress fiber formation and membrane ruffling.

As can be seen in Fig. 7, none of these anti-Crk reagents had any influence on basal rates of DNA synthesis. Microinjection of the anti-Crk reagents did not inhibit insulin-stimulated BrdUrd labeling, whereas all three anti-Crk reagents partially inhibited IGF-I, EGF, and serum stimulated BrdUrd labeling (Fig. 7, A and B). Thus, ongoing c-CrkII function as an adapter protein is necessary for IGF-I, EGF, and serum to exert their mitogenic stimulatory effects, but not for insulin in HIRc cells.

**DISCUSSION**

The adapter protein p130<sup>cas</sup> was originally identified as a prominent tyrosine-phosphorylated protein in v-Crk- and v-Src-transformed cells (20). p130<sup>cas</sup> contains an SH3 domain, two proline-rich regions, and a substrate domain consisting of 15 potential SH2-binding motifs (20). In fact, 9 of these 15 tyrosine phosphorylation sites conform to the SH2-binding motif for Crk ((p)Y<sub>XXP</sub>), suggesting that Crk is the primary adapter protein for p130<sup>cas</sup>. Paxillin also has three (p)Y<sub>XXP</sub> motifs at tyrosine positions 31, 118, and 181. Tyrosines at positions 31 and 118 of paxillin may be important in binding the v-Crk-SH2 domain (14). In the present study, c-CrkII is shown to bind to p130<sup>cas</sup>, but not to paxillin, although both p130<sup>cas</sup> and paxillin are members of focal adhesions and tyrosine phosphorylated in the basal state. c-CrkL competes with c-CrkII to bind to paxillin at phosphorylated tyrosine residues 31 and 118 in rat uterus cells, because c-CrkL is more abun-
We could not detect any association between paxillin and c-CrkII, even after growth factor stimulation, thus, paxillin does not seem to have an important function through binding to c-CrkII, in contrast to p130cas.

Several studies have recently suggested that Crk may regulate the actin cytoskeleton through activation of the Rho/Rac family of small GTPases (6, 7, 22–26). In the present study, we directly demonstrated that c-CrkII plays an important role in the cytoskeletal organization of stress fiber formation and membrane ruffling. Both the SH2 domain and (N)SH3 domain of c-CrkII are required for these effects. Although c-CrkII transiently binds to c-cbl after EGF stimulation, stress fiber formation was correlated with the association between p130cas and c-CrkII via the SH2 domain of c-CrkII. Our results are also consistent with a role for p130cas in the maintenance of stress fibers. Furthermore, a recent report showed that the p130cas knockout embryo exhibits decreased stress fiber formation (27). Taken together with the previous reports noted above, c-CrkII is now implicated in the regulation of the Rho/Rac family of small GTPases via p130cas and Crk-(N)SH3 interaction.

The introduction of activated Rho into cells induces tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin, and p130cas, placing one or more tyrosine kinase downstream of Rho activity (28). Disruption of the actin cytoskeleton with cytochalasin D inhibits p130cas tyrosine phosphorylation. Thus, Rho activation followed by stress fiber formation seems to be essential for p130cas tyrosine phosphorylation (29). In contrast, the treatment of cells with a tyrosine kinase inhibitor, tyrphostin, blocks Rho activation specifically at an upstream step (30). Thus, tyrosine kinases seem to be required at signaling steps both upstream and downstream of Rho. Recently, v-Crk was reported to activate Rho GTPase in PC12 cells (7). Thus, it is possible that c-CrkII binds to tyrosine-phosphorylated p130cas, and then activates Rho GTPase, which is important in the production of phosphorylated 4,5-phosphatidylinositol (PIP2) and mediates stress fiber formation (7). Therefore, Rho activation followed by stress fiber formation seems to be interdependent with p130cas/c-CrkII association.

The present study strongly suggests that a p130cas/c-CrkII complex acts in the maintenance of stress fibers. Insulin stimulation of HIRc cells induces stress fiber breakdown and dissociation of p130cas and c-CrkII. Insulin-induced stress fiber breakdown appears to involve the conversion from PIP2 to PIP3 by phosphatidylinositol 3-kinase activation (31). This conversion decreases the amount of PIP2 necessary for stress fiber maintenance, in the plasma membrane (32). Insulin stimulation causes c-Src inactivation through the activation of Csk and tyrosine dephosphorylation of focal adhesion proteins (33). Because c-Src activity is important for the tyrosine phosphorylation of focal adhesions, the disruption of c-Src activity would lead to the inactivation of focal adhesion proteins. Therefore, the activation of c-CrkII downstream of insulin would lead to the disruption of c-Src activity, resulting in the dissociation of p130cas and c-CrkII and stress fiber breakdown.

Insulin stimulation of HIRc cells also induces stress fiber breakdown and dissociation of p130cas and c-CrkII. Insulin-induced stress fiber breakdown appears to involve the conversion from PIP2 to PIP3 by phosphatidylinositol 3-kinase activation (31). This conversion decreases the amount of PIP2 necessary for stress fiber maintenance, in the plasma membrane (32). Insulin stimulation causes c-Src inactivation through the activation of Csk and tyrosine dephosphorylation of focal adhesion proteins (33). Because c-Src activity is important for the tyrosine phosphorylation of focal adhesions, the disruption of c-Src activity would lead to the inactivation of focal adhesion proteins. Therefore, the activation of c-CrkII downstream of insulin would lead to the dissociation of p130cas and c-CrkII and stress fiber breakdown.
Fig. 7. Effect of microinjections of (A and B) GST alone, GST-Crk-SH2 domain, (B) GST-Crk-(N)SH3 domain and anti-Crk antibody on DNA synthesis in HIRc cells. Serum-starved cells were microinjected as described in the legend to Fig. 5. After stabilization for 1 h, cells were stimulated with indicated concentrations of growth factors for 16 h at 37 °C. BrdUrd incorporation into the injected cells was determined as described under “Experimental Procedures.” Results are expressed as the percent of total cells, and are the mean ± S.E. of three separate experiments.

The membrane ruffling induced by insulin or IGF-I stimulation is inhibited by microinjection of Crk inhibitory reagents. These data are consistent with a previous report which showed that anti-Crk antibody microinjection inhibited insulin-induced membrane ruffling (36). The breakdown of stress fibers in the basal state induced by microinjection of anti-Crk reagents suggests that the CrkII complex may regulate activation of p130

cas (34, 35), its inactivation may lead to tyrosine dephosphorylation of p130

cas following insulin stimulation. Insulin-induced p130

cas dephosphorylation followed by disruption of the p130

cas-c-CrkII complex may also contribute to insulin-induced breakdown of stress fibers.

The membrane ruffling induced by insulin or IGF-I stimulation is inhibited by microinjection of Crk inhibitory reagents. These data are consistent with a previous report which showed that anti-Crk antibody microinjection inhibited insulin-induced membrane ruffling (36). The breakdown of stress fibers in the basal state induced by microinjection of anti-Crk reagents suggests that the CrkII complex may regulate activation
transfection of GST fusion protein constructs, the advantages using stimulation (Fig. 7), although 17 nM insulin stimulation causes sis through the regulation of Rho/Rac family small GTPases.

This effect would be expected to reduce PIP2 formation and DNA synthesis after growth factor stimulation. This effect would be expected to reduce almost complete breakdown of stress fibers (Fig. 5, (43)).

HIRc cells possess a DNA synthesis response to 17 nM insulin stimulation (Fig. 7), although 17 nM insulin stimulation causes complete breakdown of stress fibers (Fig. 5, A and B) and tyrosine dephosphorylation of focal adhesions (40). Insulin receptor overexpression in 184B5 epithelial cells led to ligand-dependent transformation, and these cells grow in an anchor-dependent growth manner (41). Thus, insulin-stimulated HIRc cells may be deficient in normal anchorage-dependent growth control (Fig. 8). This hypothesis is consistent with the ability of HIRc cells to retain high DNA synthesis activity in the absence of stress fibers, which may be accomplished through the robust activation of Shc/Grb2/Sos/Ras in this cell type (42). EGFR receptor overexpressing rat-1 fibroblasts also demonstrated growth in the absence of stress fibers after EGF stimulation (43).

We established an in vivo GST fusion protein binding system using in situ electroporation in this study. Compared with the transfection of GST fusion protein constructs, the advantages of this in vivo system are the rapid and homogeneous introduction of fusion proteins into cells, and the facility of controlled fusion protein concentration. This system also permits the subsequent assay of biological functions. Thus, this can be a powerful and simple method to analyze signal transduction pathways.

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