Insulin and Epidermal Growth Factor Stimulate a Conformational Change in Rap1 and Dissociation of the CrkII-C3G Complex*

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Insulin and epidermal growth factor (EGF) stimulation of Chinese hamster ovary cells expressing the human insulin and EGF receptors resulted in a time-dependent decrease in the ability of a Rap1 antibody (amino acid epitope 121–136) to immunoprecipitate Rap1 from whole cell detergent extracts. This was due to an apparent masking of Rap1 as heat denaturation of the whole cell detergent extracts (5 min at 100 °C) resulted in equal immunoprecipitation of Rap1 with this epitope-specific antibody. The time-dependent change in Rap1 immunoreactivity was paralleled with an insulin-stimulated dissociation of the CrkII-C3G complex. Similarly, EGF treatment also resulted in a time-dependent dissociation of the CrkII-C3G complex that occurred concomitantly with the masking of the 121–136 Rap1 epitope. Furthermore, pretreatment of the cells with the tyrosine kinase inhibitor, genistein, decreased both the basal and insulin-stimulated tyrosine phosphorylation of CrkII that directly correlated with the amount of CrkII that was immunoprecipitated with C3G. Together, these data suggest that insulin and EGF stimulation result in the dissociation of the CrkII-C3G complex, thereby inducing an apparent conformational change in Rap1.

Ras is a low molecular weight (M, 21,000) GTP-binding protein that plays an essential role in cell proliferation and differentiation (1, 2). Mutations that result in constitutive activation of Ras are associated with several types of neoplastic tissue in mammals, and expression of these alleles into cultured fibroblasts results in cellular transformation (3). The best characterized proximal downstream Ras target is the Raf serine/threonine kinase, which following growth factor stimulation, becomes activated upon association with GTP-bound Ras (4–8). In turn, the Raf kinase phosphorylates and activates the dual functional protein kinase, termed MEK1 (9–13). This kinase phosphorylates the ERK family of mitogen-activated pro-tein kinases on both threonine and tyrosine residues in a characteristic TEY motif. The subsequent activation of ERK provides an important bifurcation point for the stimulation of numerous signaling pathways, including metabolic, transcriptional, and mitogenic events (14–17).

OPPOSING THE RAS ACTIVATION PATHWAY, THE LOW MOLECULAR WEIGHT GTP-BINDING PROTEIN RAP WAS ORIGINALY OBSERVED TO REVERT OR SUPPRESS THE TRANSFORMED PHENOTYPE IN Ki-Ras-TRANSFORMED FIBROBLASTS (18, 19). THIS APPARENT ANTAGONISM BETWEEN RAS AND RAP FUNCTION MAY REFLECT THE ABILITY OF RAP AND RAS TO INTERACT WITH THE SAME DOWNSTREAM EFFECTORS, SINCE THESE PROTEINS SHARE IDENTICAL SEQUENCES WITHIN THEIR RESPECTIVE EFFECTOR DOMAINS (18–20). FOR EXAMPLE, SEVERAL STUDIES HAVE DEMONSTRATED THAT BOTH RAP AND RAS CAN BIND THE SAME REGULATOR (p120RasGAP) AND EFFECTORS (RafGDS, Raf1, and B-Raf) IN A GTP-DEPENDENT MANNER (21–25). IN PARTICULAR, ASSOCIATION OF Raf1 WITH GTP-BOUND Ras RESULTS IN THE ACTIVATION OF Raf1 protein kinase activity, whereas the association with GTP-bound Rap results in an inhibition of Raf1 protein kinase activity (25).

Although the precise molecular details remained to be established, there is also substantial similarity in the upstream signaling mechanisms that regulate both Ras and Rap activation. Ras GTP binding is stimulated upon the targeting of the Ras guanyl nucleotide exchange factor SOS to the plasma membrane of Ras (26, 27). The carboxyl-terminal domain of SOS contains a proline-rich region that directs its association with the SH3 domains of the small adapter protein, Grb2 (28–33). In analogy, the formation of active GTP-bound Rap results from the specific interaction with the Rap guanyl nucleotide exchange factor C3G (34, 35). Similarly, the proline-rich regions of C3G are responsible for the association with the central SH3 domain of the small adapter protein, CrkII (35–38). Since Rap can function as a suppressor of Ras downstream signaling, we hypothesized that in order for growth factors to activate the Raf/MEK/ERK pathway, there must be a mechanisms to rapidly inhibit Rap function. In this manuscript, we demonstrate that insulin and EGF stimulation result in a rapid dissociation of the CrkII-C3G complex that correlates with an apparent conformational change and/or masking of Rap1 immunoreactivity.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells expressing the human insulin and EGF receptors (CHO/IR/ER) were isolated and cultured as described previously (39). Cells were incubated for 6–8 h in serum-free medium and then incubated with and without 100 nm insulin or 20 nm EGF at 37 °C for various times as indicated. In general, cell extracts were prepared by solubilization in 30 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 50 mM sodium fluoride, 1.0 mM EGTA, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml leupeptin (40). In the case of Rap1 immunoprecipitations, the cell extracts were prepared by solubilization in 50 mM HEPES, 1 mM NaHPO4, pH 7.4, 100 mM NaCl, 1% Triton X-100, 20 mM MgCl2, 1 mg/ml bovine serum albumin, 0.1 mM GTP, 0.1 mM GDP, 1 mM ATP, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 5 μg/ml leupeptin (41).

Immunoprecipitation and Immunoblotting—Immunoprecipitations were performed from whole cell lysates by incubation with 2.0 μg of CHO/IR/ER, Chinese hamster ovary cells expressing the human insulin and EGF receptors.
Insulin and EGF Stimulate a Masking of Rap1 Immunoreactivity—Insulin and EGF have been well documented to stimulate Ras activation through the regulated exchange of GDP for GTP by the guanine nucleotide exchange factor SOS (42, 43). Since we have been unable to develop a GTP binding assay for the endogenous Rap1 protein, we examined the ability of antibodies to detect potential conformational changes in Rap1. Insulin stimulation had no significant effect on the total amount of Rap1 protein that was detergent-solubilized in the whole cell extracts as detected by immunoblotting with a Rap1-specific monoclonal antibody (Fig. 1, lanes 1–5). In contrast, we observed that insulin stimulation resulted in a marked reduction in the amount of Rap1 that was immunoprecipitated with a polyclonal antibody prepared against amino acids 121–136 of Rap1 (Fig. 1, lanes 6–10). This apparent time-dependent masking of the Rap1 epitope was detected following 1 min of insulin stimulation and was maximal between 5 and 15 min.

To further demonstrate that the Rap1121–136 antibody was detecting a masking of the Rap1 epitope, we compared the ability of this antibody to immunoprecipitate detergent-solubilized and heat-denatured Rap1 (Fig. 2A). Insulin stimulation for 15 min had no effect on the total amount of detergent extracted Rap1 protein as detected by immunoblotting of the whole cell lysates (Fig. 2A, lanes 1 and 2). In contrast, the ability of the Rap1121–136 antibody to immunoprecipitate Rap1 protein was markedly decreased following 15 min of insulin stimulation (Fig. 2A, lanes 3 and 4). However, heat denaturation of the same extracts restored the ability of the Rap1121–136 antibody to immunoprecipitate identical amounts of Rap1 protein from both the unstimulated and insulin-stimulated cells (Fig. 2A, lanes 5 and 6).

Similar to insulin, EGF stimulation for 15 min had no effect on the total amount of detergent extracted Rap1 protein (Fig. 2B, lanes 1 and 2). Immunoprecipitation of the detergent-extracted and heat-denatured Rap1 protein (Fig. 2B, lanes 5 and 6) was markedly decreased following 15 min of insulin stimulation (Fig. 2B, lanes 3 and 4). However, heat denaturation of the same extracts restored the ability of the Rap1121–136 antibody to immunoprecipitate identical amounts of Rap1 protein from both the unstimulated and insulin-stimulated cells (Fig. 2B, lanes 5 and 6).

RESULTS AND DISCUSSION

Inactivation of Ras back to the GDP-bound state occurs concomitant with the dissociation of the Grb2-SOS complex (41, 44–46). We therefore speculated that the apparent conformational change and/or masking of Rap1 immunoreactivity might have resulted from the inactivation of Rap1 due to an uncoupling of the CrkII-C3G complex. To examine the association state of the CrkII-C3G complex, we next determined the effect of insulin and EGF on the co-immunoprecipitation of these complexes (Fig. 3). As expected, in unstimulated cells immunoprecipitation of C3G resulted in the co-immunoprecipitation of CrkII and phosphotyrosine (PY20) or with a polyclonal antibody (Santa Cruz) directed against C3G.

Insulin and EGF Stimulation of Ras activation through the regulated exchange of GDP for GTP by the guanine nucleotide exchange factor SOS (42, 43). Since we have been unable to develop a GTP binding assay for the endogenous Rap1 protein, we examined the ability of antibodies to detect potential conformational changes in Rap1. Insulin stimulation had no significant effect on the total amount of Rap1 protein that was detergent-solubilized in the whole cell extracts as detected by immunoblotting with a Rap1-specific monoclonal antibody (Fig. 1, lanes 1–5). In contrast, we observed that insulin stimulation resulted in a marked reduction in the amount of Rap1 that was immunoprecipitated with a polyclonal antibody prepared against amino acids 121–136 of Rap1 (Fig. 1, lanes 6–10). This apparent time-dependent masking of the Rap1 epitope was detected following 1 min of insulin stimulation and was maximal between 5 and 15 min.

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Similar to insulin, EGF stimulation for 15 min had no effect on the total amount of detergent extracted Rap1 protein (Fig. 2B, lanes 1 and 2). Immunoprecipitation of the detergent-extracted and heat-denatured Rap1 protein (Fig. 2B, lanes 5 and 6) was markedly decreased following 15 min of insulin stimulation (Fig. 2B, lanes 3 and 4). However, heat denaturation of the same extracts restored the ability of the Rap1121–136 antibody to immunoprecipitate identical amounts of Rap1 protein from both the unstimulated and insulin-stimulated cells (Fig. 2B, lanes 5 and 6).

In comparison, EGF stimulation also resulted in a rapid dissociation of the CrkII-C3G complex (Fig. 3B, lanes 1–6). The time-dependent uncoupling of CrkII from C3G was similar to insulin with detectable dissociation following 1 min of EGF treatment (Fig. 3B, lane 2). The maximal EGF-stimulated dissociation of the CrkII-C3G complex occurred between 3 and 5 min and was persistent for up to 30 min (Fig. 3B, lanes 3–6). As
a control for immunoprecipitation, immunoblotting with the C3G antibody demonstrated equivalent amounts of immuno-
precipitated C3G protein under each condition (Fig. 3B, lanes 7–12). These data are consistent with the CrkII-C3G complex
functioning to maintain Rap1 in the active GDP-bound state, which is recognized by the Rap1-dependent epistopic-specific
antibody. However, the dissociation of the CrkII from C3G complex terminates this activation signal, thus allowing Rap1 to
convert to the inactive GDP-bound state.

The Dissociation of the CrkII-C3G Complex Requires Activation
of Protein Tyrosine Kinase Activity—Recently it has been observed that CrkII becomes tyrosine-phosphorylated follow-
ing growth factor stimulation (47). Based upon these previous findings and the rapid insulin- and EGF-stimulated disso-
ciation of the CrkII-C3G complex, we next examined the potential role for the tyrosine phosphorylation of CrkII. This was
assessed by use of the selective tyrosine kinase inhibitor, genistein (Fig. 4). Phosphotyrosine immunoblots of whole cell deter-
genent extracts demonstrated the insulin stimulation of IRS1/2
protein (Fig. 4). Phosphotyrosine immunoblots of whole cell deter-
genates were prepared and were immunoprecipitated with the PY20 phosphoty-
sine antibody (lanes 1–4) or immunoprecipitated with a CrkII anti-
body and immunoblotted with the PY20 phosphotyrosine antibody
(lanes 5–8) as described under “Experimental Procedures.” B, the whole
cell detergent extracts were immunoprecipitated with a C3G antibody
and immunoblotted with a CrkII antibody (lanes 1–4) or with a C3G
antibody (lanes 5–8).

FIG. 3. Insulin and EGF stimulation result in the dissociation
of the CrkII-C3G complex. A, CHO/IR/ER cells were incubated in the
absence (lanes 1 and 7) or in the presence of 100 nM insulin for 1 (lanes 2 and 8), 3 (lanes 3 and 9), 5 (lanes 4 and 10), 15 (lanes 5 and 11), and
30 (lanes 6 and 12) min at 37 °C. B, CHO/IR/ER cells were incubated in the
absence (lanes 1 and 7) or in the presence of 20 nM EGF for 1 (lanes 2 and 8), 3 (lanes 3 and 9), 5 (lanes 4 and 10), 15 (lanes 5 and 11), and
30 (lanes 6 and 12) min at 37 °C. In both cases, whole cell detergent
extracts were prepared and were immunoprecipitated with a C3G an-
tibody. The C3G immunoprecipitates were immunoblotted with a CrkII
antibody (lanes 1–6) or a C3G antibody (lanes 7–12) as described under
“Experimental Procedures.”

which decreased following insulin treatment (Fig. 4A, lanes 5
and 6). In any case, genistein pretreatment inhibited both the
basal and insulin-stimulated tyrosine phosphorylation of CrkII
as well as the tyrosine dephosphorylation of the 120–130-kDa
band (Fig. 4A, lanes 7 and 8).

The relative tyrosine phosphorylation state of CrkII also
was increased in the relative extent of CrkII that
was co-immunoprecipitated with C3G (Fig. 4B, lane 3). Fur-
thermore, genistein pretreatment also prevented the insulin-
stimulated increase CrkII tyrosine phosphorylation and con-
comitantly inhibited the insulin-stimulated dissociation of the
CrkII-C3G complex (Fig. 4B, lane 4). As controls for immuno-
precipitation, the amount of C3G immunoprecipitated under
these conditions remained unchanged (Fig. 4B, lanes 5–8).
Thus, these data indicated that the extent of CrkII tyrosine
phosphorylation was inversely related to the association state
of the CrkII-C3G complex.

In summary, we have observed that growth factor stimula-
tion results in a rapid dissociation of the CrkII-C3G complex,
which parallels or slightly precedes an apparent confor-
maisonal change and/or masking of the Rap1121–136 amino
acid epitope. Since the association state of CrkII with C3G appears
to correlate with the extent of CrkII tyrosine phosphorylation,
we speculate that receptor tyrosine kinase phosphorylation of
CrkII regulates this interaction. Further studies will be neces-
sary to determine whether CrkII is a direct substrate of recep-
tor tyrosine kinases, the site(s) of CrkII phosphorylation, and
the functional role of this tyrosine phosphorylation in modu-

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