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As indicated in our previous progress report, our initial objectives were to determine the role of SHPTP2 in the growth factor (insulin and epidermal growth factor) activation of Ras. However, these studies have lead us to uncover several novel pathways that together coordinately regulate Ras-dependent downstream signaling of the Raf/MEK/ERK cascade. Initially, we defined a feedback pathway which resulted in the serine/threonine phosphorylation of SOS and which was responsible, at least in part, for the termination of Ras activation. Currently, we are in the process of identifying the sites of phosphorylation responsible for the dissociation of Grb2 from SOS. During the past funding period, we have observed that another small GTP binding protein, termed Rap1, functions as a negative regulator of Ras by inhibiting the activation of the Raf/MEK/ERK cascade. However, growth factor stimulation derepresses this inhibition by converting active Rap1 to inactive Rap1. This occurs concomitant with the activation of Ras leading to a net activation of Raf/MEK/ERK pathway. In addition, our preliminary data suggests that the inhibition of Rap1 results from a rapid dissociation of the CrkII-C3G complex due to the direct tyrosine phosphorylation of CrkII.

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5) INTRODUCTION:

As indicated in our previous progress report, our initial project was focused on the role of the SHPTP2 tyrosine specific protein phosphatase in the control of mitogenesis. However, during the course of these studies, we made several fundamental observations with regard to the regulation of Ras and the MAP kinase cascade. These findings have now been further expanded to encompass the dynamic interaction between both Ras and Rap function in the regulation of Raf activity and thereby MAP kinase activation. In the current funding period, we have successfully investigated the insulin and EGF receptor intracellular signaling pathways leading to the upstream regulation of Rap1 function. A general introduction of these signaling pathways and their relationship to breast cancer research is summarized below.

The formation and proliferation of breast tumors is a complex process in which the normal growth suppressing and/or apoptotic properties of the tumor cells are lost. Although the mechanism(s) by which this occurs is not known, during the past several years substantial progress has been made. Recently, over expression of the erbB2 gene has been found in numerous human neoplasm's and is associated with approximately 20% of human breast tumors (1-3). The erbB2 is highly related to the EGF receptor both having intrinsic tyrosine kinase activity with the erbB2 receptor displaying greater transformation potential than the EGF receptor (1-6). The basic mechanism by which the EGF receptor and erbB2 induce the proliferative response results from the homo and/or heterodimerization of these receptors with other members of this receptor family (erbB3 and erbB4), activation of tyrosine kinase activity followed by auto and substrate tyrosine phosphorylation. These tyrosine phosphorylation events create recognition sites for SH2 domain containing proteins (7,8). In this regard, the adapter protein Grb2 is basally associated with the Ras guanylnucleotide exchange factor SOS and the tyrosine phosphorylation of Shc and/or the receptor generates functional receptor-Shc-Grb2-SOS and receptor-Grb2-SOS complexes that target SOS activity to the plasma membrane location of Ras (7-15). Once in the active GTP-bound state Ras activates a series of serine/threonine kinase collectively referred to as the MAP kinase cascade and include Raf/MEK/ERK (16). Activation of this cascade results in the phosphorylation of numerous proteins controlling macromolecular biosynthesis and thereby stimulating cell growth and if constitutively active leads to cellular transformation (17-19). These findings are directly related to epithelial derived breast tumors in that both ERK protein and kinase activity are constitutively elevated but not in normal or benign conditions (20).

Although much progress has been made in our understanding of the activation of the Ras/Raf/MEK/ERK kinase cascade, the mechanism by which signaling through this pathway is terminated has not been extensively investigated. Previously, we and others have provided evidence for a Ras/Raf/MEK/ERK-dependent feedback phosphorylation of SOS which induces the dissociation of the Grb2-SOS complex limiting

the duration and extent of Ras activation (21-26). However, during the course of these studies will also observed that a parallel signaling pathway exists for the regulation of another small GTP binding protein termed Rap1. Depending upon the cell context, Rap1 can functions as a potent suppressor the Ras activation pathway by sequestering Raf1 from Ras and preventing its activation.

During the past funding period, we have made excellent progress in our molecular understanding of the upstream signaling cascades regulating MAP kinase activation. We have cloned and characterized a 66 kDa Shc isoform which appears to function as a negative regulator of the well established 52 and 46 kDa Shc isoforms (27). In addition, we have determined that the CrkII-C3G complex is required for the regulation of the small GTP binding protein Rap1. Rap1 appears to function as a negative regulator of Raf1 thereby modulating the activation of MAP kinase. The functional integrity of the CrkII-C3G complex, which is analogous to the Grb2-SOS complex regulating Ras, appears to be controlled by the tyrosine phosphorylation of CrkII.

6) BODY:

1. Experimental Procedures:

Cell Culture - Chinese hamster ovary cells expressing the human insulin and EGF receptors (CHO/IR/ER) were isolated and cultured as previously described (28). Cells were incubated for 6-8 h in serum-free media 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 phenylmethylsulfonylfluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin and 5 μg/ml leupeptin (26). In the case of Rap1 immunoprecipitations, the cell extracts were prepared by solubilization in 50 mM HEPES, 1 mM Na_2HPO_4, pH 7.4, 100 mM NaCl, 1% Triton X-100, 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.1 mM GTP, 0.1 mM GDP, 1 mM ATP, 0.4 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin and 5 μg/ml leupeptin (25).

Immunoprecipitation and immunoblotting - Immunoprecipitations were performed from whole cell lysates by incubation with 2.0 μg of Rap1(121-136) or C3G polyclonal antibodies (Santa Cruz) for 2 h at 4°C. The resulting immune complexes were precipitated by incubation with protein A-Sepharose for 1 h at 4°C and washed as described above. The pellets were then resuspended in SDS-sample buffer (0.188 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 15% (w/v) SDS, 15% 2-mercaptoethanol, 0.01% bromophenol blue) and heated at 100°C for 5 min. Whole cell lysates or immunoprecipitates were separated on 10% SDS-polyacrylamide gels (30:0.4 acrylamide to bis-acrylamide) and transferred to PVDF membranes at 4°C. Immunoblotting was performed with monoclonal antibodies (Transduction Laboratories) directed

against Rap1, CrkII and phosphotyrosine (PY20) or with a polyclonal antibody (Santa Cruz) directed against C3G.

2. Results:

Insulin and EGF stimulate a conformational change in Rap1

Insulin and EGF have been well documented to stimulate Ras activation through the regulated exchange of GDP for GTP by the guanylnucleotide exchange factor SOS (29,30). 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 conformational change in Rap1 was detected following 1 min of insulin stimulation and was maximal between 5 and 15 min.

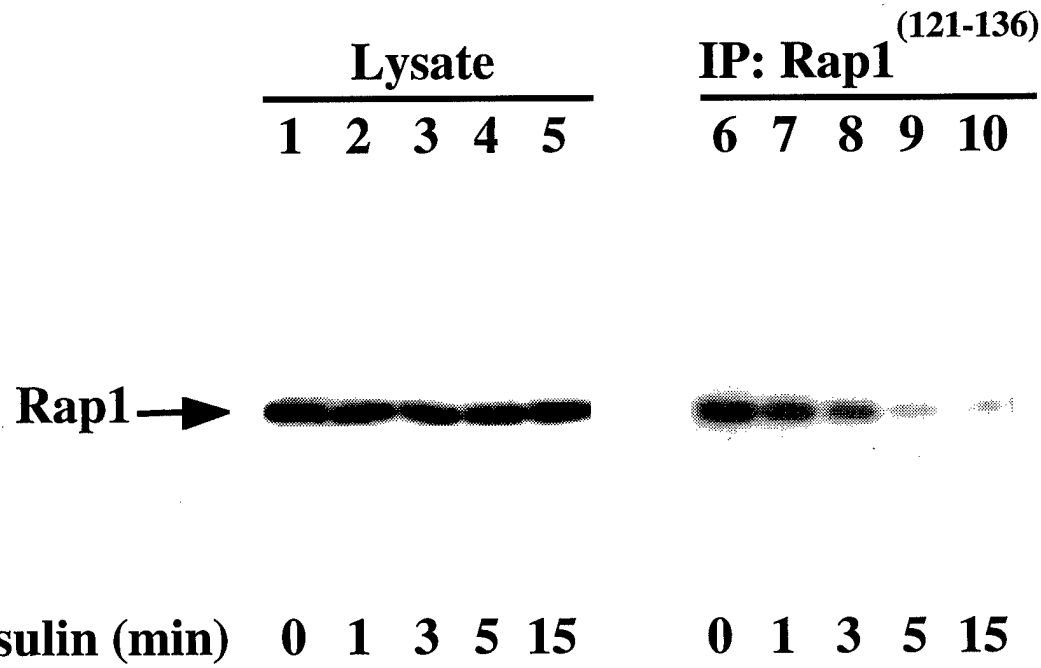

Figure 1. Insulin stimulation result in a time-dependent decrease in Rap1 immunoprecipitation. CHO/IR/ER cells were either untreated (lanes 1 and 6) or stimulated with 100 nM insulin for 1 (lanes 2 and 7), 3 (lanes 3 and 8), 5 (lanes 4 and 9) and 15 (lanes 5 and 10) min at 37°C. Whole cell detergent extracts (Lysate) were prepared and either directly subjected to Rap1 immunoblotting (lanes 1-5) or were initially immunoprecipitated with the Rap1 polyclonal antibody directed against amino acids 121-136 followed by Rap1 immunoblotting (lanes 6-10).

To further demonstrate that the Rap1$^{121-136}$ antibody was detecting an insulin-stimulated conformational change in Rap1, 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 Rap1$^{121-136}$ antibody to immunoprecipitate Rap1 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 Rap1$^{121-136}$ antibody to immunoprecipitate identical amounts of Rap1 protein from both the unstimulated and insulin-stimulated cells (Fig. 2A, lanes 5 and 6).

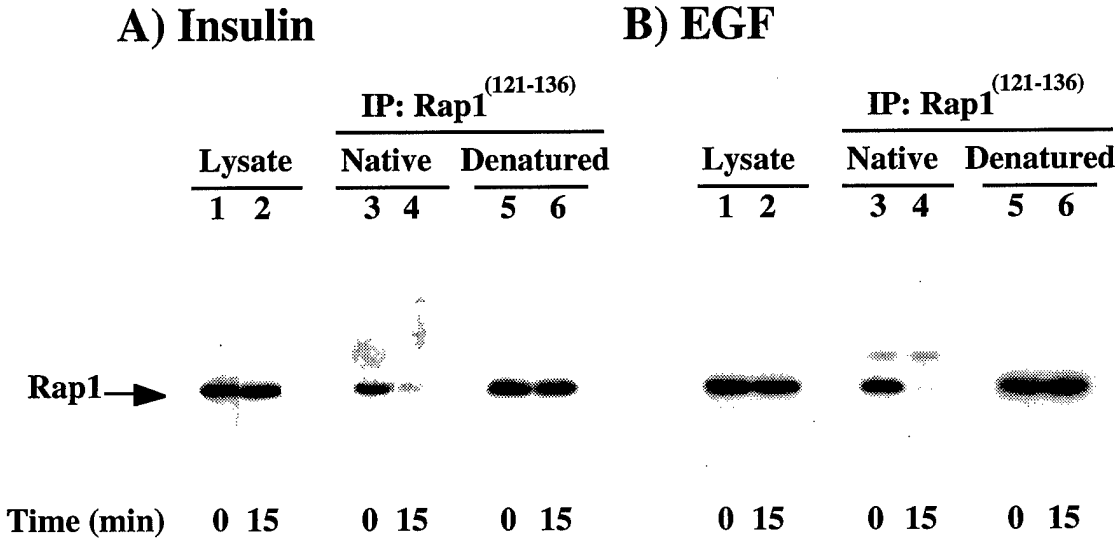

Figure 2. Insulin and EGF stimulate a conformational change in Rap1. A) CHO/IR/ER cells were either untreated (lanes 1, 3 and 5) or incubated with 100 nM insulin (lanes 2, 4 and 6) for 15 min at 37°C. B) CHO/IR/ER cells were either untreated (lanes 1, 3 and 5) or incubated with 20 nM EGF (lanes 2, 4 and 6) for 15 min at 37°C. In each case, whole cell detergent extracts (Lysate) were immunoblotted for Rap1 (lanes 1 and 2). The extracts were either then incubated with the antibody directed against the amino acid 121-136 Rap1 epitope prior to (Native) or following heat denaturation (Denatured) by incubation for 5 min at 100°C. The resulting immunoprecipitates were then immunoblotted with a Rap1 monoclonal antibody.

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 extracts with the Rap1$^{121-136}$ antibody demonstrated a marked reduction in the amount of Rap1 protein following EGF stimulation Fig. 2B, lanes 3 and 4). The decreased immunoreactivity of Rap1 was completely reversed following heat denaturation of the detergent cell extracts (Fig. 2B, lanes 5 and 6). Together, these data demonstrate that growth factor stimulation (insulin and EGF) induce a conformational change in Rap1

that is detectable by the masking of the amino acid 121-136 epitope. Although the molecular basis for this apparent conformational change in Rap1 is not known, it is likely to represent the conversion of active GTP-bound Rap1 to the inactive GDP-bound state.

Insulin and EGF stimulate the dissociation of the CrkII-C3G complex

We and others have also recently observed that the inactivation of Ras back to the GDP-bound state occurs concomitant with the dissociation of the Grb2-SOS complex (21,23-25). We therefore speculated that the conformational change in Rap1 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 (Fig. 3A, lane 1). However, following insulin stimulation, there was a time-dependent decrease in the amount of CrkII that could be co-immunoprecipitated with C3G (Fig. 3A, lanes 1-6). The dissociation of CrkII from C3G was detectable as early as 1 min and was maximal between 3 to 5 min (Fig. 3A, lanes 3 and 4). The insulin-stimulated decrease of C3G immunoprecipitated CrkII protein was not due to differences in C3G immunoprecipitation as assessed by C3G immunoblotting of the C3G immunoprecipitates (Fig. 3A, lanes 7-12). Furthemore, the time-dependence of CrkII-C3G dissociation was similar to or slightly preceded the insulin-stimulated decrease in Rap1 immunoreactivity (Fig. 1).

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 to 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 immunoprecipitated C3G protein under each conditon (Fig. 3B, lanes 7-12). These data are consistent with the CrkII-C3G complex functioning to maintain Rap1 in the active GTP-bound state, which is recognized by the $Rap1^{121-136}$ epitope 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.

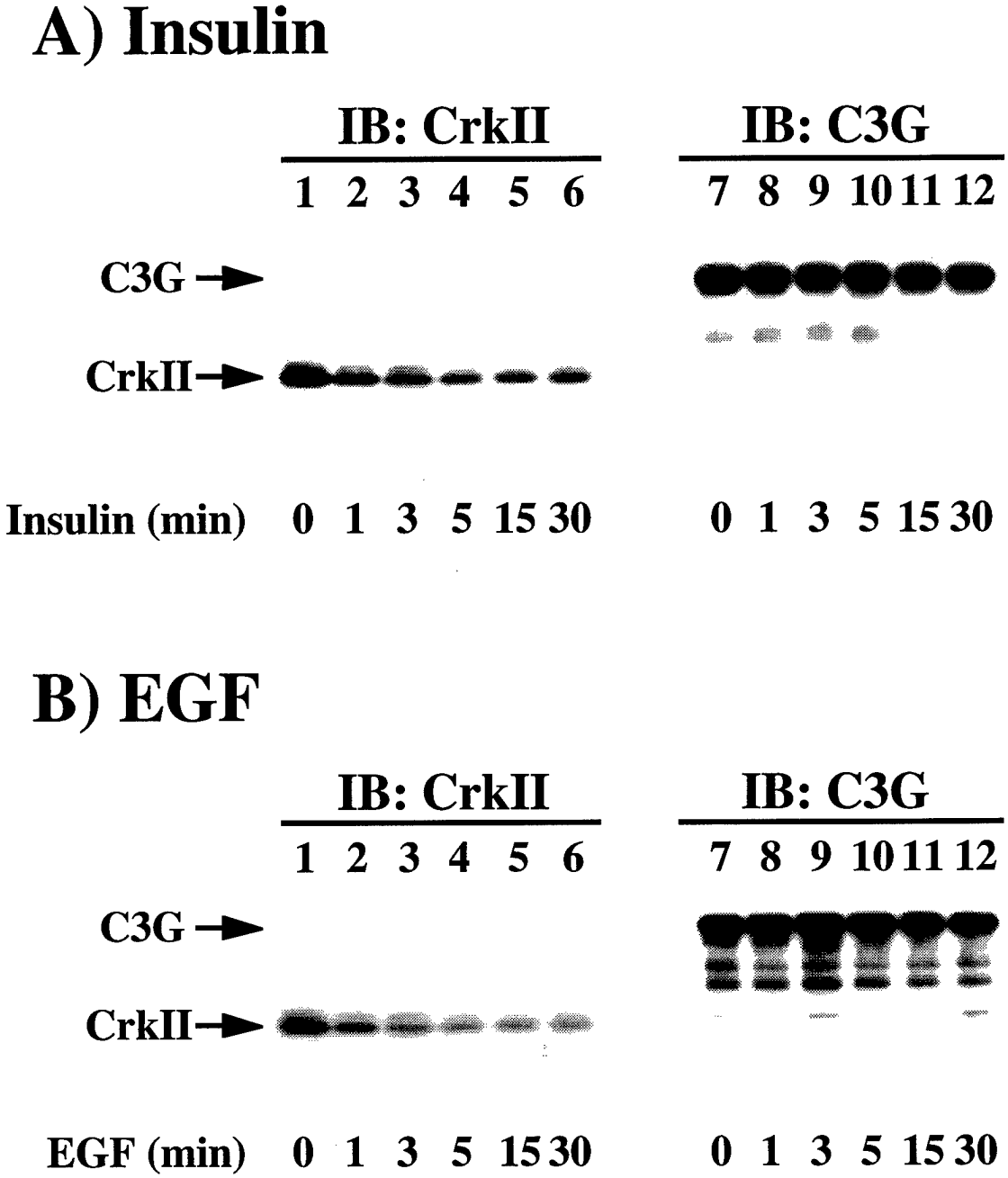

Figure 3. Insulin and EGF stimulation result in the dissociation of the CrkII-C3G complex. A) CHO/IR/ER cells were incubated in the absence (lanes 1 and 7) or in the presence of 100 nM insulin for 1 (lanes 2 and 8), 3 (lanes 3 and 9), 5 (lanes 4 and 10), 15 (lanes 5 and 11) and 30 (lanes 6 and 12) min 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 antibody. The C3G immunoprecipitates were immunoblotted with a CrkII antibody (lanes 1-6) or a C3G antibody (lanes 7-12).

The dissociation of the CrkII-C3G complex requires activation of protein tyrosine kinase activity

Recently it has been observed that CrkII becomes tyrosine phosphorylated following growth factor stimulation (31). Based upon these previous findings and the rapid insulin- and EGF-stimulated dissociation 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 detergent extracts demonstrated the insulin stimulation of IRS1/2 and insulin receptor β subunit tyrosine phosphorylation (Fig. 4A, lanes 1 and 2). Genistein pretreatment of the cells reduced both the basal level of tyrosine phosphorylated proteins as well as decreased the extent of insulin-stimulated IRS1/2 and insulin receptor β subunit tyrosine phosphorylation (Fig. 4A, lanes 3 and 4). It should be noted that genistein only partially inhibited insulin-stimulated tyrosine phosphorylation probably due to the high levels of insulin receptors expressed in this cell line. As expected, insulin stimulation resulted in an increased tyrosine phosphorylation of CrkII (Fig. 4A, lanes 5 and 6). Interestingly, a tyrosine phosphorylated protein in the 120-130 kDa range was also found to co-immunoprecipitate with CrkII 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).

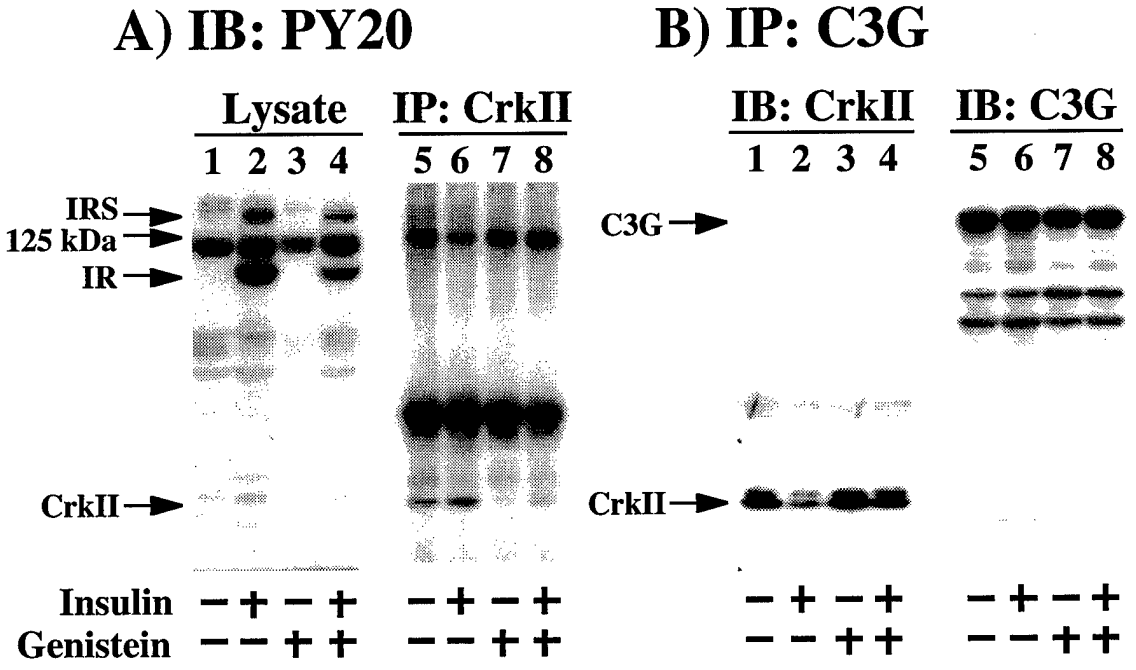

Figure 4. The amount of CrkII co-immunoprecipitated with C3G is inversely proportional to the extent of CrkII tyrosine phosphorylation. CHO/IR/ER cells were preincubated in the absence or presence of 300 μM genistein for 1 h at

37°C. The cells were then either unstimulated or incubated in the presence of 100 nM insulin for an additional 15 min at 37°C. A) Whole cell detergent extracts (Lysates) were prepared and directly immunoblotted with the PY20 phosphotyrosine antibody (lanes 1-4) or immunoprecipitated with a CrkII antibody 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).

The relative tyrosine phosphorylation state of CrkII also correlated with the extent of association between CrkII and C3G (Fig. 4B). As previously observed, immunoprecipitation of C3G resulted in the co-immunoprecipitation of CrkII which was decreased following insulin stimulation (Fig. 4B, lanes 1 and 2). In the unstimulated cells, although pretreatment with genistein decreased the amount of tyrosine phosphorylated CrkII there was an increase in the relative extent of CrkII that was co-immunoprecipitated with C3G (Fig. 4B, lane 3). Furthermore, genistein pretreatment also prevented the insulin-stimulated increase CrkII tyrosine phosphorylation and concomitantly inhibited the insulin-stimulated dissociation of the CrkII-C3G complex (Fig. 4B, lane 4). As controls for immunoprecipitation, 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.

7) CONCLUSIONS:

At present, our efforts remain focused on determining the regulation of MAP kinase through the interactive functions of Ras and Rap1. We have observed that growth factor stimulation results in a rapid dissociation of the CrkII-C3G complex which parallels or slightly precedes an apparent conformational change in Rap1. 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 necessary to determine whether CrkII is a direct substrate of receptor tyrosine kinases, the site(s) of CrkII phosphorylation and the functional role of this tyrosine phosphorylation in modulating the interaction between CrkII and C3G. In any case, based upon the ability of Rap1 to suppress Ras signaling, the ability of growth factors to activate the Raf/MEK/ERK cascade requires a mechanism for the inactivation of Rap1 function. Our data suggests that the growth factor-stimulated uncoupling of the CrkII-C3G complex may be one such mechanism.

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9) APPENDICES:

None

10) PUBLICATIONS/PERSONNEL:

A) Publications:

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