

Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2–SOS and CrkII–C3G complexes

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Insulin stimulation of Chinese hamster ovary cells expressing the human insulin receptor resulted in a time-dependent decrease in the amount of GTP bound to Rap1. The inactivation of Rap1 was associated with an insulin-stimulated decrease in the amount of Rap1 that was bound to Raf1. In parallel with the dissociation of Raf1 from Rap1, there was an increased association of Raf1 with Ras. Concomitant with the inactivation of Rap1 and decrease in Rap1–Raf1 binding, we observed a rapid insulin-stimulated dissociation of the CrkII–C3G complex which occurred in a Ras-independent manner. The dissociation of the CrkII–C3G was recapitulated *in vitro* using a GST–C3G fusion protein to precipitate CrkII from whole cell detergent extracts. The association of GST–C3G with CrkII was also dose dependent and demonstrated that insulin reduced the affinity of CrkII for C3G without any effect on CrkII protein levels. Furthermore, the reduction in CrkII binding affinity was reversible by tyrosine dephosphorylation with PTP1B and by mutation of Tyr221 to phenylalanine. Together, these data demonstrate that insulin treatment results in the de-repression of Rap1 inhibitory function on the Raf1 kinase concomitant with Ras activation and stimulation of the downstream Raf1/MEK/ERK cascade.

Keywords: C3G/CrkII/insulin/Rap1/Ras

Introduction

The Rap proteins (Rap1A, Rap1B, Rap2A and Rap2B) are members of the Ras superfamily of low molecular weight monomeric GTP-binding proteins (Bokoch, 1993; Burgering and Bos, 1995). Rap1A was identified originally based upon its homology with the *Drosophila* Ras-related gene (*Dras3*) and independently from its ability to induce a flat phenotype in v-Ki-Ras-transformed fibroblasts (Pizon *et al.*, 1988; Kitayama *et al.*, 1989). However, the physiological role of Rap1 in regulating intracellular signaling events appears to be enigmatic and can be either positive or negative dependent upon the particular cell context. Nevertheless, in multiple cell types, several studies have demonstrated that Rap1 can function as a suppressor of

Ras-mediated downstream signaling. For example, both the Ras- and T-antigen-dependent transformation can be reversed by Rap1 expression (Kitayama *et al.*, 1989; Jelink and Hassell, 1992). In addition, constitutively active Rap1 prevents Ras-induced germinal vesicle breakdown in *Xenopus* oocytes, and in mammalian cells antagonizes the Ras-dependent activation of the MAP kinase pathway, *c-fos* gene expression and the Ras-mediated inhibition of muscarinic potassium channel activity (Yatani *et al.*, 1990; Campa *et al.*, 1991; Sakoda *et al.*, 1992; Cook *et al.*, 1993). More recently, elevation of cAMP levels has been reported to activate Rap1 whereas, in several cell systems, cAMP functions to inhibit Ras downstream signals by blockade of the Raf/MEK/ERK activation pathway (Altshuler *et al.*, 1995; Burgering and Bos, 1995). This apparent antagonism between Ras and Rap1 function may reflect the ability of Rap1 and Ras to interact with the same downstream effectors, since these proteins share identical sequences within their respective effector domains (Pizon *et al.*, 1988; Kitayama *et al.*, 1989; Zhang *et al.*, 1990). In this regard, several studies have demonstrated that both Rap and Ras can bind the same regulators (p120RasGap) and effectors (RalGDS, Raf1 and B-Raf) in a GTP-dependent manner (Frech *et al.*, 1990; Hata *et al.*, 1990; Spaargaren and Bischoff, 1994; Nassar *et al.*, 1995; Vossler *et al.*, 1997).

Over the past several years, substantial progress has been made in our understanding of the proximal molecular events controlling Ras activation and its coupling to downstream effector pathways. In the case of receptor tyrosine kinase activation, the tyrosine phosphorylation of the Shc proteins generates high affinity recognition motifs for the Src homology 2 (SH2) domain of Grb2 (Rozakis-Adcock *et al.*, 1992; Medema and Bos, 1993; Downward, 1994; Chardin *et al.*, 1995; van der Geer *et al.*, 1996). Grb2 is a small adaptor protein (~23 kDa) consisting of a single SH2 domain flanked by two Src homology 3 (SH3) domains (Lowenstein *et al.*, 1992). In contrast to the SH2 domain which is responsible for its association with tyrosine-phosphorylated Shc, the Grb2 SH3 domains direct a basal state interaction with SOS, the 150 kDa guanylnucleotide exchange factor for Ras (Perrimon, 1994; Downward, 1996). Thus, receptor tyrosine kinase phosphorylation of Shc generates the formation of a Shc–Grb2–SOS ternary complex which provides a functional complex necessary for Ras activation. Once in the active GTP-bound state, Ras directly associates and activates the Raf1 kinase. In turn, activated Raf1 phosphorylates MEK which is an immediate upstream activator of ERK, a mitogen-activated protein (MAP) kinase (Avruch *et al.*, 1994; Blumer and Johnson, 1994; Marshall, 1994).

In an analogous paradigm, it is becoming clear that Rap1 activation (GTP-bound state) occurs through interaction with the Rap1-specific 140 kDa guanylnucleotide

exchange factor C3G (Tanaka *et al.*, 1994; Gotoh *et al.*, 1995). In addition, increased expression of C3G can also suppress the v-Ki-Ras-transformed phenotype in a manner similar to increased expression of Rap1 (Gotoh *et al.*, 1995). Furthermore, Crk represents another family of small adaptor proteins that are composed of SH2 and SH3 domains (Mayer *et al.*, 1988; Matsuda *et al.*, 1992; Reichman *et al.*, 1992; ten Hoeve *et al.*, 1994). In particular, CrkII is composed of a single N-terminal SH2 domain and two tandem SH3 domains. Under basal conditions, C3G is associated with CrkII through interactions of the central SH3 domain of CrkII with several proline-rich regions in C3G (Knudsen *et al.*, 1994; Matsuda *et al.*, 1994; Tanaka *et al.*, 1994; Feller *et al.*, 1995). Similarly to Grb2, the N-terminal SH2 domain can then direct the association of CrkII with several tyrosine-phosphorylated proteins including the epidermal growth factor (EGF) and nerve growth factor (NGF) receptors, IRS1/2, Cbl, paxillin and pp130Cas (Matsuda *et al.*, 1990; Birge *et al.*, 1992, 1993; Hempstead *et al.*, 1994; Sakai *et al.*, 1994; Teng *et al.*, 1995; Beitner-Johnson *et al.*, 1996; Ribon *et al.*, 1996). Whether targeting of the CrkII-C3G complex to these tyrosine-phosphorylated proteins is responsible for the positive and/or negative signaling properties of Rap1 remains to be determined.

In any case, since Rap1 can function as a suppressor of Ras downstream signaling, there must be a cellular mechanism that rapidly inhibits Rap1 function to allow for Ras activation of downstream signals. Recently, several studies have suggested that the activation of Ras can be limited by a negative feedback loop which results in the serine/threonine phosphorylation of SOS and dissociation of the Grb2-SOS complex (Cherniack *et al.*, 1995; Langlois *et al.*, 1995; Waters *et al.*, 1995b; Holt *et al.*, 1996a). We therefore hypothesized that a more proximal signaling pathway leading to the dissociation of CrkII from C3G may also exist to inactivate Rap1 prior to the activation of the Ras/Raf/MEK/ERK pathway. Here we demonstrate that insulin stimulation results in a rapid dissociation of the CrkII-C3G complex, inactivation of Rap1 and decreased association of Rap1 with the Raf1 serine kinase. In addition, the uncoupling of the CrkII-C3G complex occurs in a Ras-independent manner but is directly dependent upon the phosphorylation of CrkII at Tyr221.

Results

Insulin induces a time-dependent uncoupling of Raf1 from Rap1 with a concomitant association of Raf1 with Ras

It has been well established that growth factor activation of Ras results in GTP-dependent association and activation of the Raf1 kinase (Moodie *et al.*, 1993; Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). In addition, Rap1 can also associate with Raf1 in a GTP-dependent manner (Ruggieri *et al.*, 1994; Sprang, 1995; Herrmann *et al.*, 1996; Hu *et al.*, 1997; Vossler *et al.*, 1997). To examine the reciprocal relationship between Raf1 and these two GTP-binding proteins, we immunoprecipitated Raf1 from control and insulin-stimulated cells and compared the relative co-immunoprecipitation of Rap1 and Ras (Figure 1). In the absence of

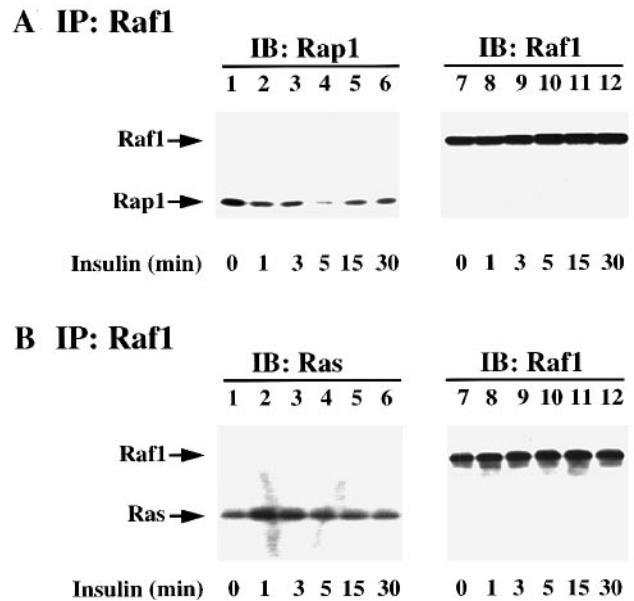


Fig. 1. Insulin stimulation decreases the amount of Raf1 associated with Rap1 concomitant with an increase in the amount of Raf1 associated with Ras. CHO/IR cells were incubated in the absence (lanes 1 and 7) or presence of 100 nM insulin for 1 (lanes 2 and 8), 3 (lanes 3 and 9), 5 (lanes 4 and 10), 15 (lanes 5 and 11) and 30 min (lanes 6 and 12) at 37°C. Whole cell detergent extracts were prepared and were immunoprecipitated with a Raf1 antibody as described in Materials and methods. (A) The Raf1 immunoprecipitates were then immunoblotted with a Rap1 antibody (lanes 1-6) or a Raf1 antibody (lanes 7-12). (B) The Raf1 immunoprecipitates were also immunoblotted with a Ras antibody (lanes 1-6) or a Raf1 antibody (lanes 7-12).

insulin, Rap1 was co-immunoprecipitated specifically with Raf1 (Figure 1A, lane 1). However, following various times of insulin stimulation, there was a time-dependent decrease in the amount of Raf1 associated with Rap1 (Figure 1A, lanes 1-6). Following 1 min of insulin stimulation, there was a detectable decrease in the amount of Raf1-associated Rap1 protein (Figure 1A, lanes 1 and 2). The maximal decrease in the amount of Rap1 co-immunoprecipitated with Raf1 occurred by 5 min and remained at reduced levels from 15 to 30 min (Figure 1A, lanes 5 and 6). These differences were not due to changes in Raf1 immunoprecipitation, as Raf1 immunoblots of these precipitates demonstrate identical amounts of Raf1 protein (Figure 1A, lanes 7-12).

Concomitant with the insulin-stimulated decrease in association between Rap1 and Raf1, there was a reciprocal increase in the amount of Raf1 that was associated with Ras (Figure 1B). Insulin stimulation for 1-3 min resulted in an increased amount of Ras co-immunoprecipitated with Raf1 compared with the unstimulated cells (Figure 1B, lanes 1-3). However, at longer times, there was a subsequent decline back towards basal levels (Figure 1B, lanes 4-6). Following 30 min of insulin stimulation, the amount of Ras associated with Raf1 was similar to that observed in the unstimulated cells. As a control for immunoprecipitation, the amount of Raf1 immunoprecipitated at all these time points was essentially constant, as determined by Raf1 immunoblotting of the Raf1 immunoprecipitates (Figure 1B, lanes 7-12).

Insulin stimulation results in a rapid inactivation of Rap1

Insulin has been well documented to stimulate Ras activation through the regulated exchange of GDP for GTP by the guanylnucleotide exchange factor SOS (Maassen *et al.*, 1992; Kahn, 1994; Roth *et al.*, 1994). To examine the GTP-binding status of Rap1, we utilized the Rap1-binding domain (RBD) of RalGDS to precipitate GTP-bound Rap1 as recently described by Franke *et al.* (1997). In the absence of insulin, GST-RBD precipitated Rap1 from whole cell detergent extracts (Figure 2, lane 1). However, insulin stimulation resulted in a time-dependent decrease in the amount of Rap1 that was precipitated with GST-RBD (Figure 2, lanes 2–5). There was a detectable decrease in the amount of Rap1 precipitated by GST-RBD following 1 min of insulin stimulation, and this was maximally reduced by 5 min. This was not a result of differences in the amount of Rap1 present in the cell extracts as determined by direct immunoblotting (Figure 2, lanes 6–10). Thus, in contrast to Ras in which insulin

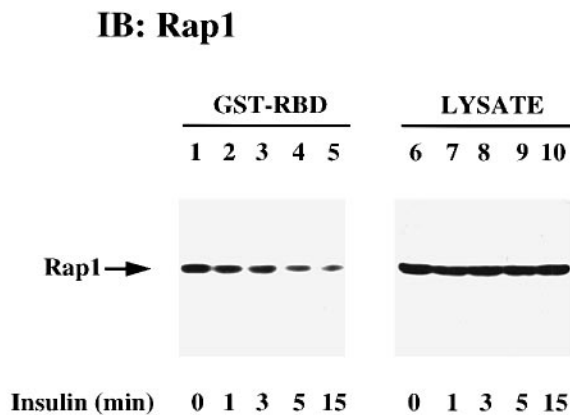


Fig. 2. Insulin stimulation results in a time-dependent inactivation of Rap1. CHO/IR cells were incubated in the absence (lanes 1 and 6) or presence of 100 nM insulin for 1 (lanes 2 and 7), 3 (lanes 3 and 8), 5 (lanes 4 and 9) and 15 min (lanes 5 and 10) at 37°C. Whole cell detergent extracts were prepared and incubated in the presence (lanes 1–5) or absence (lanes 6–10) of the GST-RBD fusion proteins as described in Materials and methods. The samples were then immunoblotted with a Rap1-specific antibody.

stimulation results in rapid activation, Rap1 is apparently converted to the inactive GDP-bound state.

Expression of Rap1 reduces insulin-stimulated Ras–Raf interaction

If insulin induces an inactivation of endogenous Rap1, then overexpression of Rap1 would be expected to saturate the system and thereby prevent this inactivation pathway. Therefore, to determine if Rap1 can antagonize Ras function, we expressed the wild-type Rap1 and determined the extent of insulin-stimulated Ras–Raf1 association (Figure 3). In empty vector-transfected cells, insulin stimulation for 1 min resulted in an increased amount of Ras co-immunoprecipitated with Raf1 (Figure 3A, lanes 1 and 2). Similarly, insulin stimulation for 5 min also resulted in a greater extent of Ras co-immunoprecipitated with Raf1 compared with unstimulated cells, but this was somewhat less than that observed at 1 min (Figure 3A, lane 3). In contrast, expression of Rap1 resulted in a marked diminution in the amount of Ras that was co-immunoprecipitated with Raf1 in unstimulated cells (Figure 3A, lane 4). In addition, the ability of insulin to increase the extent of Ras–Raf1 association was greatly attenuated (Figure 3A, lanes 5 and 6). To ensure equal immunoprecipitation of Raf1 under these conditions, the Raf1 immunoprecipitates were also immunoblotted for Raf1 (Figure 3B, lanes 1–6). Similarly, there was no significant change in the total cellular expression of Raf1 following Rap1 transfection as determined by Raf1 immunoblots of the same whole cell extracts (data not shown). Interestingly, although insulin had no effect on the amount of Ras protein present in the cell extracts, expression of Rap1 resulted in a small compensatory increase in the cellular content of Ras (Figure 3C, lanes 1–6). Nevertheless, the inhibition in Ras–Raf1 interaction directly correlated with a reduction in insulin-stimulated ERK activation (data not shown). The Rap1 inhibition of ERK activation occurred to the same extent and with the same time frame as previously reported (Cook *et al.*, 1993). Thus, these data demonstrate that in this cell system Rap1 functions as a competitor for the association of Raf1 with Ras.

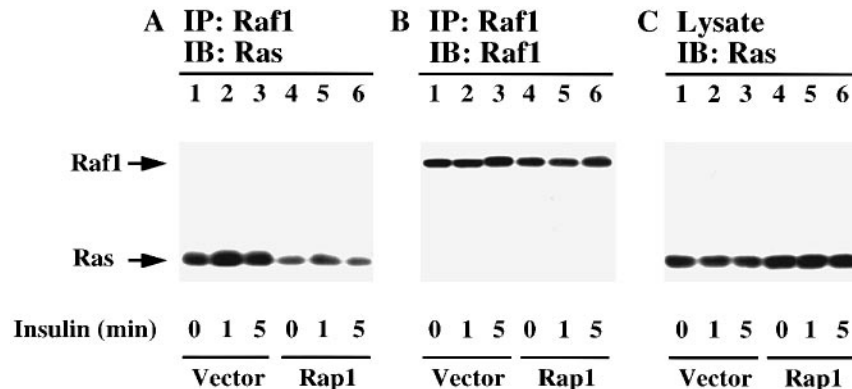


Fig. 3. Increased expression of Rap1 reduces the amount of Raf1 associated with Ras. CHO/IR cells were transfected by electroporation with 100 µg of the empty vector (Vector, lanes 1–3) or with the same vector coding for Rap1 (Rap1, lanes 4–6). At 48 h following transfection, the cells were either left untreated (lanes 1 and 4) or stimulated with 100 nM insulin for 1 (lanes 2 and 5) and 5 min (lanes 3 and 6) at 37°C. Whole cell detergent extracts were prepared, immunoprecipitated with a Raf1 antibody and immunoblotted for Ras (A) or Raf1 (B) as described in Materials and methods. (C) The whole cell extracts (lysates) were also immunoblotted directly for Ras.

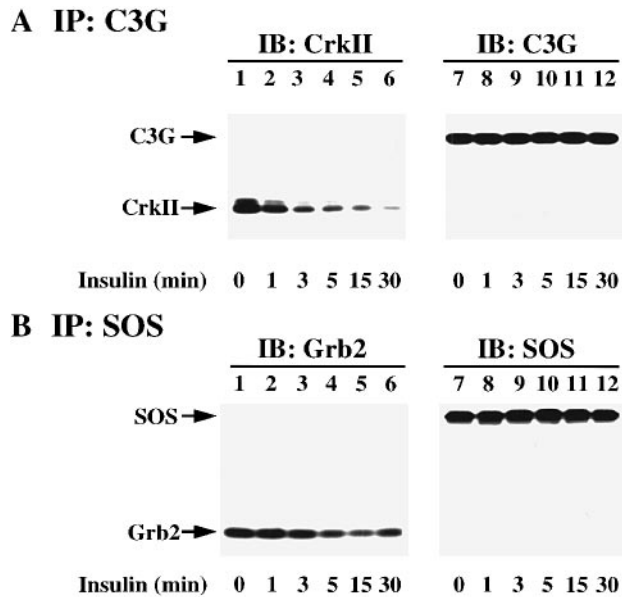


Fig. 4. Insulin stimulation results in the dissociation of the CrkII–C3G complex. CHO/IR cells were incubated in the absence (lanes 1 and 7) or presence of 100 nM insulin for 1 (lanes 2 and 8), 3 (lanes 3 and 9), 5 (lanes 4 and 10), 15 (lanes 5 and 11) and 30 min (lanes 6 and 12) at 37°C. Whole cell detergent extracts were prepared and were immunoprecipitated with a C3G antibody (A) or a SOS antibody (B) as described in Materials and methods. The C3G immunoprecipitates (upper panel) were immunoblotted with a CrkII antibody (lanes 1–6) or a C3G antibody (lanes 7–12). The SOS immunoprecipitates (lower panel) were immunoblotted with a Grb2 antibody (lanes 1–6) or a SOS antibody (lanes 7–12).

Insulin stimulation results in the dissociation of CrkII from C3G which precedes the dissociation of the Grb2–SOS complex

We and others recently have observed that the inactivation of Ras back to the GDP-bound state occurs concomitant with the dissociation of the Grb2–SOS complex (Cherniack *et al.*, 1995; Langlois *et al.*, 1995; Waters *et al.*, 1995a). We therefore speculated that the decrease in Rap1 GTP binding might have resulted from the inactivation of Rap1 due to a rapid uncoupling of the CrkII–C3G complex. Thus, to address the mechanism by which insulin induces an inactivation of Rap1, we compared the relationship between Grb2–SOS and CrkII–C3G interactions by co-immunoprecipitation (Figure 4). As expected, in unstimulated cells, immunoprecipitation of C3G resulted in the co-immunoprecipitation of CrkII (Figure 4A, lane 1). However, following insulin stimulation, there was a time-dependent decrease in the amount of CrkII that could be co-immunoprecipitated with C3G (Figure 4A, lanes 1–6). The dissociation of CrkII from C3G was detectable as early as 1 min, and continued to decline over the 30 min time period examined. 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 (Figure 4A, lanes 7–12). In addition, there was no change in the total amount of CrkII in the whole cell detergent extracts (data not shown).

In comparison with the CrkII–C3G complex, insulin stimulation also resulted a time-dependent dissociation of the Grb2–SOS complex (Figure 4B, lanes 1–6). However, as previously reported (Waters *et al.*, 1995b), the ability

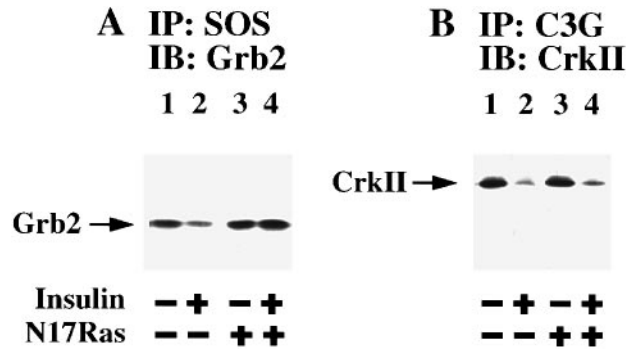


Fig. 5. The insulin-stimulated dissociation of the CrkII–C3G complex occurs through a Ras-independent mechanism. CHO/IR cells were quantitatively electroporated with an empty vector or with the vector encoding the dominant-interfering Ras mutant, N17Ras, as described in Materials and methods. The cells either were left untreated or stimulated with 100 nM insulin for 20 min at 37°C followed by preparation of whole cell detergent extracts. The extracts were then (A) immunoprecipitated with a SOS antibody and immunoblotted for Grb2 or (B) immunoprecipitated with a C3G antibody and immunoblotted for CrkII.

of insulin to induce the dissociation of Grb2–SOS was not detectable until ~5 min and was maximal by ~15 min (Figure 4B, lanes 4 and 5). As controls, the amount of SOS protein immunoprecipitated under these conditions was essentially identical (Figure 4B, lanes 7–12). Together, these data demonstrate that insulin stimulation results in a rapid dissociation of the CrkII–C3G complex which precedes the dissociation of the Grb2–SOS complex.

The insulin-stimulated dissociation of the CrkII–C3G complex is Ras independent

The dissociation of the Grb2–SOS complex results from a serine/threonine phosphorylation of SOS, which can be prevented by inhibition of Ras function or by inhibiting the downstream kinase MEK (Langlois *et al.*, 1995; Waters *et al.*, 1995a,b). As observed in Figure 4, insulin stimulation resulted in the dissociation of the Grb2–SOS complex as assessed by SOS immunoprecipitation followed by Grb2 immunoblotting (Figure 5A, lanes 1 and 2). In contrast, expression of the dominant-interfering Ras mutant (N17Ras) completely prevented the insulin-stimulated dissociation of Grb2 from SOS (Figure 5A, lanes 3 and 4). However, in the same cell extracts, the ability of insulin to dissociate the CrkII–C3G complex occurred in both the absence and presence of N17Ras (Figure 5B, lanes 1–4). Thus, unlike the insulin regulation of the Grb2–SOS complex, the insulin-stimulated dissociation of the CrkII–C3G complex occurred in a Ras-independent manner.

Dissociation of the CrkII–C3G complex results from a decreased affinity of CrkII for C3G

There are several possible mechanisms that could account for the insulin-stimulated dissociation of the CrkII–C3G complex. The most direct, and therefore the most likely, would be a rapid functional alteration of CrkII and/or C3G. To determine whether the reduced association of CrkII and C3G was due a modification in either of these two proteins, we examined the ability of GST–CrkII and GST–C3G fusion proteins to precipitate their corresponding binding partner (Figure 6). Incubation of detergent

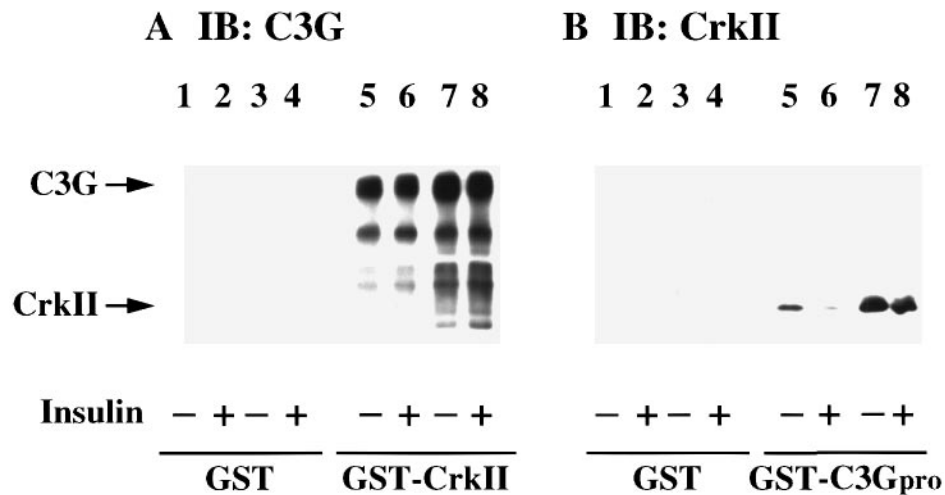


Fig. 6. The insulin-stimulated dissociation of the CrkII–C3G complex can be recapitulated *in vitro*. CHO/IR cells were incubated in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 100 nM insulin for 5 min at 37°C. Whole cell detergent extracts were prepared and the samples were then incubated for 1 h at 4°C with either 4 (lanes 1, 2, 5 and 6) or 8 µg (lanes 3, 4, 7 and 8) of GST, GST–CrkII and GST–C3Gpro fusion proteins. The resultant precipitates were immunoblotted with either a C3G antibody (A) or a CrkII antibody (B).

cell extracts isolated from control cells with 4 or 8 µg of glutathione–Sepharose demonstrated no specific binding to C3G, as determined by immunoblotting with a C3G antibody (Figure 6A, lanes 1 and 3). Similarly, there was no specific C3G binding in GST precipitates of detergent cell extracts isolated from insulin-stimulated cells (Figure 6A, lanes 2 and 4). In contrast, incubation of the detergent cell extracts with either 4 or 8 µg of GST–CrkII resulted in the precipitation of C3G (Figure 6A, lanes 5–8). Under these conditions, there was no significant difference in the amount of C3G precipitated from control and insulin-stimulated extracts (Figure 6A, compare lane 5 with 6, and lane 7 with 8).

Similarly, incubation of the detergent cell extracts with glutathione–Sepharose demonstrated no specific association with CrkII (Figure 6B, lanes 1–4). Due to the relatively large molecular weight of C3G which precluded the preparation of a full-length GST–C3G fusion protein, we prepared a truncated GST fusion protein containing the proline-rich domain of C3G (GST–C3Gpro). This domain has been shown previously to interact specifically with the middle SH3 domain of CrkII (Matsuda *et al.*, 1994). As expected, incubation of the cell extracts with GST–C3Gpro demonstrated a dose-dependent association of CrkII (Figure 6B, lanes 5–8). However, the amount of CrkII that was precipitated with GST–C3Gpro was significantly less from insulin-stimulated cell extracts compared with control cell extracts (Figure 6B, compare lane 5 with 6, and lane 7 with 8). The insulin-stimulated reduction in CrkII association was detected at both concentrations of GST–C3Gpro used, but appeared to be less at 8 µg of GST–C3Gpro compared with 4 µg. Nevertheless, there was no significant difference in the total amount of either C3G or CrkII in the detergent cell extracts isolated from control or insulin-stimulated cells (data not shown).

Since these data suggested that the reduction in CrkII association with GST–C3Gpro was concentration dependent, we next examined the binding of CrkII to various amounts of GST–C3Gpro (Figure 7). CrkII immunoblots of whole cell detergent extracts demonstrated the presence of the two typical bands migrating at ~40 and 42 kDa,

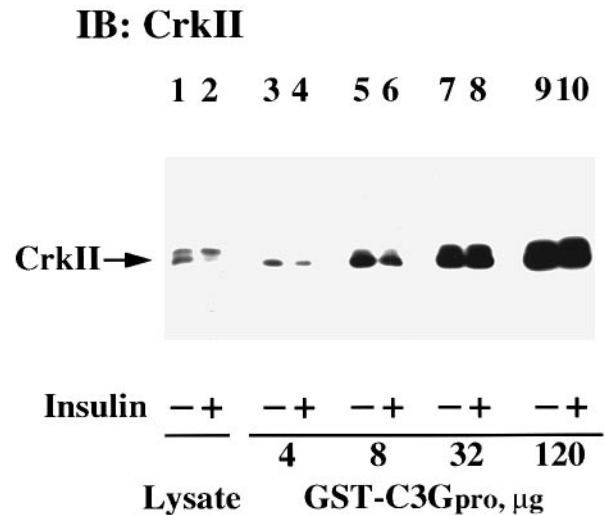


Fig. 7. The reduction of CrkII–C3G binding *in vitro* is due to a decrease in CrkII binding affinity. CHO/IR cells were incubated in the absence (lanes 1, 3, 5, 7 and 9) or presence (lanes 2, 4, 6, 8 and 10) of 100 nM insulin for 5 min at 37°C. Whole cell detergent extracts were prepared and the samples (3 mg) were then incubated for 1 h at 4°C with 4 (lanes 3 and 4), 8 (lanes 5 and 6), 32 (lanes 7 and 8) and 120 µg (lanes 9 and 10) of the GST–C3Gpro fusion protein. The original cell extracts (lysate lanes 1 and 2; 20 µg) as well as the resultant precipitates (lanes 3–10) were immunoblotted with a CrkII antibody.

respectively (Figure 7A, lane 1). Insulin stimulation resulted in a relative increase in the amount of the 42 kDa band concomitant with a reduction in the 40 kDa band (Figure 7A, lane 2). This change in the proportion of the 40 and 42 kDa CrkII proteins was due to insulin-stimulated phosphorylation which resulted in a decreased mobility of the 40 kDa band (see below). As observed in Figure 5, precipitation with 4 and 8 µg of GST–C3Gpro resulted in the appearance of only the 40 kDa band (Figure 7A, lanes 3–6). Again, there was a reduction in the amount of CrkII that was precipitated from the insulin-stimulated cell extracts compared with the control extracts (Figure 7A, compare lane 3 with 4, and lane 5 with 6). However,

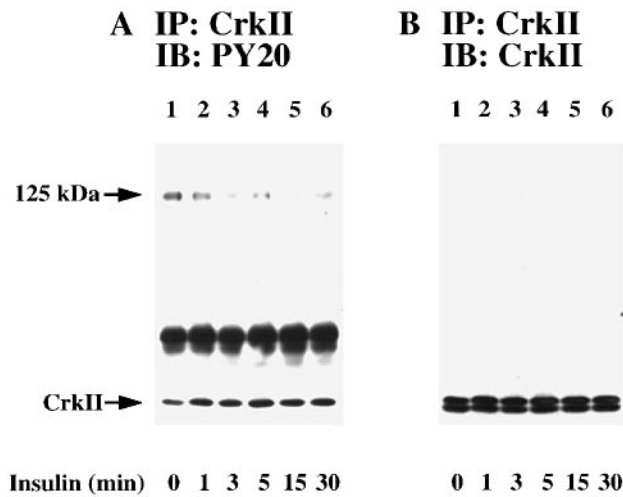


Fig. 8. Insulin stimulation increases the extent of tyrosine-phosphorylated CrkII. CHO/IR cells were incubated in the absence (lane 1) or presence of 100 nM insulin for 1 (lane 2), 3 (lane 3), 5 (lane 4), 15 (lane 5) and 30 min (lane 6) at 37°C. Whole cell detergent extracts were prepared and immunoprecipitated with a CrkII antibody as described in Materials and methods. The CrkII immunoprecipitates were then immunoblotted with the PY20 phosphotyrosine antibody (A) or a CrkII antibody (B).

with increasing concentrations of GST-C3Gpro (32 and 120 μ g), the difference between the control and insulin-stimulated cell extracts was no longer detectable (Figure 7A, compare lane 7 with 8, and lane 9 with 10). The saturation of CrkII binding was also specific at these concentrations of GST-C3Gpro as there was no detectable precipitation of CrkII by GST alone (data not shown). In addition, at these higher concentrations of GST-C3Gpro, the presence of the slower migrating 42 kDa CrkII protein was readily apparent, with no significant effect of insulin on its relative *in vitro* binding properties. Quantitation of several *in vitro* binding experiments by phosphorimager analysis demonstrated that insulin induced an ~2.5-fold reduction in binding affinity, with no change in the number of binding sites (data not shown).

In an analogous manner, incubation of a fixed amount of GST-C3Gpro with varying concentrations of detergent cell extracts also demonstrated a dose-dependent binding of CrkII (data not shown). Thus, these data demonstrate that insulin stimulation results in a modification of CrkII that reduces its ability to associate with C3G *in vitro*. Furthermore, the decreased binding between CrkII and C3G occurred due to a change in binding affinity, with no significant alteration in the number of binding sites and, hence, expression levels of either the CrkII or C3G proteins.

Insulin stimulates the tyrosine phosphorylation of CrkII

In contrast to most small SH2/SH3 adaptor proteins, CrkII undergoes tyrosine phosphorylation by the c-Abl, NGF and insulin-like growth factor 1 (IGF1) receptor tyrosine kinases (Feller *et al.*, 1994; ten Hoeve *et al.*, 1994; Beitner-Johnson and LeRoith, 1995; Ribon and Saltiel, 1996). To determine whether insulin stimulation also resulted in the tyrosine phosphorylation of CrkII, CrkII immunoprecipitates were immunoblotted with the PY20 phosphotyrosine antibody (Figure 8). In unstimulated cells, there was a

basal level of tyrosine-phosphorylated CrkII which was increased following 1 min of insulin stimulation (Figure 8A, lanes 1 and 2). The insulin-stimulated CrkII tyrosine phosphorylation was also rapid in that the maximal increase occurred within 1 min and remained constant for up to 30 min (Figure 8A, lanes 3–6).

In addition to C3G, CrkII associates with tyrosine-phosphorylated pp130Cas, Cbl and indirectly with the 125 kDa focal adhesion kinase (pp125FAK) (Polte and Hanks, 1995; Schaller *et al.*, 1995; Schaller and Parsons, 1995; Ingham *et al.*, 1996; Vuori *et al.*, 1996). A protein of ~120–130 kDa was also found to co-immunoprecipitate with CrkII (Figure 8A, lane 1). However, insulin stimulated a decrease in the amount and/or tyrosine phosphorylation of this protein that was associated with CrkII (Figure 8A, lanes 2–6). Co-immunoprecipitation and immunoblot analysis demonstrated that this protein was pp130Cas (data not shown). This is consistent with recent observations that insulin stimulation results in the tyrosine dephosphorylation of pp130Cas (Chen *et al.*, 1997; Ishiki *et al.*, 1997). As controls, the amount of immunoprecipitated CrkII was similar under these conditions (Figure 8B, lanes 1–6). As previously observed, CrkII immunoblotting demonstrated the presence of the two immunologically related proteins at 40 and 42 kDa (Figure 8B, lanes 1–6). However, phosphotyrosine immunoblotting only detected the slower migrating 42 kDa CrkII band and not the 40 kDa CrkII species (Figure 8A, lanes 1–6). These data are consistent with the presence of two electrophoretically distinct immunoreactive species that result from differences in CrkII phosphorylation (see below).

Phosphorylation of CrkII results in a reduced affinity for C3G

Having observed that insulin stimulation resulted in a rapid tyrosine phosphorylation of CrkII, we therefore examined the potential role of this event in the regulation of CrkII binding affinity for C3G. As previously observed, incubation of extracts isolated from insulin-stimulated cells demonstrated a reduction in binding to GST-C3Gpro compared with control cell extracts (Figure 9A, lanes 1 and 2). Pre-treatment of these extracts with alkaline phosphatase resulted in the disappearance of the upper band, demonstrating that the slower migrating species was due to phosphorylation (Figure 9B, lanes 1–4). Furthermore, following dephosphorylation by alkaline phosphatase, there was no significant difference in the ability of GST-C3Gpro to precipitate CrkII from the control or insulin-stimulated cell extracts (Figure 9A, lanes 3 and 4).

Alkaline phosphatase is a general phosphatase which will dephosphorylate tyrosine as well as serine/threonine phosphate. We therefore next examined the effect of the tyrosine-specific phosphatase (PTP1B) on C3G-CrkII interactions (Figure 10). As expected, incubation of cell extracts from control and insulin-stimulated cells demonstrated the expected reduction of CrkII precipitation by GST-C3Gpro (Figure 10A, lanes 1 and 2). However, incubation of the cell extracts with PTP1B resulted in a similar extent of CrkII precipitation by GST-C3Gpro (Figure 10A, lanes 3 and 4). The ability of PTP1B to tyrosine dephosphorylate CrkII was demonstrated by phosphotyrosine immunoblotting of CrkII immunoprecipitates (Figure 10B, lanes 1–4). Furthermore, PTP1B

treatment resulted in a loss of the slower migrating band, with a concomitant increase in the faster migrating CrkII species (Figure 10C, lanes 1–4). Thus, these data demonstrate that the slower migrating form of CrkII arises specifically due to tyrosine phosphorylation. In addition, these results are consistent with a direct role for CrkII tyrosine phosphorylation in mediating reduced binding affinity for C3G.

Phosphorylation at Y221 is responsible for reduced CrkII binding

It has been reported previously that the major c-Abl-dependent phosphorylation site of CrkII is Tyr221 (Feller *et al.*, 1994). To determine if tyrosine phosphorylation of this site was responsible for the insulin-stimulated dissociation of the CrkII–C3G complex, we next expressed

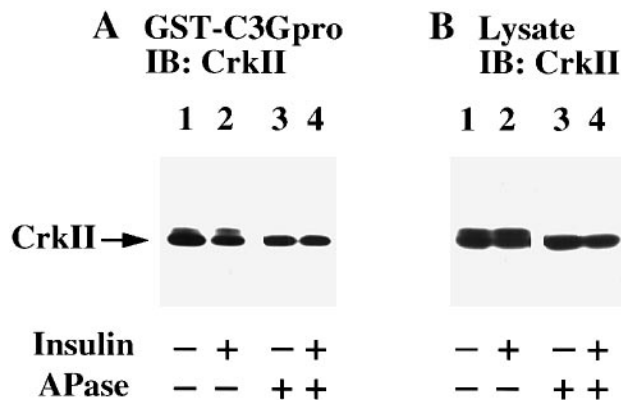


Fig. 9. The insulin-stimulated decrease in CrkII binding affinity can be reversed by dephosphorylation. CHO/IR cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 nM insulin for 5 min at 37°C. Whole cell detergent extracts were prepared and then incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of alkaline phosphatase as described in Materials and methods. (A) The samples were then incubated for 1 h at 4°C with 4 µg of the GST–C3Gpro fusion protein and the resultant precipitates immunoblotted with a CrkII antibody. (B) The alkaline phosphatase-treated cell extracts were immunoblotted directly with a CrkII antibody.

hemagglutinin epitope-tagged (HA) wild-type and Y221F mutant CrkII (Figure 11). CrkII immunoblots of the whole cell extracts from HA-CrkII-transfected cells demonstrated a similar expression level for both the HA-CrkII and endogenous CrkII proteins (Figure 11A, lanes 1–4). As previously observed, the faster migrating non-phosphorylated endogenous 40 kDa CrkII species was partially converted to the slower migrating phosphorylated 42 kDa CrkII isoform following insulin stimulation. Expression of wild-type HA-CrkII resulted in the appearance of two additional bands of ~45 and 46 kDa due to the increased molecular weight provided by the HA epitope tag (Figure 11A, lanes 1 and 2). In addition, the amount of the slower migrating 46 kDa band was also increased following insulin treatment due to tyrosine phosphorylation. Furthermore, consistent with Tyr221 as the major site of phosphorylation, mutation of this residue to phenylalanine resulted in a faster migrating species (~44 kDa) that was insensitive to the effect of insulin (Figure 11A, lanes 3 and 4). In addition, immunoprecipitation with the HA epitope antibody followed by phosphotyrosine immunoblotting demonstrated the insulin-stimulated tyrosine phosphorylation of HA-CrkII but not HA-CrkII/Y221F (data not shown).

As previously observed, incubation of extracts from insulin-stimulated cells expressing the wild-type HA-CrkII demonstrated the expected reduction in endogenous CrkII precipitation by GST–C3Gpro compared with control cell extracts (Figure 11B, lanes 1 and 2). Similarly, expression of the HA-CrkII/Y221F mutant had no significant effect on the insulin-stimulated reduction in C3G binding of the endogenous CrkII protein (Figure 11B, lanes 3 and 4). The expressed wild-type HA-CrkII also displayed an insulin-stimulated decrease in binding to GST–C3Gpro (Figure 11B, lanes 5 and 6). In contrast, not only did the HA-CrkII/Y221F mutant have a faster mobility than wild-type HA-CrkII, but insulin stimulation had no effect on the ability of the HA-CrkII/Y221F mutant to bind to GST–C3Gpro (Figure 11B, lanes 7 and 8). Together, these data directly demonstrate that insulin stimulation of CrkII

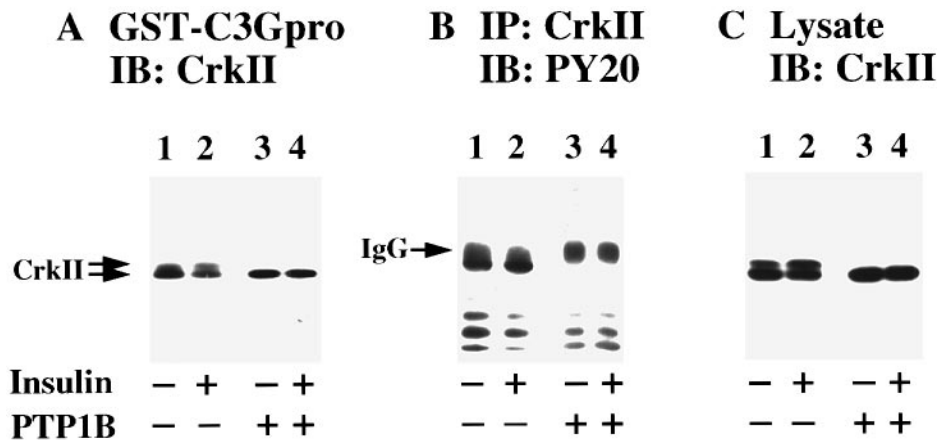


Fig. 10. The insulin-stimulated decrease in CrkII binding affinity can be reversed by tyrosine dephosphorylation. CHO/IR cells were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 nM insulin for 5 min at 37°C. Whole cell detergent extracts were prepared and then incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of the tyrosine-specific protein phosphatase PTP1B as described in Materials and methods. (A) The samples were then incubated for 1 h at 4°C with 4 µg of the GST–C3Gpro fusion protein and the resultant precipitates were immunoblotted with a CrkII antibody. (B) The PTP1B-treated cell extracts were immunoprecipitated with a CrkII antibody and immunoblotted with the PY20 phosphotyrosine antibody. The diffuse band migrating above the 42 kDa tyrosine-phosphorylated CrkII species is due to the presence of the precipitating antibody IgG heavy chain. (C) The cell extracts were immunoblotted directly with a CrkII antibody.

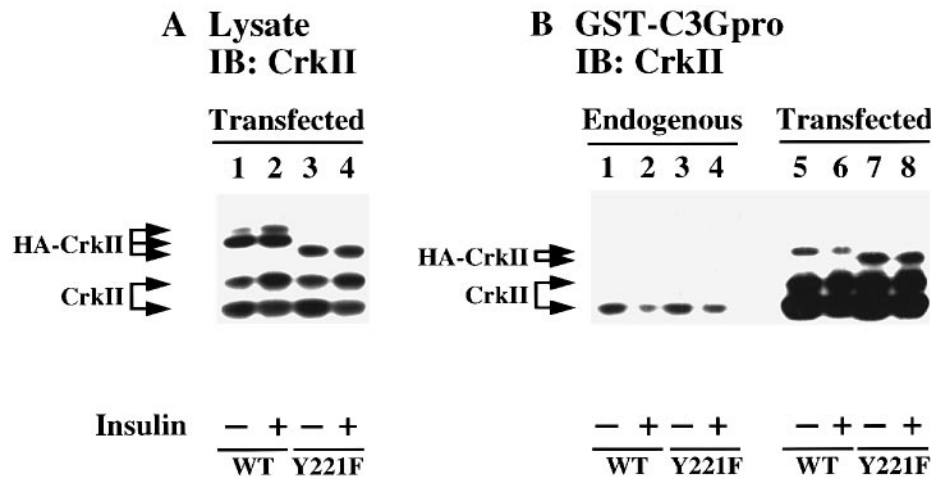


Fig. 11. Insulin-stimulated phosphorylation of Tyr221 is required for the dissociation of CrkII from C3G. CHO/IR cells were transfected by electroporation with 2 μg of the HA epitope-tagged wild-type (WT) CrkII or with an HA-tagged CrkII mutant in which Tyr221 was replaced with phenylalanine (Y221F). At 48 h following transfection, the cells were incubated in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 100 nM insulin for 5 min at 37°C. (A) Whole cell detergent extracts were prepared and immunoblotted with a CrkII antibody. (B) The whole cell detergent extracts were then incubated for 1 h at 4°C with 4 μg of the GST-C3Gpro fusion protein and the resultant precipitates immunoblotted with a CrkII antibody. A short exposure (lanes 1-4) and a longer exposure (lanes 5-8) are presented in order to visualize both the endogenous CrkII and the expressed HA-tagged CrkII proteins.

phosphorylation on Tyr221 is a required event in the dissociation of the CrkII-C3G complex.

Discussion

It is generally accepted that one pathway leading from receptor tyrosine kinases to Ras activation occurs through the tyrosine phosphorylation of Shc and formation of a Shc-Grb2-SOS ternary complex. The conversion of Ras from the inactive GDP-bound to the active GTP-bound state results in the association with the Raf1 serine kinase. Although the binding of Raf1 to activated Ras appears to be required for Raf1 activation, binding *per se* is not sufficient, and several lines of evidence have suggested that an additional event such as serine and/or tyrosine phosphorylation of Raf1 may be required (Williams *et al.*, 1992; Fabian *et al.*, 1993; Dent *et al.*, 1995; Jelinek *et al.*, 1996; Diaz *et al.*, 1997). An immediate downstream target of Raf1 is the dual functional tyrosine/threonine kinases MEK which, upon serine phosphorylation, becomes active and phosphorylates ERK on a TEY motif (Avruch *et al.*, 1994; Blumer and Johnson, 1994; Marshall, 1994). In this manner, receptor tyrosine kinase activation results in the conversion of tyrosine kinase signals to a serine/threonine kinase pathway leading to the regulation of metabolic, translational and transcriptional events (Blenis, 1993; Davis, 1993; Treisman, 1994; Hill and Treisman, 1995).

In addition to Ras, the Rap family of low molecular weight GTP-binding proteins have also been implicated in the regulation of MAP kinase pathways. For example, activation of Rap1 has been reported to stimulate DNA synthesis in Swiss 3T3 cells and to induce differentiation of PC12 cells by activation of the ERK pathway (Yoshida *et al.*, 1992; Vossler *et al.*, 1997). However, in other cell systems, Rap1 has also been observed to suppress ERK pathway-dependent signaling events (Kitayama *et al.*, 1989; Yatani *et al.*, 1990; Campa *et al.*, 1991; Jelink and Hassell, 1992; Sakoda *et al.*, 1992; Cook *et al.*, 1993). Although a unifying mechanism accounting for these

apparently contradictory observations has not been established, it appears that this may reflect cell context differences in Rap1 downstream targets. Generally, the positive signaling properties of Rap1 appear to correlate with the coupling of Rap1 to B-Raf, which results in the activation of B-Raf kinase activity and subsequent activation of MEK and ERK. In contrast, the negative signaling functions of Rap1 are more closely related to the inactivation of Raf1 which occurs upon its association with Rap1. For example, in the same cell context, expression of constitutively active Rap1 with either B-Raf or Raf1 resulted in the stimulation of B-Raf but inhibition of Raf1 activity (Vossler *et al.*, 1997).

Since B-Raf is relatively abundant in neuronal and endocrine cells whereas most other cell types predominantly express Raf1, the negative signaling properties of Rap1 are typically observed in fibroblast cell lines (Barnier *et al.*, 1995). Consistent with these observations, the Chinese hamster ovary cells expressing the insulin receptor (CHO/IR cells) used in this study express relatively high levels of Raf1 compared with B-Raf (data not shown). Thus, Rap1 would be expected to function in a suppressive manner for Ras downstream signaling by sequestering and maintaining Raf1 in an inactive state. Consistent with this hypothesis, overexpression of Rap1 was found to sequester Raf1 from Ras and to reduce the insulin stimulation of the ERK activation. Based on these findings, we therefore speculated that in order for insulin to activate the Raf/MEK/ERK pathway, the Rap1 inhibition of Raf1 activation would have to be de-repressed. In support of this hypothesis, Raf1 was associated with Rap1 in the basal state and, following insulin stimulation, there was a rapid dissociation of this complex. The uncoupling of Raf1 from Rap1 occurred in concert with the formation of the Ras-Raf1 complex responsible for the downstream activation of MEK and ERK.

Recently, we and others have observed that activation of the Raf1/MEK/ERK pathway leads to a feedback serine/threonine phosphorylation of SOS and dissociation of the

Grb2-SOS complex (Cherniack *et al.*, 1995; Langlois *et al.*, 1995; Waters *et al.*, 1995b). Since blockade of the insulin-stimulated SOS phosphorylation resulted in a persistent association of the Grb2-SOS complex and prolongation of Ras in the active GTP-bound state, it has been hypothesized that this may be one mechanism accounting for the inactivation of Ras (Langlois *et al.*, 1995; Waters *et al.*, 1995a). In analogy to the Grb2-SOS complex, C3G appears to function as a relatively specific guanylnucleotide exchange factor for Rap1 and associates with the small adaptor protein CrkII in the basal state (Knudsen *et al.*, 1994; Matsuda *et al.*, 1994; Tanaka *et al.*, 1994; Feller *et al.*, 1995; Gotoh *et al.*, 1995). Consistent with a role for the CrkII-C3G complex in regulating Rap1 function, expression of a membrane-targeted form of C3G can suppress the transformed phenotype induced by oncogenic Ras (Gotoh *et al.*, 1995). In addition, expression of membrane targeting forms of Crk enhanced the Rap1 guanylnucleotide exchange activity of C3G by the membrane recruitment of C3G (Ichiba *et al.*, 1997). In this study, we have observed that insulin stimulation induced the rapid dissociation of the CrkII-C3G complex. The time course of CrkII-C3G dissociation occurred in parallel with the inhibition of Rap1-Raf1 interaction and formation of the Ras-Raf1 complex. Based upon the temporal relationship between these events, we postulate that uncoupling of the CrkII-C3G complex de-represses Rap1 function, thereby releasing Raf1 which can then become available for activation by Ras.

To investigate the molecular basis for the insulin-stimulated dissociation of the CrkII-C3G complex, we first demonstrated that a dominant-interfering Ras mutant (N17Ras), although fully capable of preventing the dissociation of the Grb2-SOS complex, was completely without effect on the CrkII-C3G complex. These data eliminated the possibility of a Ras-dependent serine/threonine feedback phosphorylation similar to that responsible for the dissociation of the Grb2-SOS complex. Furthermore, we have not detected any insulin-dependent serine/threonine phosphorylation of either C3G or CrkII. In contrast, we have observed that insulin stimulation resulted in the tyrosine phosphorylation of CrkII, but not of C3G, in a time frame consistent with the dissociation of CrkII from C3G. Consistent with a covalent modification of CrkII, *in vitro* GST fusion protein-binding assays demonstrated that insulin reduced the binding affinity of CrkII for C3G without any detectable alterations in C3G binding properties. In addition, tyrosine dephosphorylation of CrkII restored high affinity binding *in vitro* whereas the CrkII/Y221F mutant failed to undergo tyrosine phosphorylation and was resistant to insulin-stimulated dissociation from C3G *in vivo*. These results are consistent with a model in which the tyrosine phosphorylation of CrkII creates an intramolecular binding interaction with the N-terminal CrkII SH2 domain, as has also been shown for the c-Abl-induced tyrosine phosphorylation of CrkII (Feller *et al.*, 1994; Rosen *et al.*, 1995). In this manner, a conformational change could result that either occludes or modifies the central CrkII SH3 in a manner recently described for c-Src and Hck (Anafi *et al.*, 1996; Moarefi *et al.*, 1997; Sicheri *et al.*, 1997; Xu *et al.*, 1997).

Recently, activation of the IGF receptor has also been observed to increase the tyrosine phosphorylation of a

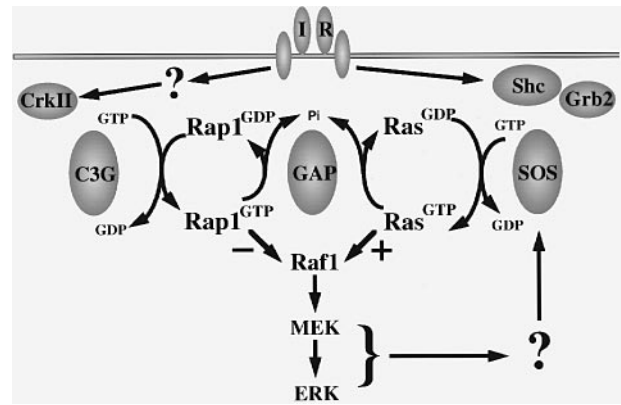


Fig. 12. Schematic model of the hypothesized dynamic relationship between the Ras and Rap1 GTP cycles. In this model, activation of the insulin receptor induces the tyrosine phosphorylation of Shc and thereby generates the formation of the Shc-Grb2-SOS ternary complex necessary for Ras activation. In parallel, the insulin receptor tyrosine phosphorylates CrkII inducing the dissociation of the CrkII-C3G complex and the inactivation of Rap1. Inactivation of Rap1 allows Raf1 to associate with Ras, and its subsequent activation results in the stimulation of the ERK kinase module. For additional details, refer to the text.

small fraction of the total cellular CrkII pool (Beitner-Johnson and LeRoith, 1995). Similarly, the extent of insulin-stimulated CrkII tyrosine phosphorylation was low compared with the total cellular content of CrkII protein. If CrkII tyrosine phosphorylation accounts for the dissociation of the CrkII-C3G complex, how do we account for the non-stoichiometric CrkII phosphorylation? Like Grb2, CrkII is not associated exclusively with C3G but is found in separate and distinct complexes with several other effector proteins such as c-Abl, p180DOCK, SOS and Eps15 (Feller *et al.*, 1994, 1995; Knudsen *et al.*, 1994; Matsuda *et al.*, 1994; Tanaka *et al.*, 1994; Schumacher *et al.*, 1995; Hasegawa *et al.*, 1996). It is therefore likely that insulin stimulation only results in the tyrosine phosphorylation of the CrkII subpopulation that is associated exclusively with C3G. Future experiments will be necessary to identify directly the fraction of tyrosine-phosphorylated CrkII and its role in regulating the binding interaction between CrkII and C3G.

In summary, based upon these data, we can propose the following mechanism for receptor tyrosine kinase activation of the ERK cascade (Figure 12). Initially, Raf1 is maintained in an inactive state due to its association with Rap1. Receptor tyrosine kinase activation results in the assembly and/or activation of the Shc-Grb2-SOS complex which then induces the exchange of GDP for GTP on Ras. At the same time, CrkII undergoes tyrosine phosphorylation which induces the dissociation of the CrkII-C3G complex. The uncoupling of CrkII from C3G allows conversion of Rap1 from the active GTP-bound conformational state to the inactive GDP form. The inactivation of Rap1 releases the bound Raf1, which can then associate with the active GTP-bound Ras, resulting in activation of the Raf1 serine kinase. The subsequent Raf1-dependent phosphorylation and activation of MEK results in phosphorylation and activation of ERK required for the downstream stimulation of various transcriptional and translational events. However, following MEK and/or ERK activation, there is a feedback serine/threonine

phosphorylation on SOS. This latter event induces the dissociation of the Grb2–SOS complex, terminating the Ras activation signal. Although this model is consistent with all available data to date, the mechanism by which these pathways are reset to the basal state following termination of receptor tyrosine kinase signals remains to be determined.

Materials and methods

Cell culture

CHO/IR cells were isolated and cultured as previously described (Frattali *et al.*, 1991; Waters *et al.*, 1995b). Cells were incubated for 6–8 h in serum-free media and then incubated with and without 100 nM insulin 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 EDTA, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml pepstatin, 10 µg/ml aprotinin and 5 µg/ml leupeptin (Waters *et al.*, 1995b). For Raf1 immunoprecipitations, the cell extracts were prepared by solubilization in 25 mM Tris, 10 mM NaH₂PO₄, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 50 mM sodium fluoride, 1 mM PMSF, 1 µg/ml pepstatin, 10 µg/ml aprotinin and 5 µg/ml leupeptin (Schieffer *et al.*, 1996). In each case, the same whole cell detergent solubilization buffer was used to wash that particular immunoprecipitate extensively prior to SDS–PAGE.

Immunoprecipitation and immunoblotting

Immunoprecipitations were performed from whole cell lysates by incubation with 2.0 µg of polyclonal SOS (Transduction Laboratories), Raf1, CrkII and C3G 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 or 12% SDS–polyacrylamide gels (30:0.4 acrylamide:bis-acrylamide) and transferred to PVDF membranes at 4°C. Immunoblotting was performed with monoclonal antibodies (Transduction Laboratories) directed against Rap1, Ras, Raf1, CrkII, Grb2, SOS and phosphotyrosine (PY20).

In vitro binding of CrkII and C3G

The GST–CrkII cDNA was obtained as previously described (Okada and Pessin, 1996). The cDNA encoding the proline-rich region of C3G (amino acids 532–779) was obtained by digestion of the C3G cDNA with *HincII* and subcloning this fragment into the *SmaI* site of pGEX-2T as previously described (Tanaka *et al.*, 1994). The GST fusion proteins were isolated and bound to glutathione–Sepharose beads as previously described (Holt *et al.*, 1996b). The bound GST fusion proteins were then incubated for 1 h at 4°C with the whole cell detergent extracts. The Sepharose beads were pelleted and washed three times with phosphate-buffered saline (PBS) and solubilized in Laemmli sample buffer. The amount of CrkII or C3G bound to the immobilized GST fusions protein was then determined by immunoblotting.

In vitro binding of active Rap1 to GST–RBP

GST–RBP was kindly provided by Dr Johannes Bos (Utrecht University) and was used to determine the relative amount of active GTP-bound Rap1 as previously described (Franke *et al.*, 1997). Briefly, whole cell detergent extracts were incubated with 5 µg of immobilized GST–RBD for 60 min at 4°C. The beads were then washed four times in lysis buffer and solubilized in SDS sample buffer, followed by SDS–PAGE and immunoblotting with a Rap1 antibody.

Quantitative transient transfection by electroporation

We have demonstrated previously that electroporation can be used to express various cDNAs with 85–100% transfection efficiency in CHO/IR cells (Yamauchi and Pessin, 1994). Briefly, CHO/IR cells were suspended in 500 µl of PBS plus the empty pcDNA3 vector or the pcDNA3 mammalian expression vector containing either the Rap1, HA–CrkII or HA–CrkII/Y221F cDNAs. The cells were then electroporated at 340 V and 960 µF and plated in α -minimal essential medium containing 10% serum. Cell debris was removed by replacing media

with fresh media 12 and 30 h later. After 48 h, the transfected cells were serum starved for 6–8 h and either left untreated or stimulated for various times with 100 nM insulin as described in the figure legends.

Phosphatase treatment of cell extracts

The isolated whole cell detergent extracts were incubated with 10 000 U of calf intestinal alkaline phosphatase (Sigma Chemical Co.) or 35 µg of protein tyrosine phosphatase 1B (Upstate Biotechnology, Inc.) for 60 min at room temperature as previously described (Kao *et al.*, 1997). The resultant cell extracts were then subjected to precipitation and immunoblotting as described above.

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