Insulin and EGF Receptors Integrate the Ras and Rap Signaling Pathways

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Abstract: Ras is a low molecular weight (Mr 21,000) GTP binding protein that plays an essential role in cell proliferation and differentiation. 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. Opposing the Ras activation pathway, the low molecular GTP binding protein Rap was originally observed to revert or suppress the transformed phenotype in Ki-Ras-transformed fibroblasts. 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. Although the precise molecular details remained to be established, there is a 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 guanylnucleotide exchange factor SOS to the plasma membrane location of Ras. The carboxyl terminal domain of SOS contains a proline-rich regions that directs its association with the SH3 domains of the small adapter protein, Grb2. Similarly, the formation of active GTP-bound Rap results from the specific interaction with the Rap guanylnucleotide exchange factor C3G which specifically associates with the central SH3 domain of the small adapter protein, CrkII. Thus, efficient regulation of receptor tyrosine kinase downstream signaling events require the coordinate interplay of these two pathways.

Key words: Ras, Rap, Tyrosine kinase, Insulin, Epidermal growth factor

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LOW molecular weight GTP binding proteins play essential roles as molecular switches controlling diverse processes including cytoskeleton organization, intracellular vesicular trafficking, protein translocation, macromolecular biosynthesis, entry into the cell cycle and mitogenesis [1, 2]. The prototypical small GTP binding protein, Ras, is a 21 kDa membrane anchored protein that was originally identified as an oncogene in human bladder, lung and colon carcinomas [3–5]. The best characterized proximal downstream Ras target is the Raf1 serine/threonine kinase which specifically associates with GTP-bound Ras [6–10]. The interaction of Raf1 with Ras results the activation

of the Raf1 protein kinase activity which in turn phosphorylates and activates the dual functional protein kinase, termed MEK [11–15]. This kinase phosphorylates the ERK family of MAP 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 [16–19].

Opposing the Ras activation pathway, the low molecular GTP binding protein Rap was originally observed to revert or suppress the transformed phenotype in Ki-Ras-transformed fibroblasts [20, 21]. Although Rap can function as a positive regulatory of the Raf/MEK/ERK cascade in certain cell contexts, in multiple cell types several studies have demonstrated that Rap can function as a

suppressor of Ras-mediated downstream signaling. For example, both Ras and T-antigen dependent transformation can be reversed by Rap1 expression [20, 22]. 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 [23–26]. This apparent antagonism between Ras and Rap function may reflect the ability of Rap and Ras to interact with the same downstream effectors since they share identical sequences within their respective effector domains [20, 21, 27–31]. Consistent with this concept of effector competition, the 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 [32].

In addition to the similarity in downstream signaling pathways, the mechanisms that regulate both Ras and Rap activation are highly related. Ras GTP binding is stimulated upon the targeting and/or activation of the Ras guanylnucleotide exchange factor SOS [33–36]. The carboxyl terminal domain of SOS contains a proline-rich regions that direct its association with the SH3 domains of the small adapter protein, Grb2 [37–42]. In analogy, the central SH3 domain of the small adapter protein CrkII associates with the proline rich regions of Rap guanylnucleotide exchange factor C3G [43–46]. Similar to Grb2, Crk represents another family of small adapter proteins that are comprised of SH2 and SH3 domains [47–50]. CrkII is composed of a single amino terminal SH2 domain and two tandem SH3 domains. Under basal conditions, the Rap guanylnucleotide exchange factor C3G is associated with CrkII through interactions between the central SH3 domain of CrkII with several proline rich regions in C3G [44–46, 51].

Recently we and others have demonstrated that the activation of Ras can be limited by a negative feedback loop which results in the serine/threonine phosphorylation of SOS [52–55]. This phosphorylation event appears to coincide with the dissociation of the Grb2-SOS complex and desensitization of Ras as measured by the amount of GTP versus GDP bound to immunoprecipitated Ras protein. These data suggest a model in which enhanced SOS activity is maintained until the Grb2-

Fig. 1. Schematic model for the activation/inactivation GTP/GDP cycle of Ras. In the basal state, Ras predominantly exists in the inactive GDP-bound state due to the GTPase activity of GAP. However, following growth factor stimulation the guanylnucleotide exchange factor SOS becomes targeted to and/or becomes activated toward Ras, resulting in the exchange of GDP for GTP. Once Ras is in the active GTP-bound state it associates and activates the Raf1 serine kinase which in turn phosphorylates and activates MEK. MEK then phosphorylates ERK which can then phosphorylate and activate a variety of cytosolic and nuclear factors. In addition, either ERK and/or another MEK-dependent kinase phosphorylates SOS which reduces its affinity for Grb2. The dissociation of the Grb2-SOS complex then inhibits any further Ras activation allowing GAP to restore Ras back to the inactive GDP-bound state.

SOS complex dissociates causing either decreased nucleotide exchange activity or prevention of the targeting of SOS to the plasma membrane location of Ras (Fig. 1).

Based upon the antagonistic function of Rap, we would expect that there also exists a cellular mechanism for the inactivation of Rap. We therefore hypothesized that a more proximal signaling pathway leading to the dissociation of CrkII from C3G may also exist to inactivate Rap prior to the activation of the Ras/Raf/MEK/ERK pathway. To explore this possibility, we have recently observed a rapid (<1 min) insulin or EGF stimulated dissociation of the CrkII-C3G complex (Fig. 2). In unstimulated cells, immunoprecipitation of C3G resulted in the co-immunoprecipitation of CrkII (Fig. 2A, lane 1). However, following insulin

A) Insulin **B) EGF**

Fig. 2. Insulin and EGF stimulation result in the dissociation of the CrkII-C3G complex. **A)** Chinese hamster ovary cells engineered to over express both the human insulin and EGF receptors (CHO/IR/ER) 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). Reprinted from reference [56].

stimulation, there was a time-dependent decrease in the amount of CrkII that could be co-immunoprecipitated with C3G (Fig. 2A, 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. 2A, 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. 2A, lanes 7–12).

Similarly, activation of the EGF receptor also resulted in a rapid dissociation of the CrkII-C3G complex (Fig. 2B, lanes 1–6). The time-dependent

Fig. 3. Hypothesized model depicting the predicted relationship between the Ras and Rap GTP cycles. In this model, activation of growth factor receptors (in this case 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 stimulates the tyrosine phosphorylates CrkII inducing the dissociation of the CrkII-C3G complex and the inactivation of Rap. Inactivation of Rap allows Raf1 to associate with Ras and its subsequent activation results in the stimulation of the ERK kinase cascade.

uncoupling of CrkII from C3G was similar to insulin with detectable dissociation following 1 min of EGF treatment (Fig. 2B, 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. 2B, lanes 3–6). As a control for immunoprecipitation, immunoblotting with the C3G antibody demonstrated equivalent amounts of immunoprecipitated C3G protein under each condition (Fig. 2B, lanes 7–12). These data are consistent with the CrkII-C3G complex functioning to maintain Rap1 in the active GTP-bound state. 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 tyrosine kinase receptor mediated dissociation of the CrkII-C3G complex was also temporally correlated with an apparent conformational change and/or masking of Rap immunoreactivity [56]. Together, these data provide for a compelling model in which growth factor activation leads to the simultaneous activation of Ras and inactivation of Rap through the control of the guanylnucleotide exchange factors SOS and C3G (Fig. 3). In this model we propose

that initially, Raf1 is maintained in an inactive state due to its association with Rap. 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 CrkII from C3G allows conversion of Rap from the active GTP-bound conformational state to the inactive GDP form. The inactivation of Rap 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 later event induces the dissociation of the Grb2-SOS complex terminating the Ras activation signal. Based upon the critical role of the Raf/MEK/ERK pathway in controlling the relative balance between apoptosis and mitogenesis, this yin-yang model provides for a highly tuned mechanism that can tightly maintain these activities within normal physiological limits.

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