**Communication**

Nerve Growth Factor-mediated Activation of the Mitogen-activated Protein (MAP) Kinase Cascade Involves a Signaling Complex Containing B-Raf and HSP90*

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The nerve growth factor (NGF)-mediated activation of the mitogen-activated protein (MAP) kinase cascade is an obligatory step in the morphological differentiation of PC12 cells. Signal transduction through the MAP kinase cascade is dependent upon activation of p21ras which binds directly to Raf family protein kinases, mediating their association with the membrane and activation. PC12 cells express two Raf isoforms, c-Raf and B-Raf. The activation of the MAP kinase cascade in response to NGF is due principally to the action of B-Raf. NGF treatment of PC12 cells resulted in the enhanced phosphorylation of B-Raf and c-Raf, and both exhibit reduced electrophoretic mobilities following stimulation of the cells. The NGF-stimulated phosphorylation of B-Raf was correlated with its enzymatic activation as measured by the phosphorylation of its substrate MEK. However, c-Raf does not exhibit significant levels of activity. B-Raf was present as a component of a high molecular mass complex, which included the molecular chaperone, heat shock protein 90 (HSP90). Importantly, c-Raf did not participate in the formation of such complexes. The B-Raf containing HSP90 complexes were normally present in PC12 cells, and their assembly was not dependent upon NGF stimulation. These data suggest that the ability of B-Raf to activate the MAP kinase cascade is due to its association with a large signaling complex, which is likely to impart signaling pathway specificity.

The activation of TrkA upon NGF binding results in the stimulation of a multiple independent signaling pathways (1, 2). One of the most important of these pathways is the MAP kinase cascade (3, 4). The activation of this signaling pathway in response to NGF requires the conversion of p21ras into an active conformation, permitting its interaction with serine kinases of the Raf family. PC12 cells express two members of this family c-Raf and B-Raf, both of which have been reported to be enzymatically activated by NGF treatment of the cells as a consequence of their translocation to the membrane (5–10). The Raf isoforms phosphorylate and activate the dual specificity kinase MEK, which in turn phosphorylates and activates the MAP kinases, ERK1 and ERK2. The MAP kinases and pp90rsk are translocated to the nucleus where they act to regulate gene expression through the phosphorylation of transcription factors (11).

One of the cardinal lessons gained from the study of intracellular signaling has been that formation of large complexes of proteins is essential for signal transmission (12). The cells must be able to selectively assemble such complexes and regulate their intracellular localization in response to extracellular stimuli. With respect to the MAP kinase cascade, stable complexes containing p21ras, c-Raf, B-Raf, MEK1, and 14-3-3 proteins have been detected in vitro (13–18). The central role of the MAP kinase signaling cascade in mediating the NGF-stimulated differentiation of these cells has been demonstrated through expression of constitutively activated forms of p21ras, c-Raf, MEK, and ERK2, which mimic the action of NGF (4, 19–21). Similarly, dominant negative mutants of p21ras, c-Raf, MEK, and the MAP kinases act to block the ability of NGF to produce the morphological differentiation of PC12 cells into a neuronal phenotype (20, 22). The identity of the constituents of the MAP kinase cascade that participate in NGF signaling has been controversial. There is evidence that NGF treatment of PC12 cells results in the phosphorylation and activation of c-Raf; however, overexpression of an activated form of c-Raf failed to stimulate the activity of the MAP kinases (20). Significantly, c-Raf isolated from NGF-stimulated PC12 cells was unable to activate the MAP kinase cascade in vitro (23), consistent with the in vivo findings reported by Wood et al. (20). Moreover, it has been demonstrated that in PC12 cells the activation of the MAP kinase cascade is mediated principally by B-Raf (23). It remains unclear how the cell is able to selectively employ only B-Raf but not c-Raf to drive the MAP kinase cascade in response to growth factor stimulation.

Heat shock proteins have diverse roles as molecular chaperones to stabilize protein structure and mediate stress tolerance. One of these proteins, heat shock protein 90 (HSP90), forms specific complexes with steroid hormone receptors and several protein kinases, including Raf (18, 24–26) and Src (27, 28). HSP90 has been demonstrated to play a critical role in the regulation of the activity and intracellular translocation of these proteins (29). Importantly, HSP90 has recently been shown to mediate intracellular signal transduction events (30, 31). We report here that the capacity of B-Raf to activate the MAP kinase cascade is correlated with its ability to form a stable association with HSP90.

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The abbreviations used are: NGF, nerve growth factor; MEK, mitogen-activated/extracellular signal-regulated kinase kinase; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; IP, immunoprecipitation; PVDF, polyvinylidene difluoride.
Materials and Methods

Materials—NGF was purchased from Austral Biologicals (San Ramon, CA). c-Raf1 antiserum was generously provided by Dr. R. Schatzman (Syntex, Palo Alto, CA). Rabbit antiserum to B-Raf were generated against a peptide comprising the C-terminal 12 amino acids of B-Raf. A monoclonal anti HSP90 antibody (SPA830) was obtained from Stressgen (Vancouver, British Columbia). A MEK1 antibody and the MEK1 expression vector were gifts of Dr. R. Erickson (Harvard University). Recombinant ERK2 was prepared as described previously (23).

Cell Culture and Preparation of Lysates—PC12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum and 5% fetal calf serum in an atmosphere of 10% CO2. NIH3T3 cells were grown in DMEM with 10% fetal calf serum. The medium was replaced with serum-free DMEM 24 h before treatment.

Immunoprecipitation—After appropriate treatments with growth factors, the cells were lysed with immunoprecipitation (IP) buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM p-nitrophenyl phosphate, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 20 mM NaF, 0.25 mM phenylmethylsulfonyl fluoride, 50 mM okadic acid, and protease inhibitors (10 μg/ml aprotinin and leupeptin). In experiments in which kinase activity of the immunoprecipitates was assayed, SDS and deoxycholate were omitted from the IP buffer. Cellular lysates were preclarified by incubation with Pansorbin (50 μl/ml) for 1 h at 4 °C and processed as described earlier (23).

MEK Phosphorylation and MEK Activation Assay—The ability of column fractions to activate MEK was measured by a coupled assay as described previously (23).

Results

NGF has been reported to stimulate the activity of both c-Raf and B-Raf (5–10); however, our inability to detect c-Raf activity in PC12 lysates (23) led us to directly compare the activity of these two enzymes. PC12 cells were incubated for 5 min with NGF and then B-Raf and c-Raf were immunoprecipitated from soluble lysates. The activity of the enzymes was measured by the phosphorylation of recombinant MEK. NGF dramatically stimulated the activity of B-Raf; however, we were unable to detect significant levels of activity associated with the c-Raf immunoprecipitates when assayed under identical conditions (Fig. 1). A positive control for c-Raf immunoprecipitations was performed using EGF-treated NIH3T3 cells which demonstrated a significant level of activity when assayed in parallel reactions. We also directly measured B-Raf and c-Raf activities in cellular lysates fractionated by Mono Q chromatography. NGF-treated cells resulted in the dramatic stimulation of B-Raf activity (control, 7199 cpm; NGF, 95,875 cpm), while c-Raf was only modestly stimulated (control, 7166 cpm; NGF, 21,461 cpm).

B-Raf and c-Raf enzymes have been reported to undergo NGF-stimulated phosphorylation in PC12 cells (5–10). We wished to independently evaluate the effect of NGF on the phosphorylation level of B-Raf and c-Raf. PC12 cells were metabolically labeled with 32P2 and c-Raf and B-Raf were immunoprecipitated from the same lysates following 5 min of NGF treatment (Fig. 2). NGF stimulated the phosphorylation of B-Raf with the appearance of B-Raf species exhibiting a lower electrophoretic mobility. Similarly, the phosphorylation of c-Raf was significantly stimulated; however, the relative amount of radiolabeled phosphate associated with c-Raf was significantly less than that of B-Raf. These data demonstrate that both c-Raf and B-Raf are phosphorylated in response to NGF treatment and that our failure to detect c-Raf activity was not due to the inability of c-Raf to undergo ligand-stimulated phosphorylation. We also detected a 90-kDa band corresponding to HSP90 in NGF-stimulated B-Raf immunoprecipitates, which was subsequently confirmed by Western blot analysis (data not shown). HSP90 was not found to be present in c-Raf immunoprecipitates performed under identical conditions.

We initiated studies to investigate the basis of the differential coupling of these two Raf family members to the MAP kinase cascade. We have previously discovered that B-Raf, isolated from PC12 cells, eluted from gel filtration columns with a much greater apparent molecular mass than expected from the size of the 95-kDa primary translation product (23), suggesting it was a component of a larger complex. Soluble lysates from NGF-stimulated PC12 cells were fractionated by chromatography on a Mono Q column. Fractions containing B-Raf and c-Raf were identified by Western analysis. The B-Raf-containing fraction was then independent pooled, applied to a Superose 12 gel filtration column, and the resulting column fractions were analyzed for their ability to activate the MAP kinase cascade using a coupled assay (Fig. 3, A and B) and by Western analysis (Fig. 3, C and D). B-Raf eluted from the gel filtration column with an apparent molecular mass > 300 kDa (Fig. 3, A and C). Importantly, HSP90
was detected within the same fractions and coeluted with B-Raf. Identical results were obtained when untreated PC12 cells were analyzed (data not shown), indicating that B-Raf and HSP90 complexes normally exist in the cytoplasm (Fig. 4). To verify that the HSP90 present in these fractions was associated with B-Raf, the Superose 12 fractions containing B-Raf were immunoprecipitated with anti-B-Raf antibody and the immunoprecipitates probed with B-Raf and HSP90 antibodies (E). Positive controls were run in parallel lane using purified HSP90 and recombinant B-Raf expressed as a 120-kDa GST fusion protein.

HSP90 was investigated by performing immunoprecipitations under stringent conditions. PC12 cells were incubated in the absence or presence of NGF for 5 min and soluble lysates of the cells subjected to immunoprecipitation using antibodies to B-Raf and c-Raf. The immunoprecipitates were washed in a buffer containing the ionic detergents SDS and deoxycholate. Western analysis revealed that the antibodies specifically immunoprecipitated B-Raf (Fig. 4B) and c-Raf (Fig. 4C); however, HSP90 was found to be associated only with B-Raf (Fig. 4A). These findings are consistent with the detection of HSP90 in immunoprecipitates of B-Raf, but not c-Raf in metabolically labeled PC12 cells (Fig. 2). We performed control experiments to verify that we could efficiently detect c-Raf/HSP90 complexes.

FIG. 3. Gel filtration chromatography of B-Raf and c-Raf. Cellular lysates obtained from PC12 cells incubated for 5 min with NGF (100 ng/ml) were applied to a HR5/5 Mono Q column and the resulting B-Raf- and c-Raf-containing fractions were identified and independently pooled. The B-Raf (A) and c-Raf (B) fractions were applied to a Superose 12 gel filtration column and assayed for MAP kinase activity in the absence (open circles) or presence (closed circles) of recombinant MEK1. The individual column fractions were probed by Western blot using anti-B-Raf antibody and the immunoprecipitates probed with B-Raf and HSP90 antibodies. These data are consistent with a previous report, to verify that we could efficiently detect c-Raf/HSP90 complexes. In NIH3T3 cells c-Raf activates the MAP kinase cascade in response to EGF. Immunoprecipitation of c-Raf from EGF-treated 3T3 cells, under conditions identical to those used in PC12 cells, revealed the presence of HSP90 in the immune complexes (Fig. 4D). These data demonstrate that in PC12 cells only B-Raf forms a stable association with HSP90, and the ability to form such complexes is correlated with the capacity of B-Raf, but not c-Raf to activate the MAP kinase cascade. Moreover, these complexes are found at equivalent levels in both untreated and NGF-treated cells, indicating that they normally exist as preformed complexes in the cytoplasm.

DISCUSSION

We have demonstrated that NGF stimulated the phosphorylation of B-Raf, which was correlated with the activation of the enzyme. NGF also stimulated the phosphorylation of c-Raf, resulting in decrease in electrophoretic mobility; however in contrast to B-Raf, c-Raf exhibited little enzymatic activity. These data indicate that the phosphorylation state of c-Raf is not reflective of its level of enzymatic activation, consistent with previous observations (34, 35). There are a number of reports documenting the NGF-mediated activation of c-Raf (6, 8–10); however, a direct comparison of the relative activities of B-Raf and c-Raf reveal that c-Raf activity is quite low relative to B-Raf. These data are consistent with a previous report,
which estimated c-Raf could account for only approximately 5% of MEK kinase activity detected in NFG-stimulated PC12 cells (33). We have demonstrated previously in PC12 cells that c-Raf is capable of interacting with p21ras, thus its inability to drive the MAP kinase cascade is not a consequence of its failure to interact with its upstream regulator (23). These findings raise the question of how the cell is able to selectively employ only one Raf isoform to activate the MAP kinase cascade and thus functionally segregate the activities of these two highly related enzymes.

The ability of B-Raf to participate in high molecular mass complexes, which include HSP90, is correlated with the capacity of this protein kinase to activate the MAP kinase cascade. Importantly, in PC12 cells c-Raf is not found to be stably associated with HSP90 or other molecules. This finding is of particular interest as Raf-1 has been shown to associate with HSP90 in other cell types (18, 25, 26, 36). c-Raf-containing complexes have also been shown to contain other proteins in addition to HSP90, including p50 and an FK506-binding protein (24, 25). We have been unable to detect the stable association of other proteins (e.g. MEK, 14-3-3, and p50) with B-Raf in PC12 cells following anion exchange chromatography or immunoprecipitation. The association of B-Raf with HSP90 is remarkably stable as interaction of these proteins was unaffected by detergents and geldanamycin (37). The present data also demonstrate that B-Raf exists in a complex with HSP90 in resting cells; thus its participation in the complex is not likely to be dependent upon its activity.

HSP90 functions as a molecular chaperone, interacting with a diverse array of proteins including steroid hormone receptors and v-Src (30, 31). HSP90 has been postulated to play a critical role in promoting the association of multiprotein complexes through conformational stabilization of the constituent proteins. There is substantial evidence that one of the primary functions of HSP90 is to mediate transport of proteins that associate in such complexes from one intracellular loci to another (29). HSP90 has also been shown to associate with a number of protein kinases, including casein kinase II (38) and other (29). HSP90 has also been shown to associate in such complexes from one intracellular loci to another (29).

The existence of a number of parallel cascades that utilize structurally homologous enzymes underscores the complexity of these signaling events. It is of some significance that in yeast the STE5 gene product acts as a molecular scaffold to physically organize elements of the MAP kinase cascade into a functional module (44, 45). This modular organization imparts efficiency to the signal transduction system, but more importantly provides a rational basis for establishing pathway specificity.

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