Intersectin Can Regulate the Ras/MAP Kinase Pathway Independent of Its Role in Endocytosis*

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We previously identified intersectin, a multiple EH and SH3 domain-containing protein, as a component of the endocytic machinery. Overexpression of the SH3 domains of intersectin blocks transferrin receptor endocytosis, possibly by disrupting targeting of accessory proteins of clathrin-coated pit formation. More recently, we identified mammalian Sos, a guanine-nucleotide exchange factor for Ras, as an intersectin SH3 domain-binding partner. We now demonstrate that overexpression of intersectin’s SH3 domains blocks activation of Ras and MAP kinase in various cell lines. Several studies suggest that activation of MAP kinase downstream of multiple receptor types is dependent on endocytosis. Thus, the dominant-negative effect of the SH3 domains on Ras/MAP kinase activation may be indirectly mediated through a block in endocytosis. Consistent with this idea, incubating cells at 4 °C or with phenylarsine oxide, treatments previously established to inhibit EGF receptor endocytosis, blocks EGF-dependent activation of MAP kinase. However, under these conditions, Ras activity is unaffected and overexpression of the SH3 domains of intersectin is still able to block Ras activation. Thus, intersectin SH3 domain overexpression can effect EGF-mediated MAP kinase activation directly through a block in Ras, consistent with a functional role for intersectin in Ras activation.

Src homology 3 (SH3) domains are 50- to 70-amino acid protein modules that mediate protein-protein interactions through binding to specific proline-rich peptide sequences. The recruitment to the plasma membrane of the Ras guanine-nucleotide exchange factor, mammalian son-of-sevenless (mSos), via the actions of the adapter protein Grb2, is a well characterized example of a functional role for SH3 domain-mediated interactions (1–6). Through its SH3 domains, Grb2 interacts with proline-rich sequences in mSos, and through its SH2 domain, Grb2 interacts with SHC or activated growth factor receptors, thereby recruiting mSos to the membrane where it can activate Ras (7, 8). Activated Ras initiates a phosphorylation cascade culminating in phosphorylation and activation of the p42/p44 MAP kinases (Erk-1 and Erk-2).

SH3 domain-mediated protein-protein interactions also function broadly in vesicular trafficking, particularly in clathrin-mediated endocytosis (9). For example, several laboratories have recently identified and cloned a novel protein, variously referred to as intersectin (frog and man) (10, 11), Dup160 (fruit fly) (12), Ese (mouse) (13), and EHS (rat) (14), which contains two Eps15 homology (EH) domains and four or five tandem SH3 domains. Through its SH3 domains, intersectin interacts with the endocytic enzyme dynamin, and through its EH domains, intersectin binds the epsins (10), which are recently identified EH domain-interacting proteins that localize to clathrin-coated pits (15). Intersectin is also localized to clathrin-coated pits in neurons and non-neuronal cells (16), and it has been proposed that the protein functions as a molecular scaffold in clathrin-coated pit formation (13, 16, 17). In support of this model, overexpression of full-length intersectin blocks constitutive endocytosis of the transferrin receptor (13), and overexpression of different SH3 domains of intersectin block transferrin receptor endocytosis at distinct steps in the endocytic pathway (18).

Very recently, we identified one of the SH3 domain-binding partners for intersectin as mSos (19). Coinmunoprecipitation analysis confirmed the interaction in vivo, and interestingly, intersectin and Grb2 were found to compete for binding to mSos. In fact, in brain, the tissue with the strongest intersectin expression (16), we were able to identify an intersectin/mSos complex that could be partially purified from Grb2 (19). These data suggest that intersectin may function as a scaffold for protein components of both the endocytic machinery and signal transduction pathways.

We were thus interested to study the link between intersectin and activation of the Ras/MAP kinase signaling pathway. Here, we demonstrate that overexpression of intersectin’s five SH3 domains blocks EGF-dependent activation of Ras and MAP kinase in HEK-293 cells and COS-7 cells. SH3 domain overexpression also blocks NGF-dependent activation of Ras in PC12 cells. For many receptors that signal through the Ras/MAP kinase pathway, active endocytic function has been suggested to be a pre-requisite for MAP kinase activation (20–27). Overexpression of intersectin’s SH3 domains blocks endocytosis.

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The abbreviations used are: SH3, Src homology 3; mSos, mammalian son-of-sevenless; MAP, mitogen-activated protein; Erk-1/2, p42/p44 MAP kinase; EH, Eps15 homology domain; EGF, epidermal growth factor; NGF, nerve growth factor; GFF, green fluorescence protein; PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PAO, phenylarsine oxide; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
sis (18), and thus, their effect on the activation of the Ras/MAP kinase pathway could be mediated indirectly through blocking endocytosis. Therefore, we examined the activation of this pathway in cells in which EGF receptor endocytosis was blocked and found that, although MAP kinase activation was attenuated, Ras activation was not affected. Interestingly, overexpression of the SH3 domains of intersectin blocked Ras activation under these conditions, suggesting that they have a direct role in disrupting Ras activation, likely through binding to mSos. Thus, in addition to its role in endocytosis, intersectin may also function in Ras/MAP kinase signaling and may provide an important link between these two processes.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antibodies against MAP kinase (Erk-1 and Erk-2) and against activated, phosphorylated MAP kinase, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Promega (Madison, WI), respectively. A monoclonal antibody against Ras was purchased from Transduction Laboratories (Lexington, KY).

**Ras Assays**—Recombinant adenoviruses encoding GFP or the five tandem SH3 domains of intersectin fused to GFP (GFP-SH3) were generated as described (19). To generate adenovirus encoding the 170-kDa isoform of synaptojanin (synaptojanin<sub>170</sub>) (28), a full-length synaptojanin<sub>170</sub> cDNA was ligated into the Eco<sub>R</sub>I site of pDNA3.1+ (Invitrogen) (29), was used in polymerase chain reaction reactions with Vent DNA polymerase and the forward primer 5′-GCGAACGTTCGTTAAATAAATGGAGGCTTG, corresponding to nucleotides 4026–4046, and the reverse primer 5′-CCGCCTGAGATTCATTTCTGAATGAGTATTG, corresponding to nucleotides 4821–4799. The resulting polymerase chain reaction product was digested with BsmBI and Eco<sub>R</sub>I and subcloned into the synaptojanin cDNA following a complete XhoI and partial HindIII digest (HindIII site at nucleotide 4015). The resulting clone was digested with KpnI and XhoI, and the insert, encoding synaptojanin<sub>170</sub>, was subcloned into the corresponding sites of pAdTrack-CMV (30). The resulting plasmid, which allows for the expression of synaptojanin<sub>170</sub> and GFP from separate cytomegalovirus promoters, was recombined with pAdEasy-1 in Bst XI sites and recombination plasmids were selected on kanamycin and identified by EcoRI and BseXI digests (30). Recombinant adenovirus was produced, amplified, and subsequently purified on CsCl<sub>2</sub> gradients as described (30).

The viruses were used to infect HEK-293, COS-7, or PC12-6-24 (31) cells, which had been plated on poly-l-lysine-coated 10-cm<sup>2</sup> dishes. For HEK-293 cells, the dishes were exposed to virus for 2 h at a multiplicity of infection of 100 before the medium was changed. Following an additional 48-h incubation, the cells were transfected into serum-free media for 5 h and then challenged with EGF as described in the figure legends. For COS-7 and PC12-6-24 cells, the viruses were used at a multiplicity of infection of 100–200 and were incubated with the cells for 24 h before the media was changed to serum-free media. Following an additional 24-h incubation, the cells were transfected into fresh serum-free media and challenged with EGF or NGF as described in the figure legends. In some cases, cells were treated with 50 μM PD-089059 (Calbiochem, La Jolla, CA) or 10 μM phenylarsine oxide (PAO, Sigma Chemical Co., St. Louis, MO) for the last hour or last one-half hour, respectively, of the serum-free treatment. The drugs were also included in the media at the same concentration during the time of growth factor addition. In other experiments, similar treatments were performed on non-infected cells. In all cases, the cells were washed in PBS, collected by scraping, and lysed in 1 ml of ice-cold Ras assay buffer (20 mM Tris, pH 7.5, containing 1 mM EDTA, 10% glycerol, 1% Triton X-100, 100 mM KCl, 5 mM sodium fluoride, 0.2 mM sodium vanadate, 5 mM MgCl<sub>2</sub>, 0.05% b-mercaptoethanol, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin). The extracts were centrifuged at maximal speed for 5 min, and the bulk of the supernatant was incubated with glutathione-Sepharose beads complexed with approximately 25 μg of a GST fusion protein encoding the Ras-GTP binding domain of Raf-1 (32, 33). Following a 2-h incubation, the beads were washed four times with 1 ml of ice-cold Ras assay buffer, and bound material was eluted with gel sample buffer and processed for SDS-PAGE, along with aliquots of starting material and unbound material. The samples were processed for Western blots with the monoclonal antibody against Ras.

**MAP Kinase Assay**—Various cells lines, plated on poly-l-lysine substrate in 6-well multi-well culture dishes, were left uninfected, or were infected with recombinant adenovirus and then treated with EGF and various drugs as described above. In all cases, the cells were then washed in PBS (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.9% NaCl, pH 7.4) and incubated for 20 min at room temperature with lysis buffer (20 mM Tris-CI, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 1% glycerol, 0.2 mM sodium vanadate, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin). The samples were collected and centrifuged at maximal speed for 5 min before the supernatants were collected, assayed for protein, and prepared for Western blot analysis with an antibody against the active, phosphorylated form of MAP kinase.

**EGF Receptor Endocytosis Assays**—COS-7 cells, plated on poly-l-lysine-coated glass coverslips, were left untreated or were treated with PAO as described above. At the appropriate time, the cells were washed with serum-free media and then incubated for 1 h at 4 °C or were left untreated. This treatment was followed by a 3.5% paraformaldehyde, washed, mounted on glass slides, and observed by epifluorescence microscopy.

**RESULTS AND DISCUSSION**

The endocytic scaffolding molecule, intersectin, displays SH3 domain-dependent interactions with the Ras guanine-nucleotide exchange factor, mSos1, and overexpression of the SH3 domains of intersectin blocks Ras activation in HEK-293 cells (19). To further investigate the potential role of intersectin in Ras function, we examined the effect of overexpressing the five SH3 domains of intersectin on Ras activation in EGF-stimulated HEK-293 cells and COS-7 cells as well as NGF-stimulated PC12-6-24 cells (31). For these experiments, we measured the levels of Ras-GTP in cells by performing Ras pull-down assays with a GST-Raf-1 fusion protein that has an increased affinity for Ras-GTP versus Ras-GDP (32, 33).

In non-stimulated (0 EGF), serum-starved HEK-293 cells, infected with the green fluorescence protein (GFP), with GFP fused to the five tandem SH3 domains of intersectin (GFP-SH3), or with a virus encoding the 170-kDa isoform of synaptojanin (synaptojanin<sub>170</sub>) (28), little binding of Ras to GST-Raf-1 was observed (Fig. 1A). However, following treatment of GFP-overexpressing cells with 100 ng/ml EGF for 2 min, a dramatic increase in Ras binding to GST-Raf-1 was observed, indicative of an increase in the cellular levels of the GTP-bound form of Ras (Fig. 1A). A similar increase is seen in cells overexpressing synaptojanin<sub>170</sub> (Fig. 1A). In contrast, cells infected with GFP-SH3 show much lower levels of active Ras following EGF treatment (Fig. 1A). To quantitate this effect, Western blots from multiple experiments were scanned and the density of Ras reactivity in the bead lanes was expressed as a ratio of reactivity in the starting materials (relative activation; Fig. 1B). Although EGF stimulated cells overexpressing GFP alone demonstrated an average ratio of 5.34 ± 0.98 (mean ± S.E., n = 9), cells overexpressing GFP-SH3 showed an average ratio of 1.65 ± 0.35. This 3.2-fold decrease in Ras activation caused by GFP-SH3 overexpression was significant with a p value of 0.001 in a two-tailed paired t test. In contrast, there was no significant difference in the levels of active Ras following EGF treatment between cells overexpressing GFP and cells overexpressing synaptojanin<sub>170</sub> (mean ± S.E. for synaptojanin = 5.15 ± 0.45, n = 7) (Fig. 1B). Interestingly, cells overexpressing GFP-SH3 also appeared to have slightly lower levels of active Ras than cells overexpressing GFP in the absence of stimulation (Fig. 1A). The values for relative Ras activation (mean ± S.E., n = 11) were 1.09 ± 0.22 for GFP and 0.71 ± 0.12 for GFP-SH3. The p value for significance between these two groups was 0.06. These data suggest that SH3 domain overexpression lowers the basal level of active Ras. Interestingly, intersectin and mSos1 are associated at the membrane in the absence of stimulation in brain (19). However, the fold activation induced by EGF is still greater for GFP-infected cells.
GFP fused to the five tandem SH3 domains of intersectin (Ras activation in HEK-293 cells. A lysate (starting material, (bead) was processed for SDS-PAGE, along with an aliquot of the cell incubation, the beads were washed, and material bound to the beads domain of Raf-1 coupled to glutathione-Sepharose beads. Following incubation, the beads were washed, and material bound to the beads (bead) was processed for SDS-PAGE, along with an aliquot of the cell lysate (starting material, SM) (equal to one-tenth the amount added to the beads) and an equal aliquot of the unbound material (void). The samples were processed for Western blots with a monoclonal antibody against Ras as indicated.

We next sought to examine the effect of the overexpression of intersectin’s SH3 domains on Ras activation in different cell lines. Serum-starved PC12-6-24 cells and COS-7 cells have approximately the same in any given cell line under the various infection conditions, indicating that the decreases in the amount of activated Ras following ligand stimulation in the

(4.9-fold) than for GFP-SH3-infected cells (2.3-fold), suggesting an additional role for intersectin following EGF stimulation.

We next sought to examine the effect of the overexpression of intersectin’s SH3 domains on Ras activation in different cell lines. Serum-starved PC12-6-24 cells and COS-7 cells have little active Ras when left unstimulated but demonstrate increased Ras activation upon stimulation with NGF or EGF, respectively (Fig. 2). As for HEK-293 cells, overexpression of GFP-SH3 attenuates Ras activation in both PC12-6-24 cells and COS-7 cells. For all of the Ras assay experiments, the levels of Ras in the crude cell lysates (starting material) were approximately the same in any given cell line under the various infection conditions, indicating that the decreases in the amount of activated Ras following ligand stimulation in the

SH3 domain expressing cells is not due to changes in the cellular levels of Ras.

We next sought to explore the potential role for intersectin in MAP kinase activation. Overexpression of the SH3 domains of intersectin with an epitope-tagged MAP kinase construct in a co-transfection system reveals that the SH3 domains of intersectin can attenuate EGF-dependent MAP kinase activation (34). In contrast, overexpression of the SH3 domain of the GTPase accelerating protein, p120RasGAP, had no effect on MAP kinase activation in the same system (34). To further characterize this result, and to extend it to our system, we examined the activation of endogenous MAP kinase in HEK-293 cells and COS-7 cells infected with GFP or GFP-SH3. At 10 or 100 ng/ml, EGF treatment strongly activates MAP kinase in GFP-infected cells as determined by Western blots with an antibody against the active, phosphorylated form of Erk-1/2 (PO4 MAPK) (Fig. 3). In contrast, MAP kinase activation is greatly reduced at both EGF concentrations in the GFP-SH3-infected cells (Fig. 3). Furthermore, GFP-SH3 domain overexpression attenuates MAP kinase activation in response to 10 ng/ml EGF stimulation of COS-7 cells (Fig. 3). In all cases, the levels of MAP kinase under the various treatments were the same as determined by Western blots with an antibody that recognizes non-phosphorylated Erk-1/2 (MAPK) (Fig. 3).

Several lines of investigation have suggested that, for several classes of receptors, endocytosis is necessary to couple receptor stimulation to MAP kinase activation. For example, both the EGF receptor and the TrkA NGF receptor appear to require endocytic function to activate MAP kinase (20, 21). Overexpression of the isolated SH3 domains of intersectin blocks transferrin receptor endocytosis (18) and EGF receptor endocytosis (data not shown). Thus, it is possible that the attenuation of growth factor-stimulated MAP kinase activation seen following intersectin SH3 domain overexpression is due to a block in endocytosis. To examine this issue further, we decided to investigate the activation of Ras and MAP kinase under conditions where endocytosis is blocked. To block endocytosis, we incubated cells at 4 °C or at 37 °C in the presence of 10 μM PAO, a well established inhibitor of EGF receptor endocytosis (35, 36). As seen in Fig. 4, both of these treatments effectively block endocytosis of the EGF receptor as determined by examining the uptake of Texas Red-labeled EGF, which
appears in vesicular endosomal structures within the cells at 37 °C but remains on the surface at 4 °C or at 37 °C in the presence of PAO. We then examined Ras and MAP kinase activity under these conditions.

In the continuous presence of EGF at 100 ng/ml and at 37 °C, Ras and MAP kinase are activated at 2 min, but the levels of the active forms of both proteins are significantly lower at 10 min (Fig. 5). The attenuation of Ras activity at 10 min appears to be due to a MAP kinase kinase (MEK)-dependent feedback inhibition of Ras activation as the addition of PD-098059, a known inhibitor of MEK (37), prevents this effect (Fig. 5). This result is consistent with previously published data from several laboratories demonstrating that MEK-dependent feedback phosphorylation of mSos, either via MAP kinase or other MEK-dependent kinases, causes Ras desensitization by inducing mSos to dissociate from Grb2 (38–42). Interestingly, we have also observed that mSos phosphorylation causes a decrease in the mSos1/intersectin interaction (data not shown). At 4 °C, EGF appears to stimulate Ras to the same extent as it does at 37 °C (Fig. 5). As is the case with PD-098059, treatment of cells at 4 °C completely blocks MAP kinase activation and prevents the attenuation of Ras activity seen after 10 min of EGF treatment at 37 °C (Fig. 5). Finally, in cells treated with PAO, Ras is fully active at 2 and 10 min, yet MAP kinase activation is strongly attenuated (Fig. 5). Thus, under conditions where endocytosis is blocked, Ras can be activated without a subsequent activation of MAP kinase.

Endocytosis appears to be important for the ability of multiple receptor types, including the EGF receptor, TrkA NGF receptor, insulin and insulin-like growth factor receptors, β2-adrenergic receptor, delta opioid receptor, and m1-muscarinic receptor to activate MAP kinase in response to ligand (20–26, 43). However, other receptors and receptor subtypes, including the α2a-, α2b-, and α2c-adrenergic receptors, the m3-muscarinic receptor, and the kappa-opioid receptor, appear endocy-
Intersectin Regulation of Ras/MAP Kinase

FIG. 6. Overexpression of intersectin’s SH3 domains blocks Ras activation independent of their effect on endocytosis. HEK-293 cells, infected with recombinant adenovirus encoding GFP or GFP-SH3, were serum-starved and then challenged with 100 ng/ml EGF for 2 min. EGF challenge was at 37, 4, or 37°C in the presence of PAO. Cells were then washed, and lysates were prepared and incubated with a GST fusion protein encoding the Ras-GTP binding domain of Raf-1 coupled to glutathione-Sepharose beads. Following incubation, the beads were washed and material bound to the beads (bead) was processed for SDS-PAGE, along with an aliquot of the cell lysate (starting material, SM) (equal to one-tenth the amount added to the beads) and an equal aliquot of the unbound material (void). The samples were processed for Western blots with a monoclonal antibody against Ras as indicated.

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May have a direct function in Ras activation via interactions with mSos.

Intersectin is concentrated at clathrin-coated pits in both neurons and in non-neuronal cells (16). In response to ligand binding, the cytoplasmic tails of receptor tyrosine kinases, including the EGF receptor, autophosphorylate on tyrosine residues and interact with signaling complexes (49). The receptors are also rapidly targeted to clathrin-mediated endocytic pathways (50). Perhaps intersectin plays a role in EGF-stimulated Ras activation specifically at clathrin-coated pits. This is especially interesting in light of the fact that active EGF receptor signaling complexes undergo endocytosis and can continue to signal on endosomes (51–53). Furthermore, it has been recently demonstrated that signaling complexes composed of Ras-1 and activated Erk-1/2, linked to clathrin-mediated endocytic vesicles through β-arrestin, are critical in regulating the subcellular localization of MAP kinase activation downstream of the Gαq-coupled proteinase-activated receptor (54). Regardless of the precise mechanisms, the data presented here further support a role for intersectin in Ras/MAP kinase activation and strengthen the growing connection between endocytosis and signaling.
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