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Hepatocyte Growth Factor/Scatter Factor Stimulates the Ras-Guanine Nucleotide Exchanger*

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Hepatocyte growth factor/scatter factor (HGF/SF) induces mitogenesis and cell dissociation upon binding to the protein-tyrosine kinase receptor encoded by the *MET* proto-oncogene ($p190^{MET}$). The signal transduction pathways downstream from the receptor activation are largely unknown. We show that HGF/SF activates Ras protein. HGF/SF stimulation of metabolically labeled A549 cells raised the amount of Ras-bound radiolabeled guanine nucleotides by over 5-fold. Furthermore, following HGF/SF stimulation of these cells, 50% of Ras was in the GTP-bound active state. The uptake by Ras of radiolabeled GTP was also increased by 5-fold following HGF/SF stimulation in digitonin-permeabilized A549 cells. Moreover, HGF/SF treatment of A549 cells leads to stimulation of the cytosolic Ras-guanine nucleotide exchange activity, measured as accelerated release of [^{3}H]GDP from purified recombinant Ras protein *in vitro*, in a dose- and time-dependent manner. Likewise, treatment with the protein-tyrosine kinase inhibitor 3-(1′,4′dihydroxytetralyl)methylene-2-oxindole of GTL-16 cells (featuring a $p190^{MET}$ receptor constitutively active) significantly decreased the cytosolic Ras-guanine nucleotide exchange activity. These data demonstrate that HGF/SF activates Ras protein by shifting the equilibrium toward the GTP-bound state and increases the uptake of guanine nucleotides by Ras, through mechanism(s) including the activation of a Ras-guanine nucleotide exchanger.

Hepatocyte growth factor (HGF),[1] also known as scatter factor (SF), is a heterodimeric protein secreted by cells of mesodermal origin (1–3) as an inactive single chain precursor (4). This molecule induces a spectrum of biological activities in epithelial cells, including mitogenesis, stimulation of cell motility, and promotion of matrix invasion (1, 3, 5, 6). HGF/SF is also a morphogen (7–9) and a potent angiogenic factor *in vitro* and *in vivo* (10).

HGF/SF is the ligand for $p190^{MET}$, the receptor tyrosine kinase encoded by the *MET* proto-oncogene (11–13). $p190^{MET}$ is a heterodimeric receptor made up of an extracellular α and a transmembrane β subunit (14). The β subunit (145 kDa) contains the extracellular ligand binding site (13) and the cytoplasmic tyrosine kinase domain (15, 16). HGF/SF binding triggers tyrosine autophosphorylation of the receptor β subunit in intact cells, up-regulating its kinase activity (17).

The pleiotropic biological response induced by HGF/SF suggests that multiple transduction pathways may be activated. Previous work has shown that $p190^{MET}$ associates *in vitro*, upon autophosphorylation, with phosphatidylinositol 3-kinase, Ras-GAP, phospholipase C-γ, and Src-related tyrosine kinases (18). Association of phosphatidylinositol 3-kinase with the activated receptor has also been found *in vivo*, in cells stimulated by HGF/SF, indicating that the generation of D-3 phosphorylated inositol lipids is one of the effector mechanisms (19). A role for the Ras protein in growth factor cell signaling has recently been reported (20–22). Indeed, several growth factors can rapidly activate Ras, promoting the shift of Ras protein from the inactive GDP-bound form to the active GTP-bound form (23–26). In this paper we show that HGF/SF activates Ras by increasing the uptake of GTP through the stimulation of a guanine nucleotide exchange factor.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cell Lines—All reagents, unless specified, were purchased from Sigma; radiochemicals were purchased from Amersham Corp. HGF/SF was obtained from a recombinant source as described (10). Purified recombinant Ras protein was kindly provided by Dr. J. Downward. Monoclonal Y13-259 anti-Ras antibodies were generously provided from Dr. J. Bos. Monoclonal anti-Met antibodies were raised against the extracellular domain of $p190^{MET}$ (27). Monoclonal anti-phosphotyrosine antibodies were from UBI. TMO (3-(1′,4′dihydroxytetralyl)methylene-2-oxindole), was synthesized as described.[2] A549 and GTL-16 cell cultures were made as described (19). Procedures for receptor's immunoprecipitation and immunoblotting were performed as described previously (19).

Detection of Guanine Nucleotide Bound to Ras in Intact Cells—A549 and GTL-16 confluent cultures were serum-starved for 3 days, and subsequently labeled with 200 μCi/ml [^{32}P]orthophosphate for 6 h in phosphate-free RPMI medium (Irvine). Cells were treated as indicated, and cell lysates were made using a phase split separation as described (26). Subsequently Ras-bound radiolabeled nucleotides were detected in anti-Ras immunoprecipitates as described in Ref. 23.

Detection of Guanine Nucleotide Bound to Ras in Permeabilized Cells—Subconfluent A549 cells were serum-starved for 3 days, then treated for 5 min at 37 °C with permeabilization buffer (10 mM PIPES buffer, pH 7.4, 0.006% digitonin, and 10 μCi of [α-^{32}P]GTP) (23) in presence or in absence of the indicated concentration of HGF/SF. Cells were lysed, Ras protein recovered, and the nucleotides eluted and separated as above.

In Vitro Ras-Guanine Nucleotide Exchanger Assay—Cytosolic extracts were made as described (28). Cytosolic proteins (3 μg) were used for each assay. Ras-guanine nucleotide exchanger activity was assayed measuring the release from Ras of prebound [^{3}H]GDP, essentially as described (29). Purified, nucleotide-free, *Escherichia coli* recombinant $p21^{c\text{-}H\text{-}ras}$ (0.3 μg; 14 pmol) were preloaded with 1 μM [^{3}H]GDP (10 μCi/nmol), by a 10-min incubation at 37 °C in 20 mM

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[1] The abbreviations used are: HGF, hepatocyte growth factor; SF, scatter factor; TMO, 3-(1′,4′dihydroxytetralyl)methylene-2-oxindole; PIPES, 1,4-piperazinediethanesulfonic acid.

[2] P. dalla Zonca, F. Buzzetti, S. Penco, A. Graziani, and P. M. Comoglio, submitted for publication.

Hepes, pH 7.5, 5 mM EDTA, and 5 μg/ml bovine serum albumin. The reaction was stopped on ice, by addition of $MgCl_2$ to 25 mM final concentration. The Ras-[^{3}H]GDP complex was incubated with cell extracts for 10 min at 30 °C in presence of unlabeled 150 μM GDP in order to quench nonspecific binding of free [^{3}H]GDP, 1.5 mM GTP, and 20 mM $MgCl_2$. The reaction was stopped on ice and the reaction mixture filtered through nitrocellulose discs (Sartorius 11306, 0.45 μm), which were washed with 20 ml of ice-cold buffer containing 20 mM sodium phosphate, pH 6.5, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl. The filters were then placed in scintillation fluid and counted for ^{3}H.

RESULTS

HGF/SF Stimulates Receptor Autophosphorylation and Uptake of Radiolabeled Guanine Nucleotides by Ras—Serum-starved A549 cells were metabolically labeled with [^{32}P]orthophosphate, and the amount of radiolabeled guanine nucleotides bound to Ras was measured. In anti-Ras immunoprecipitates from unstimulated cells, radiolabeled GDP and GTP were barely detectable by autoradiography, indicating that Ras uptake of guanine nucleotides from the radiolabeled pool was very low after starvation (Fig. 1, *lower panel*). In anti-Ras immunoprecipitates from HGF/SF-stimulated cells, both radiolabeled GTP and GDP were detected, indicating that HGF triggered the uptake of radiolabeled guanine nucleotides by Ras. The uptake was increased at least 5 times over the uptake observed in unstimulated cells, was observed as early as after 2 min, lasted for at least 20 min (Fig. 1, *lower panel*) and was dose-dependent (data not shown). Furthermore, in HGF/SF-stimulated cells more than 50% of Ras protein was in the active GTP-bound state. As *in vivo* Ras preferentially uptakes GTP rather than GDP, these data show that the GTP uptake rate on Ras is slow in unstimulated cells and that it is stimulated by HGF/SF.

HGF/SF Stimulates the Uptake of [α-^{32}P]GTP by Ras in Permeabilized Cells—A549 cells were made quiescent as above and stimulated with HGF/SF at different concentrations and for different times. Cells were then permeabilized with digitonin and exposed to exogenous [α-^{32}P]GTP for 5 min before lysis. In unstimulated cells the amount of radiolabeled guanine nucleotides bound to Ras was almost undetectable. As early as 5 min after HGF/SF stimulation (80 ng/ml), the amount of radioactivity bound to Ras was increased over 5-fold (Fig. 2). The increase in uptake was dose-dependent (data not shown).

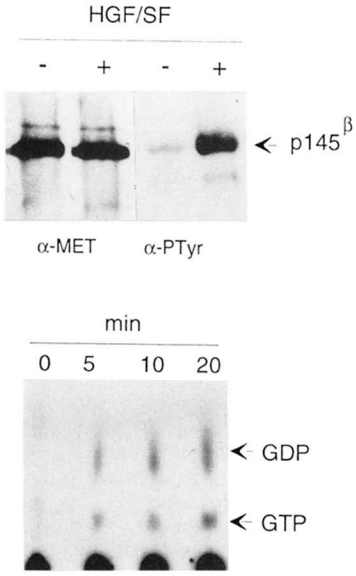

FIG. 1. **HGF/SF stimulates tyrosine phosphorylation of the receptor β subunit and Ras uptake of radiolabeled nucleotides.** *Upper panel*, A549 cells were lysed and immunoprecipitated with antibodies against the β chain of the HGF/SF receptor (anti-Met). Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Met or anti-phosphotyrosine antibodies. +, cells stimulated with 20 ng/ml HGF/SF for 15 min. −, control resting cells. *Lower panel*, A549 cells were labeled with [^{32}P] orthophosphate for 6 h and stimulated with HGF/SF as above for the times indicated. Cells were lysed and immunoprecipitated with anti-Ras antibodies. Guanine nucleotides were eluted and analyzed by TLC followed by autoradiography.

Tyrosine Phosphorylation of the HGF/SF Receptor Stimulates a Cytosolic Ras-Guanine Nucleotide Exchanger Activity—The guanine nucleotide exchanger activity was measured as percent release of radiolabeled [^{3}H]GDP from preloaded purified Ras, following a 10-min incubation with cytosolic extracts prepared from control or HGF/SF-stimulated A549 cells. The exchanger activity is expressed as percent [^{3}H]GDP released above the amount of [^{3}H]GDP released in presence of buffer only. Under these assay conditions, 8% of [^{3}H]GDP (*i.e.* 0.08 pmol/10 min/pmol Ras) was spontaneously released from Ras (data not shown). The amount of radioactivity released from Ras in the presence of cytosolic extracts prepared from HGF/SF-stimulated cells was up to 5-fold higher than the amount released in the presence of extracts from unstimulated serum-starved cells. The elevation in exchanger activity occurred within 5 min following stimulation of A549 cells with HGF/SF, reached a plateau, and lasted for at least 30 min (Fig. 3*A*). The effect was dependent upon the HGF/SF concentration used to stimulate the cells and reached saturation at 20 ng/ml (Fig. 3*B*). HGF/SF stimulation of A549 cells did not significantly affect the Ras-GTPase activity assayed in cytosolic cell extracts (data not shown).

Tyrosine Kinase Inhibition Reduces HGF/SF Receptor Autophosphorylation and Cytosolic Ras-Guanine Nucleotide Exchanger Activity—In GTL-16 cells, due to the amplification of the *MET* gene, the p190^{MET} receptor is overexpressed and constitutively phosphorylated on tyrosine (30). In these cells, similarly to A549 cells stimulated by HGF/SF, more than 50% of Ras protein was in the GTP-bound active state (data not shown). We took advantage of this model system to investigate the relationship between tyrosine phosphorylation of the receptor and the cytosolic Ras-guanine nucleotide exchange activity in greater detail. Tyrosine phosphorylation of the HGF/SF receptor in GTL-16 cells was inhibited by treatment with increasing concentrations of the tyrosine ki-

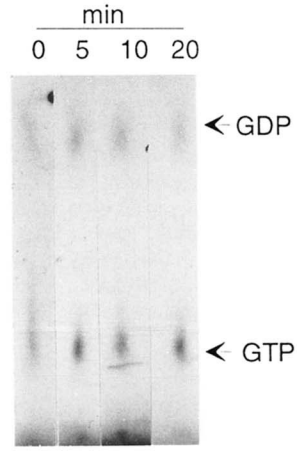

FIG. 2. **HGF/SF stimulates Ras uptake of [α-^{32}P]GTP by Ras in permeabilized cells.** Quiescent A549 cells were stimulated by HGF/SF (20 ng/ml) for the time indicated. Five min before lysis, cell were permeabilized in the presence of digitonin and [α-^{32}P]GTP. Guanine nucleotides bound to Ras were analyzed as indicated in Fig. 1.

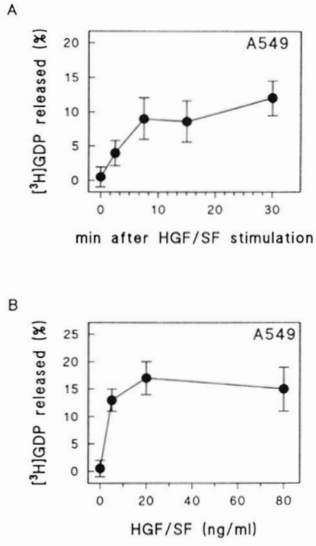

FIG. 3. **Tyrosine phosphorylation of the HGF/SF receptor stimulates a cytosolic Ras-guanine nucleotide exchanger activity *in vitro*.** Purified recombinant Ras protein was preloaded with radiolabeled GDP and incubated for 10 min in the presence of either buffer or cytosolic extracts from cells treated as indicated. The enzymatic activity is expressed as percent release of [^{3}H]GDP above Ras intrinsic release in presence of buffer only. Experimental points are the average of triplicate determinations. *A*, time course after HGF/SF stimulation (20 ng/ml) of A549 cells. *B*, dose response following HGF/SF stimulation (7.5 min) of A549 cells.

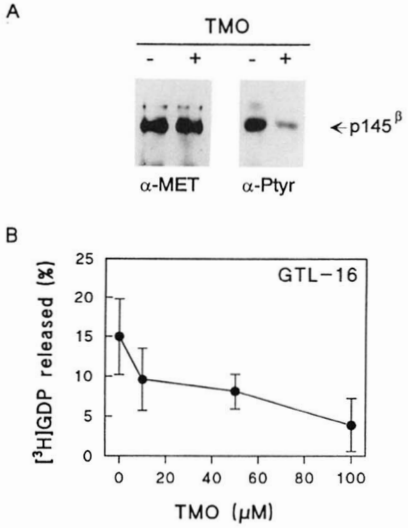

FIG. 4. **The tyrosine kinase inhibitor TMO prevents tyrosine phosphorylation of the HGF/SF receptor β subunit and reduces cytosolic Ras-guanine nucleotide exchanger activity.** *A*, GTL-16 cells lysed and immunoprecipitated with anti-Met antibodies. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Met or anti-phosphotyrosine antibodies. +, cells treated with 100 μM TMO for 3 h. −, untreated controls. *B*, dose response of Ras-guanine nucleotide exchanger activity, assayed as in Fig. 3, following treatment of GTL-16 cells with the tyrosine kinase inhibitor TMO (3 h).

nase inhibitor TMO (Fig. 4*A*). The amount of radioactivity released from Ras in the presence of cytosolic extracts prepared from TMO-treated cells was reduced up to 4-fold as compared to the amount of radioactivity released in presence of extracts from untreated cells (Fig. 4*B*). The TMO-induced inhibition was dose-dependent. The inhibitor itself, added to the cytosolic extract *in vitro*, was ineffective on the Ras-guanine nucleotide exchanger activity (data not shown).

DISCUSSION

Ras is a guanine nucleotide-binding protein associated to the plasma membrane in two functional states: as an inactive form bound to GDP and as an active form bound to GTP (20). Two enzymatic reactions regulate the equilibrium between the active and inactive form. Ras-guanine nucleotide exchange factors promote the loss of Ras-bound GDP and the subsequent uptake of GTP (31, 32). Ras-GTPase activating proteins, such as Ras-GAP and NF-1 (reviewed in Ref. 20), enhance the intrinsic Ras-GTPase activity . Cell stimulation by growth factors results in an increase of the relative amount of Ras protein in the active GTP-bound state. This may occur either by stimulation of the Ras-guanine nucleotide exchanger activity or by inhibition of Ras-GTPase activity. The mechanism by which growth factors activate Ras may depend on the regulation operating on Ras in different cell types when quiescent. In T cell and Schwannoma tumor cells, featuring a constitutively high Ras-guanine nucleotide exchange activity, Ras appears to be activated by reduction of Ras-GTPase activity (23, 33). Conversely, platelet-derived growth factor and nerve growth factor were reported to activate Ras in fibroblast and PC12 cells, respectively, by activating a Ras-guanine nucleotide exchanger (28, 34). The extent, time course, and mechanisms of Ras activation reported so far vary greatly according to the cell type and the growth factor (23–26, 28, 34).

We now report that HGF/SF activates Ras. This was observed in A549 cells after HGF/SF binding; in stimulated cells 50% of Ras was found in the active GTP-bound state. Similarly, in GTL-16 cells, featuring an HGF/SF receptor tyrosine kinase constitutively active, more than 50% of Ras protein was found in the active state. According to the current model for Ras function (20), the HGF/SF-induced Ras activation can be explained by stimulation of Ras-guanine nucleotide exchange or inhibition of Ras-GTPase activity. Indeed, HGF/SF stimulation of A549 cells, while not affecting the Ras-GTPase activity, led to a severalfold activation of the Ras-guanine nucleotide exchange activity detectable in cytosolic extracts. Accordingly, treatment of GTL-16 cells with a tyrosine kinase inhibitor decreased the cytosolic Ras-guanine nucleotide exchanger activity.

It should be noted that in the epithelial cell line studied (A549), HGF/SF also induced a sharp rise in radiolabeled guanine nucleotides bound to Ras. A similar increase in uptake has already been reported in cells stimulated by insulin (see Fig. 4 in Ref. 26) or by platelet-derived growth factor.[3]

The increased uptake may be explained either as an increased turnover or as a net increase in guanine nucleotides bound to Ras. The increased turnover, consistently with the canonical model for Ras function, is particularly noticeable in A549 cells, because of the low basal turnover of Ras-bound nucleotides. Theoretically, a putative Ras-GDP dissociation inhibitor could operate in these cells, inhibiting the Ras-intrinsic guanine nucleotide exchange activity (20). HGF/SF might activate the Ras-guanine nucleotide exchange indirectly by suppressing this inhibition. However, we obtained no evidence for Ras-GDP dissociation inhibitor activity in cytosolic extracts of A549 cells.

The possibility that HGF/SF induces a net increase of the overall guanine nucleotides binding to Ras would imply the existence of a pool of nucleotide-free Ras; however, no evidence for such a pool has been reported so far.

The molecular mechanism leading to Ras-guanine nucleotide exchanger activation by tyrosine kinases receptor is still

[3] B. Westermark, personal communication.

unknown. Exchanger proteins have been identified from mammals (35, 36) and *Drosophila* (37), but none of them features domains able to interact directly with the tyrosine phosphorylated receptor. However, genetic and biochemical evidence suggest that SH2-containing molecules, such as GRB-2/sem-5 and SHC (38–40), may mediate such an interaction.

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