Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 110-118

MACROPHAGE-STIMULATING PROTEIN ACTIVATES RAS BY BOTH ACTIVATION AND TRANSLOCATION OF SOS NUCLEOTIDE EXCHANGE FACTOR+

Bao-Qun Li[1*], Ming-Hai Wang[2], Hsiang-fu Kung[3], Christophe Ronsin[4], Richard Breathnach[4], Edward J. Leonard[2], and Tohru Kamata[1]

[1]Biological Carcinogenesis and Development Program, SAIC/Frederick, [2]Immunopathology Section, Laboratory of Immunobiology, and [3]Laboratory of Biochemical Physiology, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702

[4]Inserm U 211 Institut de Biologie-CHR, 9 Quai Moncousu 44035 Nantes Cedex 01, France

Received September 11, 1995

SUMMARY: Macrophage-stimulating protein (MSP) is a chemotactic factor that activates the receptor tyrosine kinase RON. The involvement of Ras in MSP-induced signal transduction was investigated. Here we demonstrate that, in RON-transfected MDCK cells, an active GTP-bound form of Ras was rapidly accumulated by MSP treatment and the Ras-guanine nucleotide exchange activity in SOS immunoprecipitates was concomitantly increased. GAP activity was not changed under the same conditions used. Furthermore, the SH2 domain of adaptor protein GRB2, but not Shc, associated with the activated RON-β chain, and GRB2-SOS complexes translocated from the cytosol to the membrane upon MSP treatment. These results strongly suggest that MSP activates Ras through RON, and that MSP-induced activation of Ras might be controlled by both the enhancement of catalytic exchange activity of SOS and its translocation to the membrane where its target Ras is localized. © 1995 Academic Press, Inc.

+The U.S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

*To whom correspondence should be addressed at Biological Carcinogenesis and Development Program, SAIC/Frederick, Laboratory of Biochemical Physiology, NCI-FCRDC, Bldg. 567, Room 180, Frederick, MD 21702. Fax: (301) 846-6863.

Abbreviations: MDCK, Madin-Darby canine kidney; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; NGF, nerve growth factor; (m)SOS, (mouse) son of sevenless; GRB2, growth factor receptor bound-protein 2; SH2, Src-homology 2; Shc, SH2 containing protein; GAP, GTPase activating protein; NF1, neurofibromatosis type 1 protein; GST, glutathione S-transferase; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; TLC: thin layer chromatography; PAGE: polyacrylamide gel electrophoresis.

0006-291X/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Macrophage-stimulating protein (MSP) is a new member of a kringle protein family (1) including hepatocyte growth factor/scatter factor (HGF/SF) (2,3). MSP has high sequence similarity to HGF/SF (45% identity) (1), which is the ligand for $p190^{MET}$, the receptor tyrosine kinase encoded by the MET proto-oncogene (4,5). HGF/SF acts as a mitogen, morphogen, and potent angiogenic factor in epithelia or endothelia (5-8). MSP is a chemotactic factor for mouse peritoneal resident macrophages (9,10) and also acts as a mitogenic factor in murine epithelial cells . ‡Recently, it has been shown that MSP was the ligand of RON (11), a member of the MET receptor tyrosine kinase family (12). RON has 63% amino acid sequence identity in its tyrosine kinase domain with MET (12) and, like MET, is a heterodimeric receptor made up an extracellular α (40 kDa) and a transmembrane β (150 kDa) chain (12). The β-chain of RON and MET undergoes tyrosine autophosphorylation following binding of MSP and HGF/SF, respectively (11, 13).

Ras proteins participate in the transmission of intracellular signals from various growth factors or hormones such as EGF (14), PDGF (15), NGF (16) and insulin (17). Ligand binding to such receptors leads to an increase in the level of GTP-bound Ras through mSOS, a guanine nucleotide exchanger (18-25). Adaptor proteins GRB2 (18-23) and Shc (22,24,25) play a role in coupling of receptors to mSOS. Recently, the activation of Ras by HGF/SF has been reported (13). Since MSP and HGF/SF are structurally and functionally related, we speculated that the pleiotropic biological responses induced by MSP might involve the Ras signaling pathway. In the present study, we investigated the possible role of Ras in MSP-induced signal transduction and demonstrated that the rapid activation of Ras was induced by MSP and this activation was accompanied by activation of SOS activity and translocation of SOS from the cytosol to the membrane.

MATERIALS AND METHODS: Cell lines and Reagents - MDCK cells and MDCK cells expressing human RON proteins (MDCK-RE7) were described (11). MSP was purified from human plasma (26). An anti-rabbit IgG was generated against a C-terminal peptide of RON-β chain (11). Rabbit anti-SOS antibody was generated against a C-terminal mSOS-1 peptide from residues 1321 to 1337 (27). This antibody recognized 150 kDa human, dog, and mouse SOS-1 proteins. pGEX expression plasmids carrying GST-Shc SH2, GST-GRB2 SH2 and GST proteins were provided by Dr. T. Pawson (28). The fusion proteins were produced and purified according to the protocol provided by Pharmacia Inc.

Detection of Guanine Nucleotides Bound to Ras - MDCK or MDCK-RE7 cells ($5x10^6$ cells) were labeled with 200 μCi/ml [^{32}P] orthophosphate (Amersham) in phosphate-free medium containing 0.5% FBS for 16 h. After treatment with MSP as indicated, the cells were then lysed in buffer A [20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40, 1 μg/ml Leupeptin, and 0.2 mM PMSF]. The lysates were immunoprecipitated with monoclonal

‡ M.-H. Wang. A.A. Dlugosz, Y. Sun, T. Suda, A. Skeel, and E.J. Leonard. Manuscript submitted.

antibody (Y13-259) to Ras for 90 min at 4°C. The immune complexes were collected with rabbit anti-rat IgG bound to protein A-Sepharose (Pharmacia Inc) and washed three times with 1 ml of ice-cold washing buffer [50 mM Tris-HCl (pH7.5) and 20 mM $MgCl_2$]. Radiolabeled nucleotides bound to Ras were eluted and analyzed by TLC, followed by autoradiography as described (29).

Assays for Ras-Guanine Nucleotide Exchange Activity of SOS - MDCK or MDCK RE-7 cells ($2x10^7$ cells) were serum starved in DMEM containing 0.5% FBS for 16 h and treated with MSP as indicated. The cells were then lysed in buffer B [20 mM Tris-HCl (pH7.5), 100 mM NaCl, 12 mM $MgCl_2$, 10 mM NaF, 10 mM Sodium orthovanadate, 1% NP-40, 1 μg/ml leupeptin, and 1% Aprotinin] and centrifuged at 100,000 rpm in a Beckman TL-100 centrifuge at 4°C for 15 min. The cellular extracts (300 μg of protein) were incubated with either anti-SOS rabbit serum or control IgG for 90 min at 4°C. The immune complexes retained onto the protein A-sepharose beads were washed 3 times with ice-cold buffer [20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT, 50 μg/ml BSA, and 1 μg/ml leupeptin]. Bacterially made H-Ras proteins (0.4 μg) were preloaded with 0.2 μCi of [^{3}H] GDP (10 μCi/mmol, Amersham) and incubated with the immunoprecipitates in 100 μl of reaction buffer [50 mM Tris-HCl, (pH 7.5), 200 μg/ml BSA, 1 mM DTT, 5 mM EDTA , and 0.4 mM cold GTP] at 30°C for 20 min. [^{3}H] GDP associated with Ras was determined by nitrocellulose filter binding assay as described (29).

Detection of RON-GRB2 Complex Formation - Serum-starved MDCK-RE7 cells ($5 X 10^7$ cells) were treated with or without 5 mM MSP for 10 min. The cellular extracts were then prepared with buffer B as described above. Equal amounts of proteins (300 μg) from each whole cell extract were incubated for 120 min at 4°C with GST-Shc SH2, GST-GRB2 SH2, or GST immobilized on glutathione-agarose beads. The precipitated samples were washed 3 times with ice cold washing buffer. The proteins were analyzed by 10% SDS-PAGE, followed by immunoblotting with anti-RON β chain antibody.

Assays for GRB2-SOS Complex Translocation from the Cytosol to the Membrane -Serum-starved MDCK-RE7 cells ($5X10^7$ cells) were stimulated with or without 5 nM MSP for 10 min at 37°C, and then disrupted by 50 strokes using a glass homogenizer in 1 ml of hypotonic buffer [1 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 10 mM NaF, and 10 mM sodium orthovanadate]. The homogenate was first centrifuged at 1500 rpm for 20 min at 4°C. The resultant supernatants containing cytosols and membranes were further centrifuged at 100,000 rpm for 15 min. The supernatants were used as the cytosolic fraction. The pellets were washed once with hypotonic buffer and solubilized with buffer B. The samples were then centrifuged at 100,000 rpm for 15 min. The detergent solubilized supernatants were used as the membrane fractions. Equal amounts of proteins (100 μg) from both cytosolic and membrane fractions were immunoprecipitated with either anti-SOS rabbit serum or control IgG prebound to protein A sepharose for 120 min at 4°C. The immunoprecipitated samples were subjected to SDS-PAGE, followed by immunoblotting with anti-SOS and anti-BRB2 monoclonal antibody (Transduction Laboratory Inc) respectively.

RESULTS: Accumulation of Active GTP-Bound Ras in MSP-Stimulated MDCK-RE7 Cells.

By MSP treatment, the ratio of GTP to GDP bound to Ras in MDCK-RE7 cells were increased in a dose dependent manner (Fig. 1A and 1B). The increase was observed as early as 5 min, reached to a maximal level at 10 min, and lasted at least 20 min (Fig. 1C). In contrast, no increase in the amount of Ras-GTP was detected in MSP-treated parental MDCK cells (Fig. 1D). The amount of Ras●GTP in MSP-treated MDCK-RE7 cells was about four fold higher than that in MSP-treated MDCK cells (Fig. 1D). These results indicate that MSP induced the activation of Ras through its receptor encoded by RON gene.

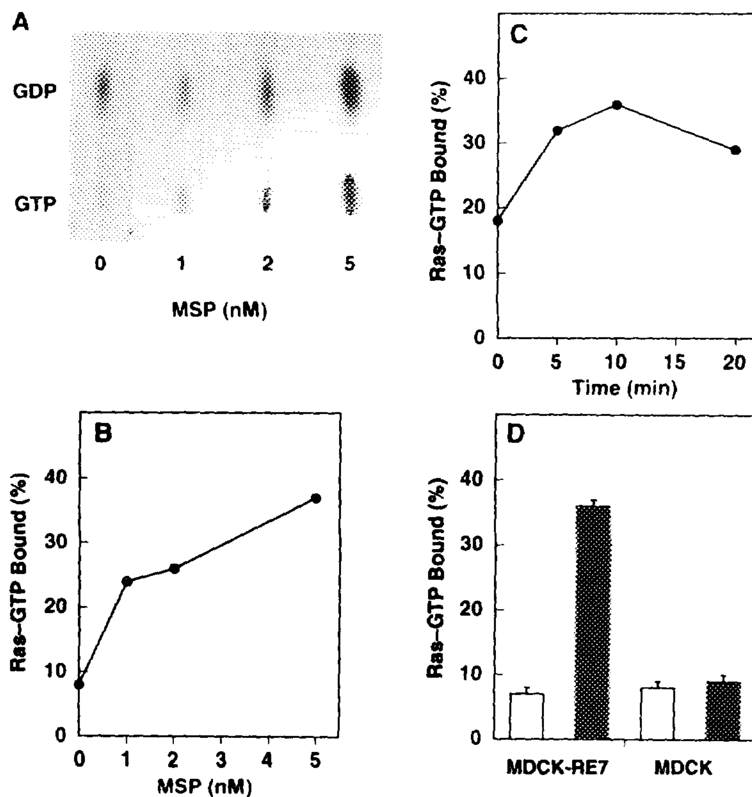

Fig. 1. Quiescent MDCK-RE7 cells were labeled with [^{32}P]orthophosphate for 16 h and treated with indicated amounts of MSP for 10 min (A and B) or with 5 nM MSP for different time intervals (C). Cell lysates were immunoprecipitated with anti-Ras antibody, and the guanine nucleotides bound to Ras were eluted and analyzed by TLC followed by autoradiography. MSP-induced Ras•GTP formation in MDCK and MDCK-RE7 cells were compared (D). Quiescent MDCK and MDCK-RE7 cells were labeled with [^{32}P]orthophosphate and treated with 5 nM MSP for 10 min (solid bars) or left untreated (open bars). The guanine nucleotides bound to Ras were analyzed as described above. In (B) through (D), the percentage of radiolabeled GTP bound to Ras [GTP/(GTP+GDP)X100] was quantitatively calculated. The mean of duplicate experiments is shown.

Activation of Guanine Nucleotide Exchange Activity of SOS by MSP Treatment. We next investigated whether MSP-induced increase in the amount of active Ras•GTP was due to the activation of SOS. In MDCK-RE7 cell, a transient increase in [^{3}H] GDP releasing activity was observed upon MSP stimulation, and the time course in the MSP-promoted GDP releasing activity correlated with that in the level of GTP•Ras (Fig. 2A). Moreover, the magnitude of MSP-promoted [^{3}H] GDP releasing activity increased depended on the concentration of MSP (Fig. 2B). Almost all of the exchange activity in the cell extracts was depleted by immunoprecipitation with anti-SOS antibody under the condition used (data not shown). In contrast to MDCK-RE7 cells, SOS activity in MDCK cells did not respond to MSP stimulation

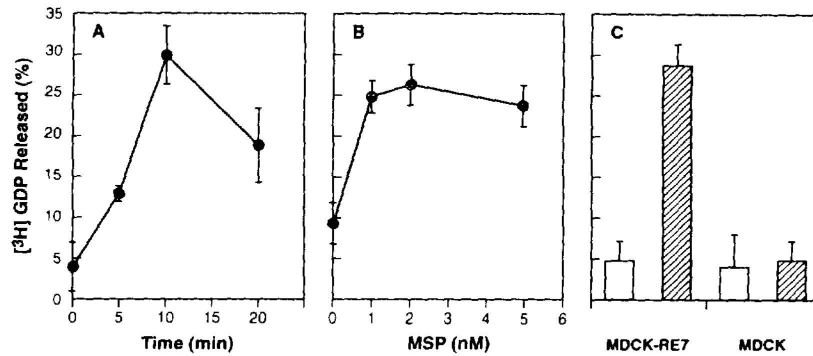

Fig. 2. Quiescent MDCK-RE7 cells were treated with or without 5 nM MSP for various time intervals (A) or with indicated concentrations of MSP for 10 min (B). Cell extracts were immunoprecipitated with either anti-SOS sera or control IgG. The immunoprecipitates were incubated with [^{3}H]GDP•Ras complex in the presence of 0.4 mM GTP at 30°C for 20 min. The amount of [^{3}H]GDP remaining bound to Ras was determined by nitrocellulose filter binding assay. MSP-induced activation of SOS activity in MDCK-RE7 and MDCK cells was compared (C). The quiescent cells were treated with (hatched bars) or without (open bars) 5 nM MSP for 10 min, and SOS activity was measured as described above. In (A) through (C), activity [the mean $\pm$ SD (n=3)] is shown as the percentage of bound [^{3}H]GDP released. [^{3}H]GDP (20,000 cpm) was bound to Ras proteins with buffer alone. The control IgG immunoprecipitate had no GDP-release promoting activity.

(Fig. 2C), and control IgG immunoprecipitates had no detectable guanine nucleotide exchange activity.

To determine whether the GAP activity was changed by the addition of MSP to cells, [α^{32}P]GTP•Ras complexes were incubated with cell extracts from quiescent MDCK or MDCK-RE7 cells treated with or without MSP. The Ras proteins were then immunoprecipited with anti-Ras antibody. The guanine nucleotides bound to Ras were analyzed by TLC as described (29). MSP treatment of cells did not alter the GAP activity, suggested that an increase in SOS activity was responsible for the MSP-induced Ras activation (data not shown).

MSP Induces the Association of GRB2 with RON-β chain. Binding of GRB2-mSOS complexes to auto-phosphorylated receptor tyrosine kinase was reported in some receptor systems (24,25) . To test whether this could occur in MSP-induced Ras activation, the cellular proteins were mixed with GST-Shc SH2, GST-GRB2 SH2 or GST fusion proteins, and then the bound protein were analyzed by immunoblotting with antibody against RON-β chain. A 150 kDa RON-β chain was found to be associated with the SH2 domain of GRB2 after 10 min treatment of cells with MSP, whereas no specific binding of proteins was detected with either GST-Shc SH2 or GST alone (Fig. 3). These results indicate that MSP-activated RON specifically bound to GRB2 but not Shc.

GRB2-SOS Complexes Translocate from the Cytosol to the Membrane upon the MSP Stimulation. To determine whether association of GRB2 with RON results in translocation of

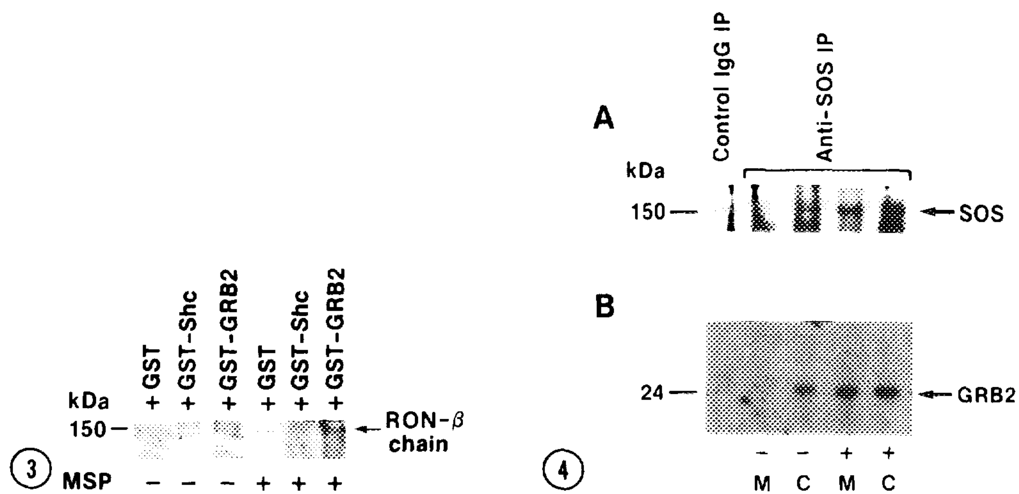

Fig. 3. Quiescent MDCK-RE7 cells were treated with (+) or without (-) 5 nM MSP for 10 min. Cell extracts were incubated with GST-Shc SH2, GST-GRB2 SH2, or GST alone immobilized on glutathione-agarose beads at 4°C for 120 min. Cellular proteins bound to the beads were analyzed by SDS-PAGE and immunoblotted with antiserum against β-chain of RON.

Fig. 4. Quiescent MDCK-RE7 cells were treated with (+) or without (-) 5 nM MSP for 10 min. The membrane (M) or cytoplasmic extracts (C) were prepared and immunoprecipitated with anti-SOS sera or nonimmune IgG. SOS or GRB2 bound to SOS in the immunoprecipitate was detected by immunoblotting with antibody against SOS (A) or monoclonal antibody against GRB2 (B).

SOS from the cytosol to the plasma membrane, membrane and cytoplasmic extracts were prepared from stimulated or unstimulated MDCK-RE7 cell. The cellular extracts were immunoprecipitated with anti-SOS antibody, and analyzed by immunoblotting with antibodies against SOS-1 or GRB2. SOS was almost exclusively found in the cytosolic fraction of unstimulated cells (Fig. 4A), but significant amounts of SOS were detected in the membrane fraction after cells were treated with MSP for 10 min. Likewise, GRB2-SOS complexes were detectable in both cytosols and membranes after MSP stimulation (Fig. 4B). Thus, our data support the idea that the translocation of GRB2-SOS complex to the membrane occurred following binding of GRB2 to MSP-stimulated RON.

DISCUSSION: Here, we demonstrate that MSP activated Ras in RON-overexpressing MDCK cells (MDCK-RE7), while such a stimulatory effect of MSP was not detectable in the untransfected MDCK cells. These results suggest that MSP-dependent stimulation of RON receptor tyrosine kinase induces the activation of Ras. Thus, MSP might exert some of its biological functions via the Ras-mediated signaling pathway. MSP physiologically acts as a chemotactic factor for macrophages and induces their motile and phagocytic activities. Since macrophages and MDCK cells are biologically different, we do not rule out the possibility that

MSP function in macrophages may involve other signaling pathways in addition to the RAS-linked pathway.

Generally, Ras activity is controlled by two classes of regulatory proteins. The guanine nucleotide exchange factor catalyzes the exchange of bound GDP for external GTP in response to growth factors, and this leads to accumulation of an active GTP-bound Ras (14-17,29). SOS has been considered as the nucleotide exchanger mediating some tyrosine kinase-related signal transmissions (27,30), because SOS formed complexes with activated growth factor receptor tyrosine kinases through GRB2 (19-23) or other nonreceptor tyrosine kinase signals through Shc (22, 24,25). In contrast, GAP (31) and NF1(32) stimulate the low intrinsic GTPase activity of Ras and thereby generate an inactive GDP-bound Ras. Thus, activation of Ras could be achieved by either the activation of mSOS or the suppression of GAP/NF1. Our results indicate that the ability of SOS to promote the nucleotide exchange on Ras was several fold enhanced in MSP-stimulated MDCK-RE7 cells, while Ras-GAP activity was not affected by MSP treatment. This implies that the activation of SOS activity is, at least in part, responsible for MSP-dependent Ras activation. Similar effects of HGF/SF on the guanine nucleotide exchange factor and GAP activities were observed in epithelial cells (13).

Since RON is a transmembrane receptor tyrosine kinase and is tyrosine phosphorylated in response to the ligand binding (11), we determined whether the activated receptor RON is associated with SOS through the adaptor proteins of GRB2 or Shc. We found that by MSP stimulation, the β chain of RON associated with the SH2 domain of GRB2 but not the SH2 domain of Shc. Thus, it is most likely that GRB2 specifically bound to the phosphorylated tyrosine residues of the RON-β chain through its SH2 domain. Therefore, the GRB2 is able to ferry SOS from cytosol to membrane where its target Ras is localized. The translocation of GRB2-SOS complexes to the membrane fraction was also detected upon MSP treatment of MDCK-RE7 cells.

According to the current model for Ras regulation, the catalytic guanine nucleotide exchange activity of mSOS is not altered in response to growth factor stimulation (33-35). The interaction of GRB2 with autophosphorylated receptor tyrosine kinases serves to translocate a constitutively active mSOS from the cytosol to the plasma membrane. This would increase the local concentration of mSOS in the plasma membrane and thus accelerate Ras activation (34,35). Our data confirmed the translocation of GRB2-SOS from the cytosol to the membrane. However, unlike published results with EGF-stimulated NIH3T3 cells (34), the nucleotide exchange promoting activity of SOS-1 was increased following MSP stimulation. Supporting our finding, previous studies showed that the Ras guanine nucleotide exchange factor activity in crude cell extracts was stimulated in response to NGF (29), EGF, PDGF (36) and HGF/SF (13). How is the specific activity of SOS increased? One possible explanation is that the

receptor activation might induce a conformational change of SOS bound to GRB2. Alternatively, the interaction of translocated SOS with a membrane component(s) could contribute to the stimulation of SOS activity. Finally, we hypothesize that the role of the stimulated MSP receptor is not only to alter the intracellular localization of SOS, but also to enhance the activity of SOS. The current model for Ras activation might be oversimplified.

ACKNOWLEDGMENTS: We wish to thank Dr. Dan Longo for his support, and Drs. Howard Young and Luigi Varesio for their critical review. We also thank Mrs. A. Rogers and Ms. Lindy Shaw for preparation of this manuscript.

REFERENCES

1. Yoshimura, T., Yuhki, N., Wang, M.-H., Skeel, A., and Leonard, J.E. (1993) *J. Biol. Chem.* **268,** 15461-15468.
2. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonish, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) *Nature* **342,** 440-443.
3. Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987) *Nature* **327,** 239-242.
4. Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.L., Kmiecick, T.E., Vande Woude, G.F., and Aaronson, S.A. (1991) *Science* **251,** 802-804.
5. Naldini, L., Vigna, E., Narsimhan, R.P., Gaudino, G., Zarnegar, R., Michalopoulos, G., and Comoglio, P.M. (1991) *Oncogene* **6,** 501-504.
6. Weidner, K.M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. (1990) *J. Cell. Biol.* **111,** 2097-2108.
7. Michalopoulos, G., Houck, A. K., Dolan, L. M., and Luetteke, C.N. (1984) *Cancer Res.* **44,** 4414-4419.
8. Montesano, R., Matsumoto, K., Nakamura, T., and Orci, L. (1991) *Cell* **67,** 901-908.
9. Leonard, J.E., and Skeel, A. (1976) *Exp. Cell. Res.* **102,** 434-438.
10. Skeel., A., and Leonard, J.E. (1994) *J. Immunol.* **152,** 4618-4623.
11. Wang, M.-H., Ronsin, C., Gesnel, M.-C., Coupey, L., Skeel, A., Leonard, J.E., and Breathnach, R. (1994) *Science* **266,** 117-119.
12. Ronsin, C., Muscatelli, F., Mattei, G.-M., and Breathnach, R. (1993) *Oncogene* **8,** 1195-1202.
13. Graziani, A., Gramaglia, D., Zonca, D.P., and Comoglio, M.P. (1993) *J. Biol. Chem.* **268,** 9165-9168.
14. Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Kaziro, Y. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7926-7926.
15. Satoh, T., Endo, M., Nakafuku, M., Nakamura, S., and Kaziro, Y. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 5993-5997.
16. Muroya, K., Hattori, S., and Nakamura, S. (1992) *Oncogene* **7,** 277-281.
17. Burgering, M.Th.B., Medema, H. R., Maassen, J.A., van de Wetering, L.M., van der Ed, J.A., McCormick, F., and Bos, L.J. (1991) *EMBO J.* **10,** 1103-1109.
18. Egan, E.S., Giddings, W.B., Brooks, W.M., Buday, L., Sizeland, M.A., and Weinberg, A.R. (1993) *Nature* **363,** 45-51.
19. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993) *Nature* 363, 83-85.
20. Gale, W.N., Kaplan, S., Lowenstein, J.E., Schlessinger, J. and Bar-Sagi, D., (1993) *Nature* **363,** 88-92.
21. Skolnik, Y.E., Batzer, A., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) *Science* **260,** 1953-1955.

22. Stephens, M.R., Leob, M.D., Copeland, D.T., Pawson, T., Greene, A. L., and Kapland, R.D. (1994) *Neuron* **12,** 691-705.
23. Lowestein, J.E., Daly, J.R., Batzer, G.A., Li, N., Margolis, B., Lammers, R., Ullich, A., Skolnik, Y.E., Bar-Sagi, D., and Schlessinger, J. (1992) *Cell* **70**, 431-442.
24. Policci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T.,and Pelicci, G.P. (1992) *Cell* **70,** 93-104.
25. Skolnik, Y.E., Lee, C.-H.,Batzer,A.,Vicentini, M.L.,Zhou, M., Daly, R., Myers, Jr. J.M., Backer, M.J., Ullrich, A., White, F.M., and Schlessinger, J. (1993) *EMBO J.* **12,** 1929-1936.
26. Skeel, A., Yoshimura, T., Showalter, D.S., Tanaka, S., Appella, E., and Leonard, J.E. (1991) *J. Exp. Med.* **173,** 1227-1234.
27. Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6511-6515.
28. Puil, L., Liu, J., Gish, G., Mbamalu, G., Bowtell, D., Pelicci, G.P., Arlinghaus, R., and Pawson, T. (1994) *EMBO J.* **13,** 764-773.
29. Li, B.-Q., Kaplan, D., Kung, H.-f., and Kamata, T. (1992) *Science* **256,** 1456-1459.
30. Chardin, P., Càmonis, H. J., Gale, W. N., Aeist, V. L., Schlessinger, J., Wigler, H. M., Bar-Sagi, D. (1993) *Science* **260,** 1338-1343.
31. Trahey, M., and McCormick, F. (1987) *Science* **238,** 542-545.
32. Martin, A.G., Viskochil, D., Bollag, G., McCabe, C.P., Crosier, J.W., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, M.R., Innis, A.M., and McCormick, F. (1990) *Cell* **63,** 843-849.
33. McCormick, F. (1993) *Nature* **363,** 15-16.
34. Buday, L. and Downward, J. (1993) *Cell* **73,** 611-620.
35. Aronheim, A., Engelberg, D., Li, N., Al-Alwn, N., Schlessinger, J., and Karin, M. (1994) *Cell* **78,** 949-961.
36. Li, B.-Q., Subleski, M., Shalloway, D., Kung, H-f., and Kamata, T. (1993) *Proc. Natl. Acad. Sci.* U.S.A. **90,** 8504-8508.