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EFFECT OF ORTHOVANADATE ON TYROSINE PHOSPHORYLATION OF P120 GTPase-ACTIVATING PROTEIN IN RAT LIVER MACROPHAGES (KUPFFER CELLS)

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GTPase-activating protein (GAP), a protein capable of regulating the activity of p21*ras* protein, is phosphorylated on tyrosine residues following the activation of tyrosine kinase(s) associated with several growth factor receptors. The present study was designed to examine potential role of phosphotyrosine phosphatase in tyrosne phosphorylation of GAP. Addition of orthovanadate, a phosphate analogue known to inhibit phosphotyrosine phosphatase, to cultured liver macrophages induced tyrosine phosphorylation of numerous cellular proteins with a range of molecular weights between 30-130 kDa; one tyrosine-phosphorylated protein was identified as the 120 kDa GAP. The effect of orthovanadate on the tyrosine phosphorylation of GAP was time- and concentration-dependent. Quantitated data indicated that approximately 4% of the total content of cellular GAP was tyrosine-phosphorylated upon orthovanadate treatment. These observations suggest a potential regulatory role of phosphotyrosine phosphatase in the tyrosine phosphorylation of GTPase-activating protein in cellular signaling mechanisms in the hepatic macrophages. © 1993 Academic Press, Inc.

It is well established that the low molecular weight GTP-binding proteins encoded by *ras* proto-oncogenes (*e.g.*, p21*ras*) play an important role in growth factor-stimulated cell proliferation (1,2). The biological activity of the p21*ras* protein relies upon its ability to maintain an active, GTP-bound form rather than an inactive GDP-bound state (3-5). The intrinsic GTPase activity of the p21*ras* protein is low but can be enhanced by a 120 kDa GTPase-activating protein (GAP) which directly interacts with the cellular p21*ras* protein (3,6). Numerous lines of evidence have indicated that GAP plays an important role in growth factor signaling mechanisms (6). GAP is tyrosine-phosphorylated upon activation of receptor tyrosine kinase and becomes physically associated with such growth factor receptors (7-9). It has been speculated that tyrosine phosphorylaiton of GAP leads to its functional alteration and which ultimately causes activation of p21*ras* (10,11).

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Abbreviations used: GAP, GTPase-activating protein; P-Tyr, phosphotyrosine; SDS, sodium dodecyl sulfate.

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Pulse experiments have suggested that the turnover of the phosphate in phosphotyrosine is rapid (12,13). It is possible that a dynamic balance exists between phosphorylation (by tyrosine kinase) and dephosphorylation (by phosphotyrosine phosphatase) reactions. It is probably this type of dynamic balance that determines the phosphorylaion status and ultimate functional utility of a signaling molecule. Therefore, it is logical to examine whether the tyrosine phosphorylation status of GAP is regulated by phosphatase that removes phosphate from phosphotyrosine.

Vanadate, an oxidized form of vanadium, has a wide range of biological and biochemical effects. Vanadate is widely distributed, in fact, all cells examined contain vanadium compounds [14]. Most of biological effects of vanadate are due to its properties as a phosphate analogue capable of inhibiting enzymes involved in phosphate transfer and release reactions. For example, vanadate has been shown to inhibit ATP-dependent ion pumps such as Na^+/K^+-ATPase (14,15). Vanadate-induced inhibition of phosphotyrosine phosphatase (16-18) leads to an accumulation of phosphotyrosine in various cellular proteins including the β-subunit of the insulin receptor and insulin-like growth factor receptor (19,20). Also, vanadate induces cellular transformation (21). In the present study, the effect of vanadate on tyrosine phosphorylation of GAP has been characterized in rat Kupffer cells.

MATERIALS AND METHODS

Isolation and Culture of Rat Kupffer Cells - Rat Kupffer cells were isolated by centrifugal-elutriation and cultured as described previously (22,23). The viability of the Kupffer cell preparation was greater than 95% as determined by trypan blue exclusion. The purity of the culltured Kupffer cells is between 90-95% based on the assay of peroxidase activity.

Subcellular Fractionation - Cultured cells were suspended in 3 ml ice-cold hypotonic lysis buffer (24), collected into a homogenizer tube with a tight-fitting pestle, and lysed with 60 strokes of the homogenizer. After centrifugation at 1000 *g* for 5 min, the supernatant fraction was ultracentrifuged at 100,000 *g* for 50 min. The cytosolic supernatant fraction (S-100) was collected and concentrated to 1 ml by ultrafiltration using a Centricon-30 concentrator. The resultant pellet was resuspended and solubilized in 1 ml Nonidet P-40 lysis buffer (7). Under these conditions, cell protein concentrations in P-100 and S-100 were approximately the same. Samples (50 µl) of each fraction were used for anti-GAP immunoblot analyses. The remainder of the lysates were collected and incubated with anti P-Tyr antibody followed by immunoblotting with anti-GAP antibody.

Immunoprecipitation - Cultured cells were lysed with 1 ml ice-cold Nonidet P-40 lysis buffer. The cell lysate was then transferred to an Eppendorff tube and rocked on a platform at 4 °C for another 20 min. The lysate was centrifuged at 10,000 X *g* for 10 min at 4 °C. The supernatant (or the subcellular fractions described above) was used for subsequent immunoprecipitation assays. Immunoprecipitation was performed by incubating 1 ml of the supernatant or 950 µl of the subcellular fractions (S-100 and P-100) with 30 µl of agarose-monoclonal anti P-Tyr antibody for 3-4 h at 4 °C. The immune complex was washed 3 times with 1 ml NP-40 lysis buffer and once with 1 ml of 10 mM Tris-HCl, pH 7.4. Finally, the immune complex was lysed in 40 µl NP-40 lysis buffer and 40 µl SDS sample buffer (2X) (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol). The mixture was heated at 95 °C for 5 min before being subjected to SDS polyacrylamide gel electrophoresis.

Immunoblotting - Immunoblotting experiments were performed as described previously (24).

RESULTS

Cultured Kupffer cells were either left untreated or treated with 10 mM vanadate for 30 min followed by immunobloting with anti P-Tyr antibody. As shown in Fig.1, *left panel*, vanadate treatment resulted in tyrosine phosphorylation of numerous cellular proteins with molecular masses ranging between 30 and 130 kDa. In order to determine whether GAP was phosphorylated on tyrosine residues in response to vanadate, cell lysates were incubated with anti P-Tyr antibody to immunoprecipitate tyrosine-phosphorylated proteins. The precipitated proteins were resolved in SDS polyacrylamide gels and probed with anti-GAP antibody. As indicated in Fig.1, *right panel*, a significant amount of GAP was recovered from the immunoprecipitates prepared from the vanadate-treated cells but not from the control cells. The 58 kDa protein bands are IgG heavy chains. The vanadate-induced tyrosine phosphorylation of GAP was time- and concentration-dependent. Compared with tyrosine phosphorylation of GAP induced by other agonists such as platelet-derived growth factor [7] and platelet-activating factor[1], the vanadate-induced phosphorylation of GAP was relatively slow, the stimulatory effect of vanadate (5 mM) was observed after 20 min of incubation reaching a maximal level within 30 min (Fig.2). A significant increase in tyrosine phosphorylation of GAP was observed when the concentration of vanadate exceeded 1 mM following a 30 minute-incubation (Fig.3). However, a much lower concentration (*e.g.*, 5-30 μM) of vanadate was capable of inducing tyrosine phosphorylation of GAP when the incubation period was prolonged to 24 h (data not shown).

To determine the subcellular localization of cellular GAP as well as the tyrosine phosphorylated GAP, cells were lysed with hypotonic buffer without detegent and the subcellular fractions (membrane and cytosol fractions) were prepared. As indicated in Fig.4, *lanes 1-4*, nearly all of the cellular GAP was associated with the cytosolic fraction (S-100). However, when the amount of sample applied to each gel lane was increased, it was apparent that approximately ~ 15% of the cellular GAP was located in the membrane fraction (data not shown). Vanatate seemed to have no significant effect on the subcellular distribution of GAP. In contrast to the predominant cytosolic location of cellular GAP, the tyrosine phosphorylated GAP in vanadate-treated cells was equally distributed in the cytosolic and membrane fractions (Fig.4, *lanes* 7 and 8). By comparing the relative signal intensity of anti-GAP immunoblots of precipitates recovered from anti P-Tyr immunoprecipitation versus total cellular lysate, it was estimated that approximately 4% of cellular GAP was tyrosine-phosphorylated upon vanadate treatment (Fig.4).

DISCUSSION

Recent studies have suggested that GTPase-activating protein plays an important role in signaling mechanisms induced by growth factors (6-8,11) and T-lymphocyte

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[1] Chao, W., Liu, H., and Olson, M.S. Manuscript submitted.

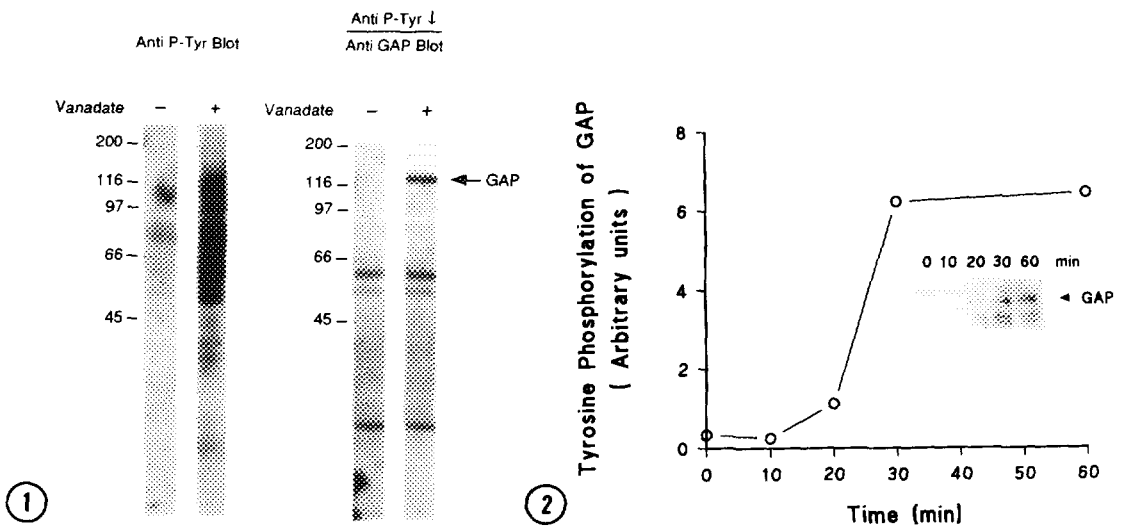

Figure 1. *Tyrosine phosphorylation of GAP in vanadate-treated cells. Left panel,* Immunoblots of whole cell lysate using anti P-Tyr antibody; *Right panel,* Immunoblots of anti-P-Tyr-recovered precipitates using anti-GAP antibody. The numbers on the left side of the autoradiogram indicate the molecular weight of standard proteins in kDa.

Figure 2. *Time course of GAP tyrosine phosphorylation in vanadate-treated cells.* Cells were incubated with 5 mM sodium orthovanadate for the indicated time intervals. The cell lysates were immunoprecipitated using anti-P-Tyr antibody followed by immunoblotting with anti-GAP antibody as described in "*Materials and Methods*". The blots were quantitated using a computer imaging system. The results are presented in arbitrary units. The inset contains the autoradiogram from which the graphics were derived.

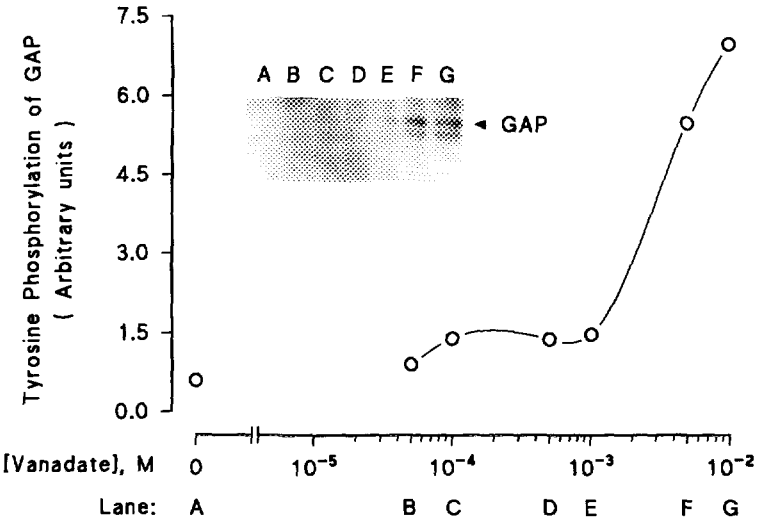

Figure 3. *Concentration dependence of vanadate-stimulated tyrosine phosphorylation of GAP.* Cells were incubated with various concentrations of vanadate for 30 min followed by cell lysis and anti-P-Tyr immunoprecipitation. The precipitates were resolved in SDS polyacrylamide gels, transferred to membranes, and probed with anti-GAP antibody. Autoradiograms were analyzed and expressed in arbitrary units. *Inset*: the autoradiogram from which the graphics were derived.

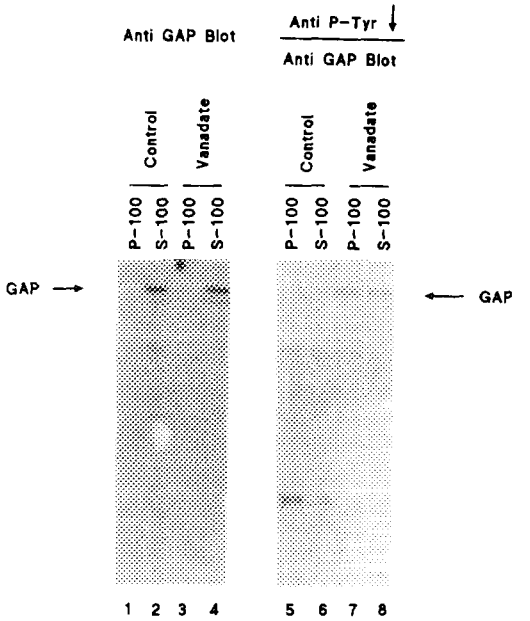

Figure 4. *Subcellular localization of GAP in control and vanadate-treated cells.* Cells were lysed and subcellular fractions [membrane (P-100) and cytosol (S-100) fractions] were prepared. *Left panel*, 50 µl fractions were pipetted and resolved using SDS polyacrylamide gels, transferred to membranes, and probed with anti-GAP antibody. *Right panel*, 950 µl fractions were incubated with anti P-Tyr to immunoprecipitate tyrosine-phosphorylated proteins followed by immunoblotting with anti-GAP.

activation (10). In response to growth factors, GAP is phosphorylated on tyrosine residues following the activation of the respective receptor-associated tyrosine kinase(s) and becomes physically associated with the receptors (7,8). The present study was designed to examine potential role of phosphotyrosine phosphatase in tyrosine phosphorylation of GAP. Vanadate has been shown to inhibit specifically phosphatase(s) acting on phosphotyrosine both in intact cells and in cell-free systems (17,21). The finding that vanadate induces tyrosine phosphorylation of GAP in a time- and concentration-dependent fashion suggests an involvement of a phosphotyrosine phosphatase in this event. This observation may indicate the existence of another mechanism for tyrosine phosphorylation of GAP since all previous studies have indicated that GAP is tyrosine-phosphorylated by activation of receptor-associated tyrosine kinases. It seems both likely and logical that the tyrosine phosphorylation status of GAP is balanced by phosphorylation and dephosphorylation. It is equally important to identify such phosphotyrosine phosphatase involved in receptor-mediated signal transduction mechanism(s).

It may be appropriate to speculate as to the significance of vanadate-induced tyrosine phosphorylation of GAP. In intact NRK-1 cells, vanadate was shown to stimulate tyrosine phosphorylation of several proteins and to induce a cellular transformation (21). Although the exact mechanism for the transformation is not clear, it was suggested that the vanadate-stimulated protein tyosine phosphorylation was causative in the cellular

transformation (21). Whether a relationship exists between cellular transformation and the tyrosine phosphorylation of GAP is not known. However, the potential role of GAP in cellular transformation has been suggested by the observation that in cells overexpressing GAP, the cellular transformation induced by $pp60^{src}$ is supressed (25). The inhibitory effect of GAP is probably a function of its negative regulatory action on endogenous *ras* activity. For this reason it would be most interesting to determine whether tyrosine phosphorylation of GAP has any effect on the GAP function and $p21^{ras}$ activity and ultimately on the cellular transformation.

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REFERENCES

1. Barbacil, M. (1987) *Ann. Rev. Biochem.* 56, 779-827
2. Grand, R.J.A., and Owen, D. (1991) *Biochem. J.* 279, 609-631
3. Trahey, M., and McCormik, F. (1987) *Science* 238, 542-545
4. DeVendittis, E., Vitelli, A., Zahn, R., and Fasano, O. (1986) *EMBO J.* 5, 3657-3663
5. Marshall, M.S., Gibbs, J.B., Scolnick, E.M., and Sigal, I.S. (1987) *Mol. Cell. Biol.* 7, 2309-2315
6. McCormick, F. (1989) *Cell,* 56, 5-8
7. Molloy, C.J., Bottaro, D.P., Fleming, T.P., Marshall, M.S., Gibbs, J.B., and Aaronson, S. (1989) *Nature* 342, 711-714
8. Kaplan, D.R., Morrison, D.K., Wong, G., McCormick, F., and Williams, L. (1990) *Cell,* 61, 125-133
9. Liu, X., and Pawson, T. (1991) *Mol. Cell. Biol.* 11, 2511-2516
10. Downward, J., Graves, J.D., Warne, P.H., Rayter, S., and Cantrell, D.A. (1990) *Nature,* 346, 719-723
11. Torti, M., Marti, K.B., Altschuler, D., Yamamoto, K., and Lapetina, E. (1992) *J. Biol. Chem.* 267, 8293-8298
12. Sefton, B.M., Hunter, T., Beemon, K., and Eckhart, W. (1980) *Cell* 20, 807-816
13. Beemon, K., Ryden, T., and McNelly, E.A. (1982) *J. Virol.* 42, 742-747
14. Simon, T.J.B. (1979) *Nature* 281, 337-338
15. Cantley, L.C., Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C., and Guidotti, G. (1977) *J. Biol. Chem.* 252, 7421-7423
16. Leis, J.F., and Kaplan, N.O. (1982) *Proc. Natl. Acad. Sci.* USA 79, 6507-6511
17. Swarup, G., Cohen, S., and Garbers, D.L. (1982) *Biochem. Biophys. Res. Commun.* 107, 1104-1109
18. Nelson, R.L., and Branton, P.E. (1984) *Mol. Cell. Biol.* 4, 1003-1012
19. Kadota, S., Fantus, I.G., Deragon, G., Guyda, H.J., and Posner, B.I. (1987) *J. Biol. Chem.* 262, 8252-8256
20. Torossian, K., Freedman, D., and Fantus, I.G. (1988) *J. Biol. Chem.* 263, 9353-9359
21. Klarlund, J.K. (1985) *Cell* 41, 707-717
22. Knook, D.L, and Sleyster, E.C. (1976) *Exp. Cell Res.* 99, 444-449
23. Chao, W., Liu, H., Hanahan, D.J., and Olson, M.S. (1989) *J. Biol. Chem.* 264, 20448-20457
24. Chao, W., Liu, H., Hanahan, D.J., and Olson, M.S. (1992) *J. Biol. Chem.* 267, 6725-6735
25. DeClue, J.E., Zhang, K., Redford, P., Vass, W.C., and Lowy, D.R. (1991) *Mol. Cell. Biol.* 11, 2819-2825