Journal of Cellular Biochemistry 38:229–236 (1988)
Growth Factors and Their Receptors: Genetic Control and Rational Application 49–56

Evidence for Autocrine Activation of a Tyrosine Kinase in a Human Gastric Carcinoma Cell Line

Silvia Giordano, Maria Flavia Di Renzo, Radha P. Narsimhan, Luca Tamagnone, Elena V. Gerbaudo, Loredana Chiadó-Piat, and Paolo M. Comoglio

Department of Biomedical Sciences and Oncology, University of Torino Medical School, Torino, Italy

Phosphotyrosine (P-Tyr) antibodies have been used to identify the phosphorylated forms of growth factor receptors and oncogene-coded tyrosine kinases. Western blot analysis of a gastric carcinoma cell line with P-Tyr antibodies revealed a tyrosine-phosphorylated protein of M_r 145,000 (P145). In addition, in vitro phosphorylation with (γ-^{32}P)ATP of P-Tyr immunoprecipitates of the same cells resulted in labelling of this protein on tyrosine. P145 appears to be a transmembrane glycoprotein, with features suggestive of a growth factor receptor. However, the in vivo or in vitro addition of known growth factors did not affect P145 tyrosine phosphorylation. We now report that P145 is rapidly dephosphorylated in vivo when cells are exposed to low pH, a condition known to dissociate ligands from their receptors. The addition of serum-free medium, conditioned by the gastric carcinoma cells, fully restores the tyrosine phosphorylation lost with acid treatment. These data suggest that the activity responsible for P145 phosphorylation on tyrosine, whether intrinsic to P145 itself or due to an associated kinase, is stimulated by a factor secreted by the tumor cells themselves.

Key words: oncogenes, growth factors, phosphotyrosine, autocriny, stomach cancer

Several independent findings support the hypothesis that the malignant behavior of some tumors is sustained by an "autocrine" loop, wherein cancer cells produce growth factors that stimulate their own growth (1–5). Many growth factor receptors are associated with a protein tyrosine kinase activity, which acts—in concert with other biochemical events—as a signal transducer, triggering the mitogenic intracellular responses (6–10). Upon the binding of their specific growth factors, the receptors endowed with tyrosine kinase activity are transiently activated and phosphorylate themselves on tyrosine. In malignant cells, these receptors may be permanently activated, even in the absence of exogenous growth factors, due to structural altera-

Received February 1, 1988; accepted July 8, 1988.

tions of the receptors [11–12] or due to an autocrine stimulation [2]. This results in their constitutive phosphorylation.

Phosphotyrosine (P-Tyr) antibodies have been used successfully to identify the autophosphorylated form of receptors for known growth factors and the oncogene-coded proteins endowed with tyrosine kinase activity (10, 13–21). Using P-Tyr antibodies, it has been shown that platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors are phosphorylated only upon the addition of exogenous growth factor and are rapidly dephosphorylated (20–21). The same technique has also shown that kinases involved in autocrine loops are permanently activated and that phosphotyrosine-containing proteins accumulate within the cells (16, 18). We recently used P-Tyr antibodies to screen a number of human tumor cells; a few contained high levels of proteins phosphorylated on tyrosine (17). A tyrosine-phosphorylated protein with the M_r of 145,000 (P145) was unique to a gastric carcinoma line. After immunoprecipitation with P-Tyr antibodies, P145 displayed a strong associated protein kinase activity in vitro, becoming phosphorylated on tyrosine. Neither immunologic cross-reactions nor similarities in phosphopeptide maps were found between P145 and known tyrosine kinases (36). The present work provides evidence that the tyrosine phosphorylation of P145 in the gastric carcinoma cell line studied may be stimulated through an autocrine loop.

MATERIALS AND METHODS

Cells and Reagents

The GTL-16 cell line is a clonal line derived from a poorly differentiated gastric carcinoma cell line (22). Normal human gastric mucosa was obtained as described previously (17). SR-BALB cell line is a mouse fibroblast line transformed by the Schmidt-Ruppin D strain of Rous sarcoma virus. P-Tyr antibodies were raised against p-amino-benzene-phosphonate and affinity purified as previously described (13). They are defined as phosphotyrosine antibodies (α-P-Tyr).

Western Blotting

Cells were washed with phosphate-buffered saline (PBS), pH 7.4, and solubilized in boiling Laemmli buffer (23) with or without reducing agents. Samples were adjusted to a protein concentration of 200 μg/well, run in SDS-PAGE and transferred onto nitrocellulose sheets as previously described (24, 25). Blots were probed with 9 μg/ml of purified P-Tyr antibodies, followed by ^{125}I-labelled *S. aureus* protein-A (Amersham). The M_r of labelled proteins was estimated using cotransferred ^{14}C-methylated myosin (200,000), phosphorylase b (92,500), bovine serum albumin (69,000), egg albumin (46,000), and carbonic anhydrase (30,000) (Amersham).

Immunocomplex Kinase Assay

After immunoprecipitation with P-Tyr antibodies or control preimmune rabbit immunoglobulins, immunocomplexes were collected on Protein-A Sepharose, washed, and phosphorylated in the presence of 10 mM $MnCl_2$ and 10 μCi (γ-^{32}P)ATP (specific activity: 7,000 Ci/mM, Amersham) at 0°C for 5 min. Samples were eluted off the protein-A Sepharose by boiling in Laemmli buffer. These samples were subjected to SDS-PAGE followed by autoradiography for 2 h using an intensifying screen.

Acid Treatment and Stimulation by Conditioned Medium

Cells were incubated with an isotonic solution containing 20 mM acetic acid (pH 3.7), 150mM NaCl, and 0.25% BSA for 5 min at 4°C. Under these conditions, known growth factors such as EGF and PDGF are dissociated from their cell surface receptors without affecting cell viability (20, 26).

For stimulation with conditioned medium, exponentionally growing cultures were incubated in serum-free medium for 24 h and then trypsinized and allowed to recover in medium without serum for an additional 48 h. Cells were treated with the above-described isotonic acid solution for 5 min, washed twice with PBS, and incubated with conditioned serum-free medium. The latter was collected from cells previously grown in serum-free medium for 48 h and trypsinized as above. Negative controls included incubation with isotonic acid solution (pH adjusted to 7.0 with NaOH) and physiological saline.

RESULTS

Detection and Localization of Proteins Phosphorylated on Tyrosine in a Gastric Carcinoma Cell Line GTL-16

Whole-cell proteins were solubilized in Laemmli buffer (23), separated by PAGE, transferred to nitrocellulose sheets, and probed with purified antibodies against P-Tyr followed by radiolabelled protein-A, as described elsewhere (11). As previously reported (17), no labelled bands were observed in normal human fibroblasts grown in vitro nor in samples harvested from the gastric mucosa of donors affected by different pathologies. A protein of the approximate M_r of 145,000 was detected in samples solubilized from the gastric tumor cell line GTL-16 (Fig. 1A). Longer exposure of the gels or the vanadate treatment of the cells allowed the detection of other phosphotyrosine-containing bands of different M_r (data not shown). The presence of authentic P-Tyr in P145 was confirmed by immunoprecipitating it from cells metabolically labelled with ^{32}P-orthophosphate (36).

In Vitro Tyrosine Kinase Activity

It was previously shown (36) that P145 exhibited an associated tyrosine kinase activity, which can be demonstrated in an in vitro kinase assay. Proteins extracted from cells by nonionic detergent were immunoprecipitated with either P-Tyr antibodies or control rabbit immunoglobulins. When immunoprecipitates were exposed to (γ-^{32}P)ATP to assay the kinase activity, P145 became phosphorylated (Fig. 1C). In analogy with the known tyrosine kinases (7-8) and in view of the fact that P145 is virtually the only protein phosphorylated on tyrosine in the assay, it seems reasonable to propose that P145 phosphorylates itself on tyrosine (Fig. 1C).

The band seen on Western blot represents the same protein that becomes autophosphorylated, because as described elsewhere (36), both proteins show an unusual shift in their electophoretic mobility when separated in SDS-PAGE under nonreducing conditions, i.e., in the absence of beta-mercapto-ethanol. This shift has never been observed when known tyrosine kinases were analyzed under the same conditions. It had been attributed to the presence of a light chain covalently linked to P145, as previously shown (36). In cells lacking P145 on Western blot, in vitro phosphorylation of proteins with similar M_r was not observed (14, 17).

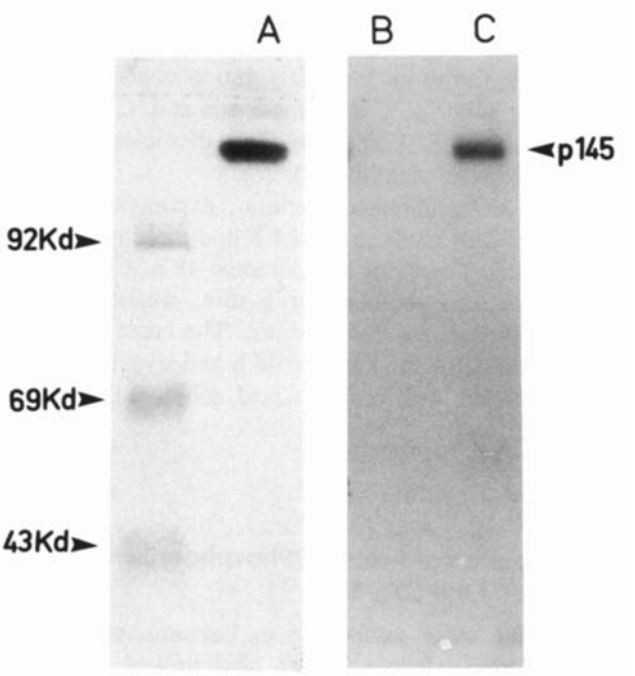

Fig. 1. Tyrosine phosphorylation of P145 in vivo and in vitro. **A**, Western blot analysis of proteins solubilized from GTL-16 cells, probed with P-Tyr antibodies. **B,C**, Immunocomplex kinase assay of P145 precipitated by P-Tyr antibodies (C) or by preimmune rabbit immunoglobulins (B).

Tyrosine Phosphorylation of P145 in Live Cells Is Sustained by an Autocrine Loop

Because it has been shown that the constitutive phosphorylation of receptors could be sustained by factors produced by the cells themselves, experiments were designed to interfere with such a putative autocrine stimulation. GTL-16 tumor cells were treated with mild acid solutions (see Methods). A short acid treatment rapidly removes bound EGF or PDGF molecules from their receptors and switches off growth-factor induced tyrosine kinase activity. The effect is rapid and reversible (20).

GTL-16 cells were incubated at pH 3.7 for different lengths of time. The extent of tyrosine phosphorylation of P145 was evaluated on Western blots probed with P-Tyr antibodies. Following acid treatment, dephosphorylation of P145 was almost complete with 5–15 min (Fig. 2).

To rule out the possibility that the drop of intracellular pH could be directly responsible for the fall of the tyrosine kinase activity, mouse fibroblasts transformed by v-src were treated with acid for the same lengths of time: the activity of $pp60^{src}$ was unaffected (Fig. 2). Thus, the acid treatment appears to interfere with events taking place at the cell surface, most likely the binding of an extracellular factor.

Evidence suggesting the presence of a factor secreted by the gastric carcinoma cells themselves was obtained by studying the effect of conditioned medium. As discussed above and shown in Figure 3, the phosphorylation of P145 is abolished by treating cells with acid. P145 is rephosphorylated if the cells are allowed to recover in serum-free medium at 4°C for a minimum of 15 min (Fig. 3). The addition of

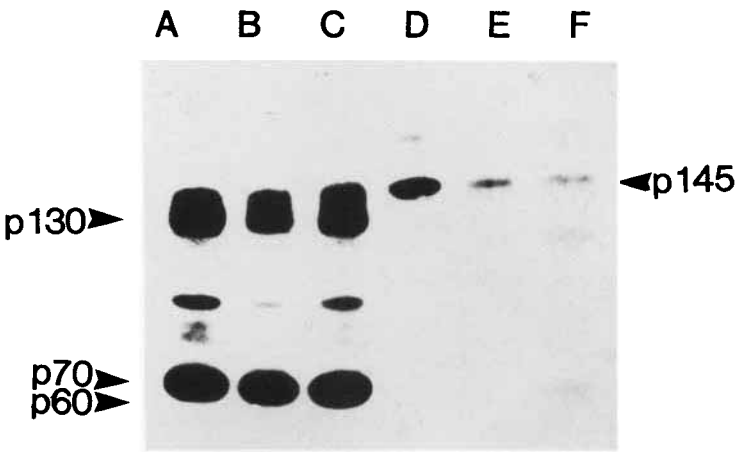

Fig. 2. Effects of mild acid treatment on P145 phosphorylation in intact cells. GTL-16 cells (**D,E,F**) or cells transformed by v-src (**A,B,C**) were treated by an isotonic acid solution pH 3.7 (see Methods) for 5 min (B,E) or 15 min (C,F). The tyrosine phosphorylation of P145 observed in untreated cells (D) is abolished by acid treatment (E,F). The treatment had no effect on the activity of $pp60^{src}$ (A,B,C). Intact cells were solubilized and protein phosphorylated on tyrosine were analyzed in Western blot with P-Tyr antibodies. In v-src transformed cells, the proteins phosphorylated on tyrosine other than $pp60^{src}$ have been described elsewhere (14).

conditioned medium restores the tyrosine phosphorylation of P145 within 5 min at the same temperature. P145 phosphorylation was not restored either by treating the cells with the same acid solution neutralized to pH 7.00 or by treating them with nonconditioned medium in presence or absence of serum (data not shown). Similarly, media conditioned by a number of cell lines other than GTL-16 did not stimulate P145 phosphorylation. These data suggest that the GTL-16 cells produce and secrete in the medium a factor responsible for stimulating the phosphorylation of P145.

DISCUSSION

The data reported in this paper show that in a gastric carcinoma cell line the tyrosine phosphorylation of a membrane protein of M_r 145,000 is sustained by an extracellular factor produced by the cells themselves.

P145 was previously identified as a glycoprotein exposed at the cell surface because it incorporated ^{3}H-D-Glucosamine, was bound by lentil lectin, was labelled by radioactive Iodine under nonpermeating conditions, and was cleaved by mild trypsin treatment of intact cells (36). After immunoprecipitation with P-Try antibodies, P145 displayed a strong associated tyrosine kinase activity in vitro. In immunocomplex-kinase assays, P145 itself was by far the major detectable substrate. In analogy with the known tyrosine kinases, which almost invariably phosphorylate themselves, it was speculated that P145 itself could be tyrosine kinase.

The transmembrane structure and the tyrosine kinase activity of P145 are features of growth-factor receptors. The possible identity of P145 and other known tyrosine kinase receptors (27–31) was disproved by data reported and discussed elsewhere (36).

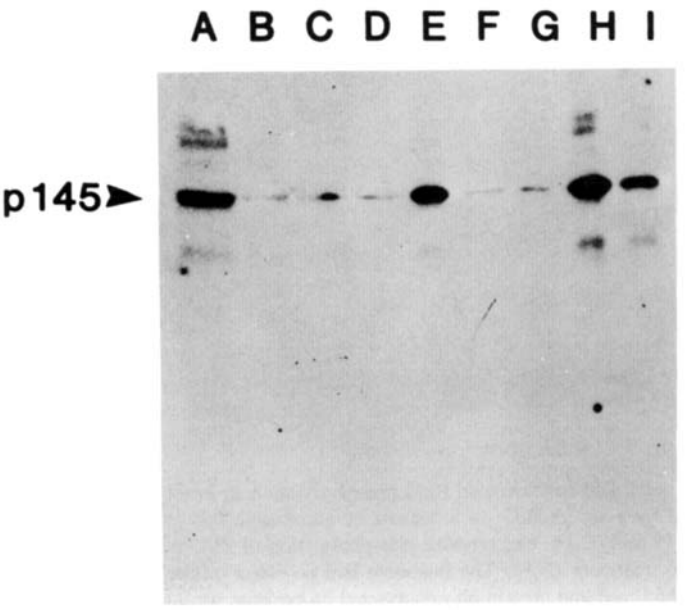

Fig. 3. Stimulation of P145 tyrosine phosphorylation by conditioned medium. Intact cells were solubilized by Laemmli buffer and analyzed by Western blot using P-Tyr antibodies. **A**, GTL-16 cells grown in serum free medium for 3 days. **B**, The same cells treated by mild acid solution as described in the Methods section. **C**, Cells treated as in B and incubated with neutralized acid solution (see Methods section) for 5 min at 4°C. **D**, Cells treated as in B and incubated with physiological saline for 5 min at 4°C. **E**, Cells treated as in B and stimulated with conditioned medium for 5 min at 4°C. **F**, Cells treated as in B and incubated in the neutralized acid solution for 10 min at 4°C. **G**, Cells treated as in B and incubated with physiological saline for 10 min at 4°C. **H**, Cells treated as in B and stimulated with conditioned medium for 10 min at 4°C. **I**, Recovery of cells as in B after 20 min in neutralized acid solution at 4°C.

The present study demonstrated that P145 is dephosphorylated when cells are exposed to a treatment known to dissociate ligands from their receptors and also that tyrosine phosphorylation of P145 is fully restored upon the addition of conditioned medium to the cells. This suggests that the kinase associated with P145 may be stimulated by binding a soluble factor secreted by the gastric carcinoma cells themselves. However, the possibility that P145 could be transphosphorylated by a different kinase representing the actual receptor for the factor present in the medium cannot be ruled out. A similar transphosphorylation mechanism has been shown to operate in the case of erbB-2 phosphorylation by the EGF-receptor (32, 33).

The existence of autocrine circuits of growth stimulation has been reported in physiologic as well as in pathologic conditions, including the autocrine loop triggered by IL-2 (34) in lymphocytes, by FGF (35) in gliomas, by PDGF (7), or by bombesin (2) in small-cell lung carcinomas. In some instances, autocrine activation of a receptor-associated tyrosine kinase also has been demonstrated (16, 18). Evidences for the existence in the cell line studied of one or more autocrine circuits include the following: (1) cell growth in the absence of serum; (2) the presence of a membrane protein (P145) with structural features common to growth-factor receptors; (3) loss of phosphorylation of P145 after acid wash, followed by spontaneous recovery in the

absence of serum; (4) stimulation of P145 phosphorylation by conditioned medium. Whether or not tyrosine kinase activity sustained by the autocrine loop(s) is relevant to the altered cell growth of the line remains to be investigated.

ACKNOWLEDGMENTS

This work was supported by funds granted by the P.F. Oncologia of the Italian C.N.R. to P.M.C. (no. 87.01241.44) and to M.F.D.R. (87.01269.44) and by the Italian Association for Cancer Research (A.I.R.C.). S.G. is the recipient of A.I.R.C. fellowships, and L.C.P. is the recipient of Ghirotti fellowship.

We thank Dr. C. Ponzetto for helpful discussion and revision of the manuscript.

REFERENCES

1. Betsholtz C, Heldin C-H, Nister M, Ek B, Wasteson A, Westermark B: Cell 39:447–457, 1984.
2. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD: Nature 316:823–826, 1985.
3. Rettenmier CW, Roussel MF, Ashmun RA, Ralph P, Price K, Sherr CJ: Mol Cell Biol 7:2378–2387, 1987.
4. Sporn MB, Roberts AB: Nature (London) 313:745–747, 1985.
5. Sporn MB, Todaro GJ: New Engl J Med 303:878–880, 1980.
6. Hunter T: Cell 50:823–829, 1987.
7. Heldin CH, Westermark B: Cell 37:9–20, 1984.
8. Hunter T, Cooper JA: Annu Rev Biochem 54:897–930, 1985.
9. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AY, Stanley ER: Cell 41:665–676, 1985.
10. Cirillo D, Gaudino G, Naldini L, Comoglio PW: Mol Cell Biol 6:4641–4649, 1986.
11. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield M: Nature (London) 307:521–527, 1984.
12. Ullrich A, Coussens L, Hayflick J, Dull T, Gray A, Tam A, Lee J, Yarden Y, Libermann T, Schlessinger J, Downward J, Mayes E, Whittle N, Waterfield M, Seeburg P: Nature (London) 309:418–425, 1984.
13. Comoglio PM, Di Renzo MF, Tarone G, Giancotti FG, Naldini L, Marchisio PC: EMBO J 3:483–489, 1984.
14. Di Renzo MF, Ferracini R, Naldini L, Giordano S, Comoglio PM: Eur J Biochem 158:383–391, 1986.
15. Frackelton AR Jr, Tremble TM, Williams LT: J Biol Chem 259:7909–7915, 1984.
16. Gaudino G, Cirillo D, Naldini L, Rossino P, Comoglio PM: Proc Natl Acad Sci USA 85:2166–2170, 1987.
17. Giordano S, Di Renzo MF, Cirillo D, Naldini L, Chiadó-Piat L, Comoglio PM: Int J Cancer 39:482–487, 1987.
18. Leal F, Williams LT, Robbins KC, Aaronson S: Science 230:327–330, 1985.
19. Ross AH, Baltimore D, Heisen HN: Nature (London) 294:654–656, 1981.
20. Sturani E, Zippel R, Toschi L, Comoglio PM, Alberghina L: Mol Cell Biol 8:1345–1351, 1988.
21. Zippel R, Sturani E, Toschi L, Naldini L, Alberghina L, Comoglio PM: Biochim Biophys Acta 881:54–61, 1986.
22. Motoyama T, Hojo H, Suzuki T, Oboshi S: Acta Med Biol (Niigata University, Niigata, Japan) 27:49–52, 1984.
23. Laemmli JK: Nature (London) 227:680–685, 1970.
24. Burnette WN: Anal Biochem 112:195–204, 1981.
25. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci USA 76:4350–4353, 1979.
26. Garrett JS, Coughlin SR, Niman HL, Tremble PM, Giels GM, Williams LT: Proc Natl Acad Sci USA. 81:7466–7470, 1984.
27. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Wande Woude G: Proc Natl Acad Sci USA 84:6379–6383, 1987.

28. Martin Zanca D, Hughes SH, Barbacid M: Nature (London) 319:743–748, 1986.
29. Martin Zanca D, Mitra G, Long LK, Barbicid M: In "Cold Spring Harbor Symposia on Quantitative Biology," 1986, pp 983–992.
30. Besmer P, Murphy JE, George PC, Qiu F, Bergold FP, Lederman L, Snyder HW, Brodeur D, Zuckerman EE, Hardy WD: Nature (London) 320:415–421, 1986.
31. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A: EMBO J 6:3341–3352, 1987.
32. Kabowaki T, Kasuga M, Tobe K, Takaku F: Biochim Biophys Res Comm 144:699–704, 1987.
33. Akiyama T, Saito T, Ogawara H, Toyoshima K, Yamamoto T, Mol Cell Biol 8:1019–1026, 1988.
34. Linch DC, Wallace DL, O'Flynn K: Immunol Rev 95:137–159, 1987.
35. Libemann TA, Friesel R, Jaye M, Lyall RM, Westermark B, Drohan W, Schimdt A, Maciag A, Schlessinger J: EMBO J 6:1627–1632, 1987.
36. Giordano S, Di Renzo MF, Chiadó-Piat L, Ferracini R, Comoglio PM: Mol Cell Biol 8:3510–3518, 1988.