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Differential sensitivity to pertussis toxin of 3T3 cells transformed with different oncogenes

I. Rey, H. Suarez*, F. Lavelle and B. Tocque

Rhône-Poulenc Santé, Centre de Recherches de Vitry, BP No. 14, 94403 Vitry-sur-Seine and **IRSC, BP No. 8, 94802 Villejuif, France*

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Pertussis toxin (PT), which blocks the activity of several G-proteins, has been found to exert a marked inhibitory effect on the DNA synthesis induced in 3T3 cells by serum or growth factors. 3T3 cells transformed with human c-*ras* oncogenes (Ha-*ras*, Ki-*ras*, N-*ras*) or with *src*, an oncogene coding for a protein kinase, have lost sensitivity to growth control by PT, even though substrates for PT can still be ADP-ribosylated in vivo. In contrast, 3T3 cells transformed with the SV40 virus behave like normal untransformed cells with respect to the ability of PT to decrease their growth rate. Oncogenes can thus likely be classified either as 'responders' or 'non-responders' to PT.

Pertussis toxin; Oncogene; Polyphosphoinositide breakdown; (3T3 fibroblast)

1. INTRODUCTION

Activated *ras* oncogenes appear to play a major role in several human cancers. In this gene family, Ha-*ras*, K-*ras* and N-*ras*, which have been well characterized [1], code for a 21 kDa guanosine triphosphate-binding protein (p21) which is related to signal transducing G-proteins [2] and is believed to play a role in growth-signal transduction. The similarities between the ras and G-proteins are based on their GTP-binding consensus region and their co-localization on the inner surface of the plasma membrane. GTP-binding proteins such as G_i and G_s regulate the adenylate cyclase system [2]. In the case of mast cells, neutrophils and hamster fibroblasts, receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) is abolished by pertussis toxin (PT) ADP-ribosylation of another G-protein called G_p [3,4]. It has been suggested that the mammalian ras family proteins could act as regulatory components of inositol production [5,6]. However, more recent work showed apparently contradictory results [7,8]. Thus, the question as to whether the ras proteins would function as authentic coupling G-proteins is still open. It has been observed [9,10] that PT, which blocks the activity of several G-proteins, exerts a dramatic inhibitory effect on the DNA synthesis induced in 3T3 cells by serum and growth factors (GFs). Here, we study the response of oncogene(s) transformed 3T3 cells to PT treatment. The data show that, after PT treatment, mitogenic signals controlled by G-proteins can no longer influence Ha-*ras* and *src*-transformed 3T3 cell growth, but are still active in SV40 virus-transformed cells. Our results also suggest that mutated ras proteins might control transduction of GFs which stimulate PIP_2 breakdown at a step subsequent to the polyphosphoinositide phosphodiesterase (phospholipase C).

Correspondence address: B. Tocque, Rhône-Poulenc Santé, Centre de Recherches de Vitry, BP No. 14, 94403 Vitry-sur-Seine, France

Abbreviations: PT, pertussis toxin; DTT, dithiothreitol; GFs, growth factors; IP, inositol phosphates; PIP_2, phosphatidylinositol 4,5-bisphosphate

2. MATERIALS AND METHODS

2.1. *Cell lines*

ESC-3T3 fibroblasts [11], 3T3/src and 3T3/SV40 were cultured in modified Eagle's medium (MEM) enriched with 10%

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newborn calf serum. 3T3/Ha-ras cells were cultured in the same medium supplemented with glucose (3.2 g/l) and tryptose phosphate broth (2.7 g/l). The 3T3/Ha-ras cells are ESC cells transformed with the high molecular mass (HMM) DNA from a stomach human tumor bearing an activated Ha-*ras* gene [11]. HMM DNA was extracted and purified according to [12]. Transfections were carried out as in [11] using the calcium phosphate precipitation method. 3T3 cells transformed with the c-*src* gene were a gift from Dr G. Goubin (Institut Curie, Paris). 3T3/SV40 cells were transformed in our laboratory by infection of 3T3 ESC cells with the complete SV40 virus (stock LP).

2.2. *Stimulation of cell growth by newborn calf serum in a PT-dependent manner*

Cell growth rates were determined by plating the different cell lines in 24-well (diameter: 15 mm) plates. PT kept in saturated ammonium sulfate was diluted to the required concentration in phosphate buffer. Control cells received the same dilution of ammonium sulfate. The medium was changed every 2 days and fresh toxin was added. Cells were counted from triplicate plates with a haemocytometer after trypsinization with 0.5 ml of 0.25% trypsin in phosphate buffer.

2.3. *Soft agar assay*

The soft agar assay was carried out as described [13]. Briefly, cell suspensions in culture medium containing 0.33% agar were seeded in 60 mm Petri dishes and allowed to solidify for 5 min at 4°C to prevent cell sedimentation. Pretreatment with PT toxin (100 ng/ml) was carried out for 20 h at 37°C. The number of colonies growing in agar was determined after 18 days of incubation at 37°C under 5% carbon dioxide. Colonies larger than 50 μm in size were counted from quadruplicate plates.

2.4. *ADP-ribosylation assay*

PT (1 mg/ml) kept at 4°C in saturated ammonium sulfate was prepared for use by 10-fold dilution with 100 mM potassium phosphate (pH 8.0) and dialysis for two 30-min periods against 100 mM potassium phosphate (pH 8.0). The toxin was then activated with an equal volume of 100 mM DTT and stored for 1 h at room temperature. Cells were treated in the presence or absence of PT (100 ng/ml) overnight at 37°C.

100 μg homogenates prepared from each cell line were assayed for pertussis toxin substrates in a total volume of 50 μl containing 250 mM potassium phosphate (pH 7.0), 3 μM [^{32}P]-NAD (10^7 cpm), 20 mM thymidine, 1 mM ATP, 0.1 mM GTP, 20 mM arginine · HCl, 5 mM DTT and 6 μg activated PT. Incubations were carried out for 1 h at 37°C and were terminated on ice by the addition of an excess of unlabeled NAD. After trichloroacetic acid precipitation, samples were subjected to SDS-PAGE and dried gels were subjected to autoradiography with an X-omat 2 Kodak film.

2.5. *Measurement of phosphoinositide breakdown*

Confluent cultures were arrested and labeled to equilibrium with *myo*-[^{3}H]inositol (4 μCi/ml) for 24 h in serum-free MEM. PT was added directly to the medium 12 h before addition of GFs. Cells were extensively washed with a buffer containing 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 8 mM $MgSO_4$, 25 mM glucose, 20 mM Tris-HCl (pH 7.4), then incubated with 10 mM LiCl for 5 min. Thereafter the cultures were stimulated with GFs at the indicated concentrations: serum (10%, v/v), thrombin (1 IU/ml) in 300 μl of fresh Li^+ containing solution for 60 min. The reaction was stopped by extracting the cells with $HClO_4$. Separation of [^{3}H]inositol phosphates was carried out as described by Bone [14].

2.6. *Reagents*

Human α-thrombin was from Sigma. PT was a gift from the Mérieux Institute, France. [α-^{32}P]NAD (28 Ci/mmol) was obtained from New England Nuclear and *myo*-[^{3}H]inositol (14.3 Ci/mmol) from Amersham.

3. RESULTS

3.1. *Pertussis toxin and cell growth*

Table 1 summarizes the results concerning the phenotypic characteristics of the 3T3 control and transformed cells. 3T3 cells, after integration in their genome of one of the oncogenes (Ha-*ras*, c-*src* or SV40), acquired a transformed phenotype as judged from their ability to clone in agar and to form tumors in nude mice. After transfection or infection, morphological transformation was judged

Table 1

Growth characteristics and tumorigenicity of 3T3 cells and derivatives

	Control cells (3T3 cells)	Transformed cells		
		3T3/Ha-ras	3T3/src	3T3/SV40
Morphological transformation	–	+	+	+
Loss of contact inhibition of cell division	–	+	+	+
Growth in semi-solid medium (% of clones)	0	12	5	7
Growth in semi-solid medium of cells pretreated with PT (% of clones)	0	12	5	2
Doubling time (h)	22	12	16	17
Tumorigenicity in nude mice	–	+	+	+

The numbers of clones in agar were, respectively, for 3T3/Ha-ras, 3T3/src and 3T3/SV40, 90 ± 7, 37 ± 4 and 52 ± 5. Data are means ± SE of quadruplicate determinations. When different cell lines were pretreated overnight with PT (100 ng/ml), the numbers of clones were 92 ± 5 for 3T3/Ha-ras, 38 ± 3 for 3T3/src and 15 ± 2 for 3T3/SV40 cells. These experiments were repeated several times

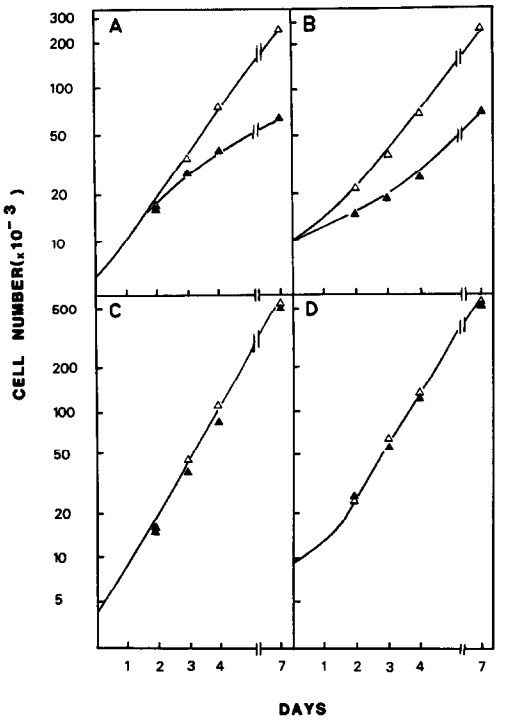

Fig.1. Effect of PT on the growth of control and transformed 3T3-cell lines. Normal 3T3 fibroblasts (A), 3T3/SV40 (B), 3T3/Ha-ras (C) or 3T3/src (D) were seeded respectively at 6×10^3, 9×10^3, 3.5×10^3 and 10×10^3 cells per dish. Pertussis toxin (10 ng/ml) was added with fresh medium at day 0, 2 and 4 and the number of cells was determined as indicated in section 2. Treated (▲—▲); untreated (△—△).

by the appearance of spherical or spindle shape cells and the refractive appearance of the transformed cells. The tumorigenicity of the transformed clones was evaluated by the appearance of 100% of tumors, without any latency, after injection of 2×10^6 cells per animal (not shown). PT does not affect the ability of 3T3/Ha-*ras* or 3T3/*src* to form clones in soft agar. In contrast, overnight treatment of 3T3/SV40 with 100 ng/ml of PT significantly decreases the number and size of the clones. We have measured the growth rate of control and transformed cells in the presence of 10 ng/ml of PT (fig.1). 3T3 fibroblasts are very sensitive to PT treatment. After 7 days of treatment, the number of cells is decreased by 80%. PT slows down normal 3T3 cell growth with an IC_{50} of about 1 ng/ml. Interestingly, 3T3 cells transformed with SV40 virus, like normal cells, retain full sensitivity to PT treatment. After 7 days treatment the number of 3T3/SV40 cells was decreased by 75%. Cells transformed with Ha-*ras* or c-*src* do not show any sensitivity to PT even at concentrations up to 100 ng/ml. These data imply that transformation with SV40 does not orientate the mitogenic signals toward steps which are completely insensitive to PT as it does following transformation with Ha-*ras* or *src* oncogenes.

3.2. *Phospholipase C activity in 3T3 cells transformed with different oncogenes*

As receptor activation of phospholipase C is mediated by a GTP-binding protein sensitive to PT in 3T3 cells [12], and as it has been demonstrated that the activation of phospholipase C is a necessary step mediating the mitogenic effect of serum and thrombin [16], we measured accumulation of inositol phosphates (IP) in the different cell lines.

Accumulation of IP was measured in the presence of Li^+ after stimulation by serum (10%) or thrombin (1 IU/ml) with or without pretreatment with PT (100 ng/ml). Serum and thrombin stimulate IP formation in control cells in the absence of PT treatment (table 2). This stimulation is significantly reduced after toxin treatment.

Table 2

Stimulation of IP formation by serum (10%) or thrombin (1 IU/ml) in the presence or absence of PT

		% stimulation	
		No pretreatment	PT pretreatment
3T3/control	serum	83	43 (47)
	thrombin	61	24 (61)
3T3/Ha-ras	serum	13	12 (7)
	thrombin	25	26 (0)
3T3/src	serum	13	13 (0)
	thrombin	35	32 (9)
3T3/SV40	serum	29	10 (64)
	thrombin	33	20 (40)

Basal activities of IP accumulation for 3T3 control, 3T3/Ha-ras, 3T3/src and 3T3/SV40 cells were respectively 260 ± 20, 237 ± 15, 293 ± 23 and 250 ± 20 fmol/10^6 cells (data are means ± SE of triplicate determinations). Data in parentheses are the percentages of inhibition elicited by pretreatment of the cells with 100 ng/ml of PT. Data represent a typical experiment. Values for % of stimulation are the average of triplicates. Experiments were repeated five times

In Ha-*ras* and *src*-transformed cells, however, the basal phospholipase C activity is not modified and the stimulated production of IP never exceeded 35% over basal, either after serum or thrombin treatment. PT equally failed to modify residual production of IP in these cells. Similar data were obtained with c-Ki-*ras* and N-*ras* transformed 3T3 cells (not shown). A kinetic study of IP accumulation or the use of different concentrations of serum or thrombin gave identical results (not shown). In 3T3/SV40 cells, we observed the same desensitization to serum and thrombin as in the other transformed cell lines but PT was still able to block partially IP formation.

3.3. *Labeling with [^{32}P]NAD of PT substrates*

PT can ADP-ribosylate several related GTP-binding proteins in 3T3 cells [10].

We compared PT catalyzed ADP-ribosylation profiles of homogenates from the different clones. PT-dependent labeling of a 40 kDa band was visualized in 3T3 control cells (fig.2). The same pattern of ADP-ribosylation was obtained for the transformed clones except in the case of the 3T3/Ha-*ras* cells which displayed a reduced incorporation of [^{32}P]ADP-ribose. The reduced amount of incorporation of [^{32}P]ADP-ribose into the 40 kDa band does not seem to be specific of either the Ha-*ras* gene or *ras* gene family since we obtained different results with Ki-*ras* or N-*ras* transformed cells and EJ/T24/Ha-*ras* cells (not shown). Preincubation of the cells with 100 ng/ml of PT abolished the in vitro labeling, showing that the toxin can still reach its substrates in every clone tested.

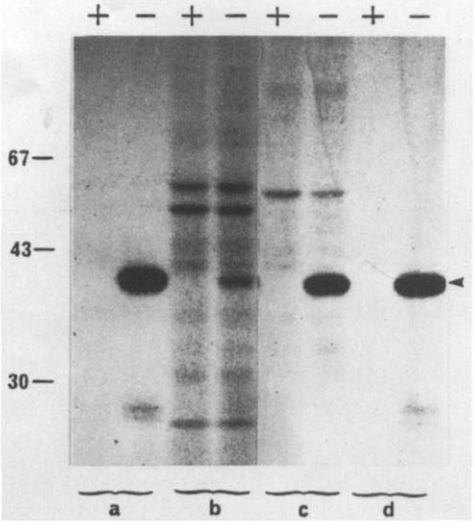

Fig.2. Pertussis toxin-dependent ADP-ribosylation of 40 kDa substrates (arrow), in homogenates prepared from (a) Swiss 3T3, (b) 3T3/Ha-ras, (c) 3T3/SV40, (d) 3T3/src cells. Cells were pretreated (+) or not (−) overnight with 100 ng/ml of toxin. ADP-ribosylation was performed and 100 μg proteins were subjected to SDS-PAGE (12.5%) and autoradiographed as described in section 2.

4. DISCUSSION

In this paper, we have studied the ability of PT to control growth rate and IP production in 3T3 fibroblasts transformed with different oncogenes. PT exerts absolutely no effect on the growth rate of *ras* and *src* 3T3 transformed cells but dramatically decreases the growth rate of SV40 virus transformed 3T3 cells and of 3T3 control cells. The important point is that transformation with SV40 does not confer a resistant phenotype to PT. SV40 virus products (T and t antigens) act at the cellular DNA level or in the cytoplasm rather than on a membrane phenomenon [15]. *src* and *ras* products are anchored in the cytoplasmic membrane [1,22]. PT discriminates between 'different transforming' oncogenes. This difference between the oncogenes is considerably reinforced by the fact that in vitro clonogenicity of 3T3/SV40 cells is diminished by PT. This means that PT blocks essential information for the complete expression of the 3T3/SV40 cell transformed phenotype. PT is known to control cell growth [9,10] as well as to block the inositol lipid pathway in 3T3 normal fibroblasts [16]. Therefore, we measured the ability of PT to decrease serum- and thrombin-induced release of IP in the presence of Li^+ in transformed cells. As reported by others [17,18] accumulation of diacylglycerol is a common phenomenon in a large number of mammalian transformed cells, suggesting increased PIP_2 turnover [19]. It has been previously shown that a direct relationship exists between *ras* proteins and phospholipase C [5,20]. Indeed, an increased production of IP has been reported in response to respectively bombesin or PDGF polypeptides, using a hormone-regulated construction of cellular *ras* genes, causing overexpression of either N-*ras* protein [5] or Ha-*ras* protein [20]. If we assume that mutated forms of c-Ha-

ras are permanently activated [18] and participate directly in the PI cycle, increased IP formation should be detectable in cells transformed specifically with this oncogene. Our data show that the basal rate of IP formation was unchanged in all the transformed cells tested and that complete depression of the serum- and thrombin-induced stimulation of IP accumulation occurs. These data are not in favor of a direct interaction between *ras* proteins and phospholipase C. Similar observations were made in Ha-*ras* 3T3 transformed cells [8,21] or Ha-*ras* CCL39 transformed cells [6]. The residual stimulation of IP formation was no longer sensitive to PT in the presence of *src* or *ras* transforming proteins, but was comparable to that of control cells when large T and small t SV40 proteins were produced. Lack of PT activity cannot be explained by the disappearance of PT substrates or PT-binding units in the transformed cells. Indeed, in vivo ADP-ribosylation assay (fig.2) and ALF_4^- stimulation of PIP_2 breakdown (not shown) were still positive in every cell line.

A decreased number of cell receptors coupled to PIP_2 breakdown cannot be ruled out even though such a phenomenon has not yet been reported. It is more likely that mutated *ras* and *src* proteins turn off the phosphoinositide mitogenic pathway by an unknown mechanism which favors the tyrosine kinase stimulation of DNA synthesis. Indeed, it has been demonstrated that *ras* proteins are necessary for *src* transformed phenotype expression [22,23]. Thus, it is not surprising that *src* and mutated *ras* confer the same resistant phenotype to PT. Our data suggest that, when oncogenes such as *src* or *ras* are present, transduction pathways sensitive to PT, such as GF-induced IP formation or GF-induced decrease of cellular cAMP, are not necessary for mitogenicity. A different situation is generated by SV40 transformation, which does not impair the mitogenic signals transduced through PT substrates. It would be of considerable importance to know how *src* and *ras* proteins desensitize PT-sensitive mitogenic pathways.

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REFERENCES

[1] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[2] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[3] Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
[4] Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.
[5] Wakelam, M.J.O., Davies, S.A., Houslay, M.D., McKay, J., Marshall, C.J. and Hall, A. (1986) Nature 323, 173–176.
[6] Fleischman, L.F., Chahwala and Cantley, L. (1986) Science 231, 407–410.
[7] Seuwen, K., Lagarde, A. and Pouysségur, J. (1988) EMBO J. 7, 161–168.
[8] Parries, G., Hoebel, R. and Racker, E. (1987) Proc. Natl. Acad. Sci. USA 84, 2648–2652.
[9] Hildebrandt, J.D., Stolzenberg, E. and Graves, J. (1986) FEBS Lett. 203, 87–90.
[10] Murayama, T. and Ui, M. (1987) J. Biol. Chem. 262, 12463–12467.
[11] Suàrez, H.G., Grosjean, L., Andéol, Y. and Nardeux, P.C. (1985) in: Retroviruses and Human Pathology (Gallo, R.C. et al. eds) pp. 509–516, Humana, Clifton, NJ.
[12] Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32–38.
[13] Krishan, A., Sauerteig, A. and Wellham, L.L. (1985) Cancer Res. 45, 1046–1051.
[14] Bone, E.A., Fretten, P., Palmer, S., Kirk, C.J. and Michell, R.H. (1984) Biochem. J. 221, 803–811.
[15] O'Reilly, D. (1986) Biol. Cell. 57. 187–196.
[16] Murayama, T. and Ui, M. (1985) J. Biol. Chem. 260, 7226–7233.
[17] Wolfman, A. and Macara, J.G. (1987) Nature 325, 359–361.
[18] Lacal, J.C., De La Pena, P., Moscat, J., Garcia-Barreno, P., Anderson, P.S. and Aaronson, S.A. (1987) Science 238, 533–536.
[19] Yu, C.L., Tsai, M.H. and Stacey, D.W. (1988) Cell 52, 63–71.
[20] Marshall, C.J. (1987) Cell 49, 723–725.
[21] Benjamin, C.W., Tarpley, W.G. and Gorman, R.R. (1987) Proc. Natl. Acad. Sci. USA 84, 546–550.
[22] Smith, M.R., De Gudicibus, S.J. and Stacey, D.W. (1986) Nature 320, 540–543.
[23] Noda, M., Selinger, Z., Scolnick, E. and Bassin, R.H. (1983) Proc. Natl. Acad. Sci. USA 80, 5602–5606.