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Effect of phosphotyrosine phosphatase over-expression on glutathione metabolism in normal and oncogene-transformed cells

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Abstract

We measured the level of reduced glutathione (GSH) and oxidized glutathione (GSSG) in normal and oncogene-transformed NIH/3T3 fibroblasts and 32D hematopoietic cells. NIH/3T3 cells transformed by the activated oncogenes *erbB*, *src*, and *raf*, showed increased levels of GSH with concomitant alterations in the levels of GSH-related enzymes. Transfection and over-expression of a synthetic gene coding for a phosphotyrosine protein phosphatase (PTPase), which inhibited the proliferation of normal and transformed NIH/3T3 cells, was accompanied by a decrease in GSH levels in normal and *erbB*-transformed fibroblasts, and by an increase in *src* and *raf* transformants. Among GSH-related enzymes, only γ-glutamylcysteine synthetase was altered in normal and *erbB*-transformed NIH/3T3 fibroblasts following PTPase transfection. Therefore, tyrosine phosphorylation could be selectively involved in the regulation of GSH metabolism in normal and oncogene-transformed NIH/3T3 fibroblasts, possibly by a dual-type effect on receptor/oncoprotein-mediated mitogenic signal transduction. However, no relationship was observed between the GSH and PTPase effect on cell growth, either after oncogene transfection or PTPase transfection. Moreover, the changes in GSH metabolism were specifically related to cell lineage. In fact GSH and related enzymes did not change in 32D hematopoietic cells transformed by the same activated *erbB* oncogene and in those – normal or transformed – over-expressing the PTPase: in these cells also, over-expression of the PTPase gene was not accompanied by growth inhibition.

Key words: Glutathione; Oncogene; Phosphatase; Neoplasia

1. Introduction

Among the different biochemical mechanisms controlling the proliferation of normal and transformed cells, protein tyrosine phosphorylation is of utmost importance. We recently demonstrated that transfection and over-expression of a synthetic gene coding for a novel phosphotyrosine protein phosphatase (hereafter referred to as PTPase) resulted in the inhibition of proliferation of normal, and of *erbB*-, *src*- and *raf*-transformed NIH/3T3 fibroblasts [1,2]. Transfection and over-expression of PTPase did not affect the growth of normal or transformed 32D hematopoietic cells, thus suggesting a selective effect on tyrosine-phophorylated protein(s) controlling the proliferation of NIH/3T3 cells [2].

NIH/3T3 fibroblasts transformed by a single activated oncogene also proved useful in studying the relationship between alterations of glutathione (GSH) metabolism and oncogenic transformation. Thus, we recently demonstrated that NIH/3T3 cells transformed by the *erbB*, *src*, *raf*, and *ras* oncogenes, but not those transformed by *sis* or *dbl*, showed peculiar alterations of GSH metabolism: GSH was elevated in *erbB*, *src*, *raf* and *ras* transformants, whereas GSSG increased significantly only in *src* transformants [3]. The activity of the synthetic enzyme γ-glutamylcysteine synthetase (γ-GCS) was inversely related to GSH content, suggesting a mechanism of down-regulation; the activity of GSH reductase (GR) was directly related to GSSG levels [3], and GSH-*S*-transferase decreased, thus indicating a decrease in the detoxification processes. Thus, neoplastic transformation by each oncogene caused specific alterations to GSH metabolism, suggesting a relationship between the biochemical mechanism(s) responsible for transformation and those causing alterations in GSH metabolism that, in turn, are related to resistance to the killing effects of anti-neoplastic drugs and ionizing radiations [4–7].

Considering these previous results, we performed a study to relate GSH metabolism with PTPase activity and transformation in order to determine whether protein tyrosine phosphorylation processes could be involved in the specific alterations of GSH metabolism in oncogene-transformed cells. Data have been reported on the involvement of GSH on the phosphorylation/dephosphorylation mechanisms by thiol disulfide exchange [8]. We monitored the metabolism of GSH in

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase (EC 5.1.18); GR, glutathione reductase (EC 1.6.4.2); γ-GCS, γ-glutamyl cysteine synthetase (EC 6.3.2.2).

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NIH/3T3 fibroblasts and in 32D hematopoietic cells, either normal or oncogene-transformed, before and after transfection and over-expression of the synthetic PTPase gene.

2. Materials and methods

2.1. Materials

All enzymes, coenzymes and substrates were from Boehringer (Mannheim, Germany). All other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Cells

Normal NIH/3T3 fibroblasts, 32D, immature myeloid murine cells, and their oncogene-transformed counterparts were obtained from the Laboratory of Cellular and Molecular Biology of the National Cancer Institute (NIH, Bethesda, MD, USA) [9–12].

2.3. Transfection and over-expression of the synthetic PTPase gene

A gene coding for the bovine isoform of PTPase was synthetized in our laboratory, and was transfected into normal and oncogene-transformed cells. Transfection experiments were performed as follows; 10 μg of the plasmid, termed pSVPTP (generated in our laboratory as described by Ramponi et al. [1]) and 0.5 μg of the plasmid termed pK0neo (i.e. the function coding for resistance to antibiotics such as neomycin and geneticin (G-418 Sigma Chem. Co.), used to select transfection-positive clones, and, when transfected alone, as control, as described in Ramponi et al. [1]) were used for transfecting 1.5 million cells on a 100-mm plate. Selection of stable antibiotic-resistant clones was performed by supplementing the medium with 500 μg/ml of antibiotic (G-418). mRNA analysis was performed using the ^{32}P-labelled PTPase synthetic cDNA as a probe, under high stringency conditions (2× SET at 65°C). Total RNA preparation and Northern blot analysis were performed as previously reported [11]. Both normal and transformed cells were also transfected with pK0neo alone. Stable over-expression of PTPase was assessed by ELISA as described in Ruggiero et al. [11], and the level of the enzyme remained approximately the same for up to 2 months. In this study we used transfected clones that expressed PTPase as follows (fold increase over control values): normal NIH/3T3, 2 fold; *erbB*-NIH/3T3, 6 fold; *src*-NIH/3T3, 44 fold; *raf*-NIH/3T3, 21 fold; normal 32D, >50 fold, *erbB*-32D, >50 fold.

2.4. Glutathione determinations

Cells (4–10 × 10^6) were centrifuged in a microcentrifuge (12,550 × *g* for 15 min at 4°C), the precipitate was directly dissolved in 0.5 ml 5% $HClO_4$ and the cells were lysed by sonication. Following centrifugation, the supernatant was assayed for GSH and GSSG by HPLC as described by Reed and Fariss [13]. Briefly, it was neutralized by 2 M K_2CO_3, then, 50 μl of 300 mM iodioacetic acid, and 200 μl of 0.7 M $KHCO_3$ were added to 0.5 ml of extract. The resulting alkaline solution was incubated for 1 h at room temperature in complete darkness. 0.1 ml of 5% (v/v) fluorodinitrobenzene was added to the supernatant, which was then stored at 4°C overnight. The solutions were analyzed by liquid chromatography (Beckman Gold System equipped with an NEC/PC8201H computer, a Shimadzu C-R6A chromatopac integrator, and a Bio-Sil NH_2 90-5S Bio-Rad column). The 2,4-dinitrophenyl derivatives were detected at 365 nm. GSH and GSSG were quantified relative to standards by integration.

2.5. Enzyme assay

Cells (4–10 × 10^6) were centrifuged and the precipitate was dissolved using 0.5 ml 150 mM Tris buffer containing 5 mM $MgCl_2$, 2 mM 1,4-dithiothreitol at pH 7.4. Cells were lysed by sonication at 4°C. The homogenate was centrifuged at 15,000 × *g* for 25 min. The activity of GR was determined by the method of Goldberg and Spooner [14]. The activity of γ-GCS was assayed by Seeling's method [15]. GST was assayed with the method of Habig et al. [16]. All enzymatic activities were measured at 37°C using a Kontron (Uvikon 710) spectrophotometer, and were expressed in terms of micromoles per min per mg of total protein. The protein concentration was determinated by Bradford's method [17]. Bovine serum albumin (Sigma Chemical Co.) was used as the control standard.

2.6. Statistical analysis

The significance of the disparities between the observed and normal means was evaluated using Student's *t*-test. A difference of $P < 0.05$ was considered significant.

3. Results and discussion

As a first step in our study of GSH metabolism, we monitored the effect of PTPase over-expression on the different components of the GSH system in normal and NIH/3T3 fibroblasts transformed by the oncogenes, *erbB*, *raf* and *src*, which code for transforming proteins interfering with signal transduction [18]: (i) v-*erbB* encodes a truncated form of the EGF receptor; (ii) the product of *src* is a non-receptorial tyrosine kinase associated with the inner layer of the membrane; (iii) *raf* codes for a cytoplasmic serine/threonine kinase bearing homology of sequence and function with protein kinase C.

Table 1 shows that GSH increased in cells transformed by the oncogenes, *erbB*, *raf*, and *src*, as compared with normal fibroblasts by 93, 133 and 66%, respectively. PTPase over-expression significantly reduced GSH levels in normal cells and in *erbB* transformants by about 50%, while in *raf* and *src* transformants the GSH level increased by 23 and 50%, respectively, as compared with normal and transformed fibroblasts not over-expressing the PTPase. Since PTPase inhibited the proliferation of all the transfected lines approximately to the same extent [1,2], these results demonstrate a dissociation between the effects of PTPase on cell growth and GSH levels. It could be hypothesized that the different oncogenes used distinct mechanisms leading to the increase in GSH; dephosphorylation of tyrosine residues proved critical for GSH levels to decrease in normal and *erbB*-transformed NIH/3T3 fibroblasts, while in *src* and *raf* transformants, tyrosine dephosphorylation appeared to favour an increase in GSH. Table 1 shows that the GSSG content also increased in all the transformed lines, but this increase was statistically significant only in *src* transformants. Transfection and over-expression of PTPase caused changes in GSSG content in normal and transformed fibroblasts, but these alterations were not statistically significant. These results indicate that over-expression of PTPase did not enhance oxidative mechanisms. These was further confirmed by the redox index (ratio of GSSG/GSH; marker of the intracellular oxidative status) that was similar in all the lines.

Having observed these variations of the components of the GSH system, we studied the activity of the enzymes involved in GSH metabolism. Table 2 shows the complex alterations of γ-GCS, GST and GR specific activities occurring in transformed cells either wild-type or over-expressing PTPase. γ-GCS activity, i.e. the activity of the synthetic enzyme which represents a rate-limiting step in intracellular GSH synthesis, significantly decreased in the transformed cells, and we previously

Table 1
Level of GSH system components in normal, transformed NIH/3T3 fibroblasts, normal and *erbB*-transformed 32D hematopoietic cells: effect of PTPase synthetic gene over-expression

Cell line	GSH	GSSG	GSSG/GSH
NIH	30 ± 2	2.0 ± 0.5	0.07 ± 0.007
NIH/PTPase	15 ± 1*	1.5 ± 0.4	0.10 ± 0.015
erbB-NIH	58 ± 6*	3.0 ± 0.3	0.05 ± 0.009
erbB-NIH/ PTPase	37 ± 4°	2.0 ± 0.3	0.05 ± 0.005
src-NIH	70 ± 6*	6.0 ± 0.6*	0.08 ± 0.006
src-NIH/PTPase	90 ± 4^{++}*	7.0 ± 0.6*	0.07 ± 0.004
raf-NIH	50 ± 5**	3.0 ± 0.1	0.06 ± 0.002
raf-NIH/PTPase	77 ± 7^{+}*	3.5 ± 0.5	0.05 ± 0.006
32D	160 ± 17	16.0 ± 2.0	0.10 ± 0.01
32D/PTPase	155 ± 30	20.0 ± 3.0	0.12 ± 0.02
erbB-32D	160 ± 24	20.0 ± 3.0	0.12 ± 0.02
erbB-32D/ PTPase	180 ± 30	17.0 ± 3.0	0.09 ± 0.01

GSH and GSSG are expressed as nmol/mg protein. Data are means ± S.E.M. of four (32D) and five (NIH/3T3) experiments, each performed on duplicate samples. Significant differences: from NIH/3T3 cells, $*P < 0.005$, $**P < 0.05$; from *erbB*-transformed cells, $°P < 0.05$; from *src*-transformed cells, $^{++}P < 0.05$; from *raf*-transformed cells, $^{+}P < 0.05$.

interpreted this decrease as a negative feedback mechanism brought about by elevated GSH content [3]. PTPase transfection and over-expression caused γ-GCS activity to increase in normal and *erbB*-transformed fibroblasts (i.e. in those cells where PTPase over-expression reduced GSH levels as compared with cells not over-expressing the PTPase), whereas *src* and *raf* transformants were unaffected. In the presence of over-expressed PTPase, however, γ-GCS activity remained low even if the GSH level increased. GST activity (which is the enzymatic activity involved in the GSH-related detoxication processes) decreased in all the transformed lines as compared with normal NIH/3T3 fibroblasts; transfection and over- expression of PTPase, however, did not significantly change the specific activity of this enzyme neither in normal nor in transformed cells. This may indicate that detoxication mechanisms decreased in transformed cells. This in turn could be a cause for the increased GSH which would be less utilized. The specific activity of GR, the enzyme involved in the reduction of GSSG to GSH, showed a significant increase in *src*-transformed fibroblasts. PTPase transfection and over-expression did not cause GR activity to change significantly as compared with either normal or transformed wild-type fibroblasts. The GR increase in *src* transformants (either over-expressing or not the PTPase gene) could be explained by substrate induction since a marked increase in GSSG content was found only in these cells. The main conclusion from these results is that only in *erbB*-transformed fibroblasts and in normal NIH/3T3 fibroblasts did over-expression of PTPase decrease the level of GSH and cause γ-GCS activity to increase, whereas in *src* and *raf* transformants over-expression of PTPase increased GSH content.

In order to clarify the observed effects of PTPase on GSH metabolism in the different transformants, we studied another cell line, i.e. the hematopoietic cell line termed 32D, either normal or transfected by the same activated *erbB* oncogene, over-expressing or not the PTPase. This immature, non-tumorigenic myeloid line is strictly dependent on interleukin-3 for proliferation and survival [12]. Interleukin-3 requirement could be abrogated by oncogene-induced transformation (i.e. by genes such as *erbB*, *abl*, and *src* [12,19]). In this cell line, PTPase over-expression did not influence cell proliferation, neither of normal nor of transformed cells [2]. Thus, it was of interest to determine whether it affected GSH metabolism in normal or *erbB*-transformed 32D cells, i.e. in the hematopoietic counterpart of those NIH/3T3 lines the GSH metabolism of which was altered by PTPase over-expression. Table 1 shows that normal and *erbB*-transformed 32D cells had identical levels of GSH, GSSG, and GSSG/GSH. Since *erbB*-transformed 32D cells are fully malignant, these data confirm that alterations in GSH metabolism observed in some oncogenic transformants were specifically correlated not only with particular oncogenes, but also with a specific cell line, as they were not trivial epiphenomena of malignant transformation. These results (i.e. alterations of GSH metabolism specifically associated with particular oncogenes and cell types) are in agreement with a previous observation reporting altered content of GSH only in certain tumors [20]. Transfection and over-expression of PTPase to a level even higher than that achieved in NIH/3T3 cells, did not alter GSH metabolism, neither in normal

Table 2
Specific activity of GSH-related enzymes in normal, transformed NIH/3T3 fibroblasts, normal and *erbB*-transformed 32D hematopoietic cells: effect of PTPase synthetic gene over-expression

Cell line	GST	GR	γ-GCS
NIH	0.064 ± 0.003	0.052 ± 0.005	0.15 ± 0.010
NIH/PTPase	0.060 ± 0.004	0.040 ± 0.003	0.20 ± 0.020*
erbB-NIH	0.035 ± 0.003**	0.060 ± 0.002	0.11 ± 0.006*
erbB-NIH/ PTPase	0.040 ± 0.003**	0.050 ± 0.004	0.15 ± 0.009°
src-NIH	0.030 ± 0.002**	0.080 ± 0.004*	0.10 ± 0.004**
src-NIH/PTPase	0.020 ± 0.001**	0.075 ± 0.004*	0.11 ± 0.010*
raf-NIH	0.035 ± 0.002**	0.060 ± 0.002	0.12 ± 0.002*
raf-NIH/PTPase	0.045 ± 0.007*	0.060 ± 0.005	0.10 ± 0.005**
32D	0.040 ± 0.004	0.130 ± 0.010	0.11 ± 0.010
32D/PTPase	0.050 ± 0.006	0.120 ± 0.020	0.10 ± 0.010
erbB-32D	0.055 ± 0.010	0.120 ± 0.020	0.11 ± 0.010
erbB-32D/ PTPase	0.040 ± 0.004	0.110 ± 0.010	0.12 ± 0.010

Specific activity of enzymes are expressed as μmol per min per mg total protein. Data are means ± S.E.M. of four (32D) and five (NIH/3T3) experiments, each performed on duplicate samples. Significant differences: from NIH/3T3 cells, $*P < 0.005$, $**P < 0.001$; from *erbB*-transformed cells, $°P < 0.05$.

nor in transformed 32D cells. Consistent with these results, PTPase over-expression did not change the activity of GSH-related enzymes as evidenced in Table 2. Taken together these data indicate that the effect of PTPase over-expression on GSH metabolism was selective for normal and oncogene-transformed fibroblasts, and was not related to the rate of proliferation per se.

In conclusion we determined a specific action of PTPase on GSH metabolism in normal and oncogene-transformed NIH/3T3 fibroblasts that could be explained by postulating that PTPase over-expression exerts a dual-type effect on receptor/oncoprotein-mediated mitogenic signal transduction: (i) direct dephosphorylation, and inhibition of growth factor receptors in normal and *erbB*-transformed fibroblasts; (ii) inidrect, downstream, inhibitory effect on proliferation in cells transformed by other oncogenes. This hypothesis is supported by our previous observation that PTPase directly dephosphorylated in vitro the autophosphorylated normal human EGF receptor [21]. Since activation of growth factor receptors is realted to the increased metabolism of GSH [22,23], we believe that the negative effect of PTPase on GSH levels in normal fibroblasts and *erbB* transformants could be ascribed to direct inhibition of growth factor receptors. These results support the hypothesis that elevated GSH content is an early response to growth factor receptor stimulation [24].

A further consideration resulting from our data regards the relationship between GSH content and pharmaco-radioresistance. Increased GSH levels in tumor cells has been correlated with resistance to anti-neoplastic agents and ionizing radiations [4–7], and, as such, it has been proposed as a marker of resistance and as a prognostic factor in neoplasia. Our data on specific oncogenes involved in human cancer indicate that inhibition of neoplastic cell proliferation (in this case achieved by PTPase over-expression) does not necessarily lead to a decrease in GSH content; in fact in *erbB* transformants GSH content decreases while in *src* and *raf* transformants it increases. Therefore, slowing the proliferation rate of tumors without taking into account GSH content might paradoxically lead to increased resistance to further anti-neoplastic treatment. These results might prove useful in devising new diagnostic and therapeutic strategies targeting the GSH system in order to achieve greater sensitivity to different anti-neoplastic treatments.

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