The importance of the GTP-binding protein tissue transglutaminase in the regulation of cell cycle progression

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Abstract Tissue transglutaminase (tTgase) is a GTP-binding Ca^{2+}-dependent enzyme which catalyses the post-translational modification of proteins via $\varepsilon(\gamma$-glutamyl) lysine bridges. Recent evidence suggests that the GTP-binding activity of tTgase may be important in intracellular signalling thus explaining some of the diverse suggested roles for the enzyme. In the following work a malignant hamster fibrosarcoma (Met B) has been stably transfected with both the full length tTgase cDNA (wild type) and a mutant form of the cDNA whereby the active site cysteine (Cys 277) has been replaced by serine. Expression of this mutant cDNA leads to a protein with GTP binding activity which is deficient of protein crosslinking activity. When synchronised into S-phase and allowed to progress through the cell cycle tTgase transfected clones (both mutant and wild type), when compared to transfected controls, show a delayed progression from S-phase to G_2/M when analysed by flow cytometry which appears to be elicited by the G-protein activity of the tTgase.

Key words: Transglutaminase; GTP-binding protein; Cell cycle

1. Introduction

Tissue transglutaminase (tTgase, Type II) is calcium-dependent enzyme wich catalyses the post-translational modification of proteins either through their crosslinking via $\varepsilon(\gamma$-glutamyl) lysine bonds or through the incorporation of amines into protein bound γ-glutamyl groups. The tissue transglutaminase is ubiquitously found in mammalian tissues both in the membrane and cytosolic fractions and belongs to a larger family of transglutaminases which have distinct genes, structures and biological functions [1]. Although the cellular function of tTgase is still not fully understood it has been implicated in receptor mediated endocytosis [2] stimulus secretion coupling [3], programmed cell death [4] extracellular matrix organisation and cell adhesion [5], cell growth and proliferation [6,7] and tumour growth [8,9].

Despite these observations the correlation between tTgase gene expression and crosslinked proteins found in cells is not always apparent suggesting that the tTgase is either very tightly regulated within the cell or the enzyme has other functions in addition to or in association with its crosslinking role. In keeping with this theory is the finding that tTgase is a GTP-binding protein with GTPase activity [10] and that GTP binding to the enzyme can inhibit its catalytic activity at physiological calcium levels [11]. However only recently has a biological role for tTgase as a functional G-protein been reported [12] whereby it has been shown to mediate α1-adrenergic receptor stimulation of phospholipase C in COS-1 cells. This ability of tTgase to modulate levels of intracellular messengers opens up an intriguing scenario about the biological functions of tTgase and may explain some of its diverse physiological roles.

In the following paper we have concentrated on the possible role of tTgase in cell growth and proliferation and its potential importance in tumour growth. In this respect we have investigated whether the tTgase G-protein has any regulatory role in the progression of the cell through the cell cycle. In order to differentiate tTgase crosslinking activity from its GTP-binding role in this process we have stably transfected a malignant hamster fibrosarcoma (Met B) with both the full length tTgase cDNA (wild type) and a mutant form of the cDNA under the control of a constitutive eukaryotic expression vector. In the mutated cDNA the active site cysteine (Cys 277) has been replaced by serine so that expression of this mutant cDNA in the tumour cells leads to a protein with GTP-binding activity but is deficient in protein crosslinking activity [13].

2. Materials and methods

The origin and cell culture of the Met B hamster fibrosarcoma has been previously described [14]. Cells were grown in supplemented DMEM and cell numbers were monitored by direct counting using a haemocytometer. Viable cells were counted as those excluding Trypan blue. Synchronisation of cells into early S-phase using a double exposure to thymidine has been previously described [15]. The degree of cell synchrony was assessed at various times following release of thymidine either by flow cytometry or by incorporation of [^{3}H]thymidine (33 mCi/mmol Amersham International Buck., UK) into DNA as described earlier [15]. Flow cytometry was carried out in cells stained with propidium iodide using an Orthocyte bench top cytometer equipped with a mercury/xenon arc lamp. DNA histograms were stored and files reanalysed using the MULTICYCLE DNA analysis package [15].

Expression constructs containing the human tissue transglutaminase cDNA were prepared as previously described [16]. Mutation of the active site cysteine (Cys 277) to serine was undertaken by site directed mutagenesis using the Clontech Kit 'Transformer'. Site-Directed Mutagenesis Kit (Clontech Palo Alto, CA) based upon the Deng and Nickoloff method [17] Cys → Ser 277 tTgase mutant was identified and confirmed by automatic DNA sequencing of the full length cDNA. A initial transient transfections of the wild type pSG5-tTgase and the Cys → Ser-mutant pSG5-tTgase (pSG5-tTgase-M) were carried out in COS-1 cells using the DEAE-dextran method and both transglutaminase activity and antigen levels were determined following procedures previously described [14].

Stable transfection of Met B cells with the expression constructs and

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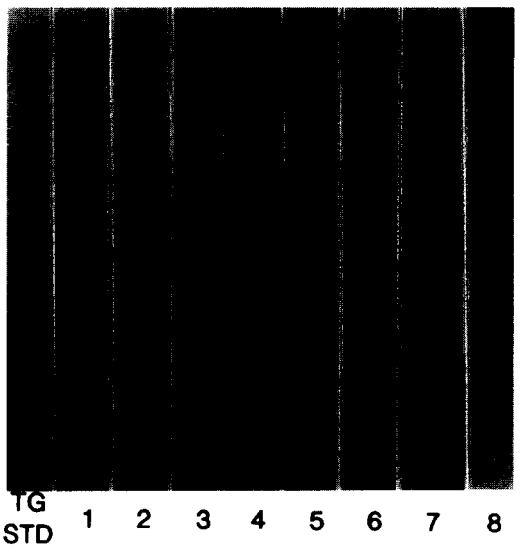

Fig. 1. Expression of tissue transglutaminase in transfected clones detected by Western blotting TG STD; purified guinea pig liver transglutaminase, Lane 1, I36; Lane 2, Clone I24; Lane 3, Clone 36; Lane 4, Clone 1; Lanes 5–8, pSVneo controls 1–4.

the pSVneo selection plasmid was undertaken using Lipofectin as described by Johnson et al. [14]. Stably transfected cells were selected and cloned in normal growth media containing 800 U per ml G418 sulphate (Geneticin, Gibco Laboratories). For monitoring the expression of tissue transglutaminase, activity was measured in cloned cell lines by the [^{14}C]putrescine incorporation into *N,N*-dimethylcasein assay [18]. Total cellular RNA was extracted using RN azol B (Cinnex Ltd). Initial screening of clones carrying the mutant cDNA was undertaken by dot blots whereby isolated RNA was hybridised to a 1.1 kb Bal 1 deletion probe constructed from a full length cDNA of the human tissue transglutaminase as previously described [14]. Enzyme antigen was measured by immunoprobing Western blots with a goat anti-guinea pig liver transglutaminase and revealing by chemiluminescence (Amersham Ltd.) Guinea pig liver enzyme (Sigma Chemical Co., Poole, Dorset, UK) or human colon carcinoma cells (HT 29) were used as sources of tTgase standard.

3. Results and discussion

3.1. Selection of transfected clones

The eukaryotic expression vector pSG5 containing a 3.3 kb cDNA (wild type pSG5 tTgase and the Cys→Ser mutant, pSG5 tTgase M) of the human endothelial cell tissue transglutaminase [19] was used in these studies. Mutation of the active site cysteine (Cys 277) to serine of the human tTgase resulted in the expression of a catalytically inactive enzyme as determined by enzymatic assays and immunoblotting analysis of transient transfection experiments carried out using the DEAE-dextran method in COS-1 cells (data not shown). Wild type pSG5-tTgase and pSG5-tTgase-M (The Cys → Ser mutant) were then used to express the enzyme in the highly malignant hamster fibrosarcoma cell line Met B. For stable clones carrying the wild type cDNA the previously reported Clones 1 and 36 were used in this study [14]. These clones carry significantly higher levels of tTgase activity and antigen (Table 1, Fig. 1) when compared to the Met B controls transfected with only the selection vector pSVneo. Stable clones of Met B cells carrying the pSG5 tTgase M and the selection vector pSVneo were obtained as described in Section 2. All stable clones including control clones carrying pSVneo alone were selected by their ability to tolerate growth on media containing G418.

Assessment of tTgase antigen in homogenates of cells transfected with pSG5 tTgase M allowed the selection of 2 clones, I24 and I36, with increased levels of tTgase antigen when compared to transfected controls (Fig. 1). These two clones contained comparable levels of tTgase antigen, when compared by immunoblotting to that found in wild type Clones 1 and 36 (Fig. 1). However unlike Clones 1 and 36 the levels of tTgase activity in Clones I24 and I36 was comparable to that found in the pSVneo transfected controls (Table 1), which is in keeping with the expression of the mutated cDNA in these cells. In Clone I24 a higher molecular weight form of the enzyme antigen (approx. 120 kDa) was also detectable when Western blots were immunoprobed with a goat anti-guinea pig liver antibody. Although it is difficult at this point to suggest reasons for expression of this higher molecular weight antigen it is interesting to note that an inactive tTgase protein of comparable size was found in the original Met B cell line when cell extracts were fractionated by anion exchange chromatography [20].

3.2. Cell cycle progression studies

Previous studies with the Met B fibrosarcoma have shown that these cells can be efficiently synchronised into early S-phase after a double exposure to thymidine such that 75–80% of the cell population is in early S-phase [15]. Extension of this procedure to the transfected clones indicated similar results could be obtained with each of the stable transfects when the cells were analysed by flow cytometry and the DNA histograms compared (Fig. 2). Following release of the thymidine drug cells progressed through S-phase into G2/M such that for control cell lines carrying pSVneo total loss of synchrony was apparent after 8 h. In contrast a comparison of the DNA histograms from cells transfected with the tTgase cDNA (both mutant and wild type) indicated that even after 8 h a large percentage of cells were still progressing from S-phase into G2/M. A comparison of cell cycle distribution for all the transfected clones at 8 h after release of drug (Table 2) indicated comparability for each of the tTgase transfected clones (wild type and mutant cDNA), while the transfected control cell lines were more comparable to the distribution shown for the wild type Met B cell line. To confirm the data shown by flow cytometry two of the transfected clones Clone 1 and pSVneo1 were further moni-

Table 1
Tissue transglutaminase activity in Met B and stably transfected clones

	Cell line		Transglutaminase activity (U/10^6 cells)
	Met B		0.14 ± 0.16
Transfected controls	Met B pSVneo	1	0.18 ± 0.13
		2	0.67 ± 0.2
		3	0.78 ± 0.39
		4	1.72 ± 0.3
Transfected with wild type cDNA	Clone	1	28.7 ± 2.7
	Clone	36	20.0 ± 1.0
Transfected with mutant cDNA	Clone I	24	1.04 ± 0.29
	Clone I	36	1.42 ± 0.34

Tissue transglutaminase activity was measured according to the procedures described in section 2.

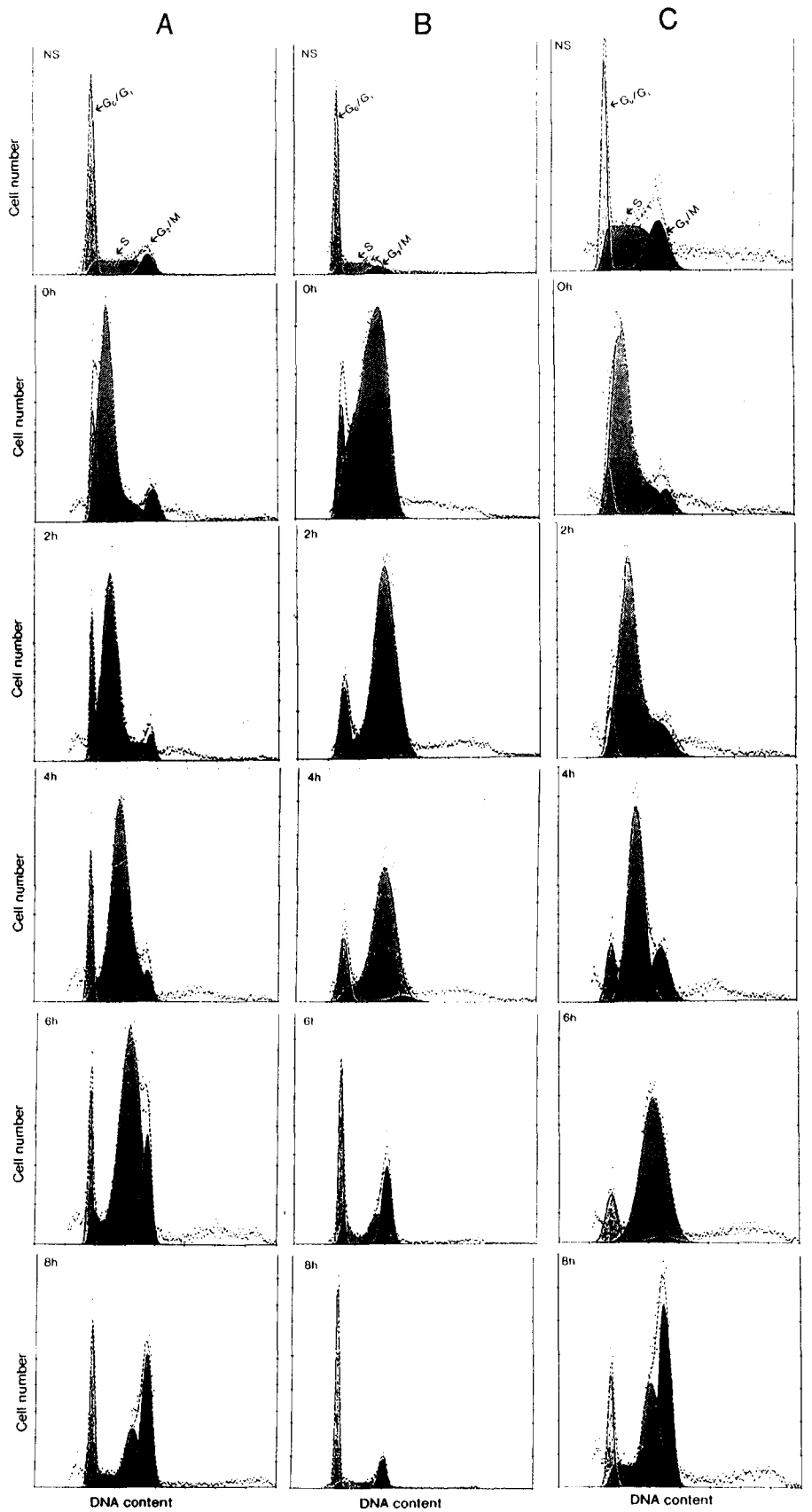

Fig. 2. DNA histograms from thymidine-synchronised transfected Met B cells (A), Clone 1; (B), pSVneo1; (C), Clone I24. At the time indicated in each figure, cells were released from thymidine blockade and analysed by flow cytometry as described in section 2. NS, non-synchronised cell population.

Table 2
Cell cycle distribution 8 h after release of drug for Met B, Met B cells transfected with pSVneo, the wild type tTgase cDNA and the mutant cDNA

		Percentage distribution of cells at 8 h after synchronisation into early S-phase				
		Cell line	G_1	S	G_2/M	S + G_2/M
tTgase-transfected clones	Mutant	Clone I24	13.3	43.7	43	86.7
		Clone I36	24.6	24.7	50.6	75.3
	Wild type	Clone 1	23.1	51.2	25.7	76.9
		Clone 36	23.9	35.9	40.2	76.1
	Controls	pSVneo 1	62.7	18.7	18.6	37.3
		pSVneo 2	44.8	17.3	37.9	55.2
		pSVneo 3	43.8	19.4	36.9	56.3
		pSVneo 4	44.9	18.4	36.7	55.1
		Met B	56.7	23.2	20.1	43.3

The distribution of cells in each phase of the cell cycle was calculated from DNA histograms using the MULTICYCLE DNA analysis package.

tored by following DNA synthesis using [^{3}H]thymidine incorporation into DNA (Fig. 3A,B). Following synchronisation and removal of the drug pSVneo1 cells move through the cell cycle such that DNA synthesis reaches a maximum at 3–4 h decreasing at 6 h with the number of viable cells nearly doubling at 8 h. In contrast for Clone 1 DNA synthesis is not completed at 6 h after release of drug and has still not fully declined after 8 h such that the number of viable cells is only 50% greater than that found at 0 h.

Taken together the results clearly indicate that increased expression of the tTgase protein in this malignant hamster fibrosarcoma can affect its progression through the cell cycle from S-phase to G2/M. Since clones transfected with either the wild type or mutant cDNA show this characteristic it may be concluded that it is the GTP binding activity of the tTgase and not its ability to cause post-translational modification of proteins which is the cause of the effect. Our results therefore confirm the potential importance of this multifunctional protein in cell growth and proliferation. It has been stated that any negative regulator of the cell cycle is a potential target for inactivation during cancer development [21]. Our findings in keeping with this observation since reduced levels of tTgase protein are generally observed in tumours [6,8,9]. This effect of tTgase on the cell cycle may also in part explain the reduced incidence of primary tumour growth observed in this hamster fibrosarcoma when stably transfected with the wild type tTgase cDNA [14].

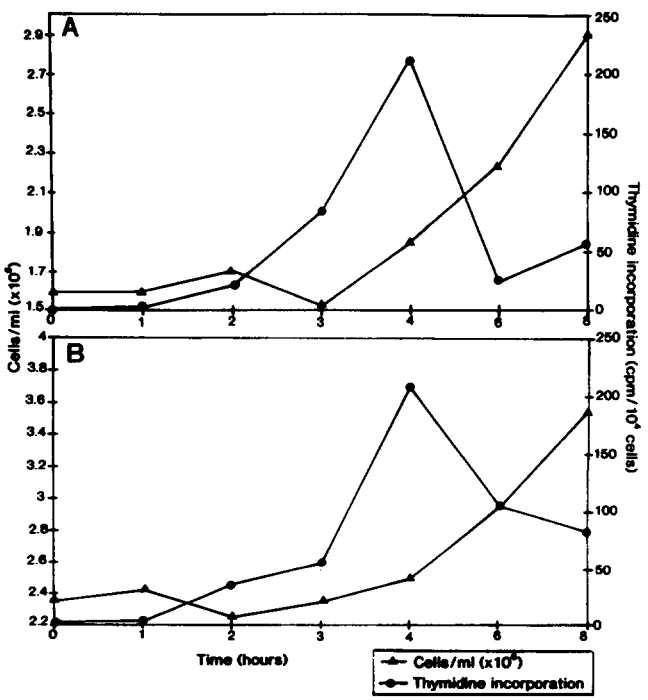

Fig. 3. DNA synthesis and cell replication in thymidine synchronised pSVneo1 cells (A) and (B) Clone 36. Cells were synchronised with a double exposure to 2 mM thymidine as described in section 2. (●) DNA synthesis was assessed by following the incorporation of [^{3}H]thymidine and cell numbers (▲) determined by microscopic counting.

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