Sodium butyrate inhibits the synthesis of the transformation related protein p 53 in 3T6 mouse fibroblasts

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Sodium butyrate, which blocks the cell cycle of many cell types in the G_1 phase, strongly inhibits the synthesis of the transformation related, 53 kDa protein in 3T6 fibroblasts but much less so in SV 40 transformed mouse cells. By several criteria, this effect of the fatty acid appears to be indirect; p 53 synthesis takes place several hours after the butyrate-sensitive step in G_1. The results are discussed in the light of a putative role of p 53 in growth control.

Cell cycle *G_1/S transition* *3T6 cell* *SV 40 transformed cell* *Transformation related protein* *Monoclonal antibody*

1. INTRODUCTION

Sodium butyrate interferes with the cell cycle progression in many cell types. It generally causes an arrest in the G_1 phase of the cell cycle and in some cell systems a concomitant induction of terminal differentiation [1–7]. 3T6 mouse fibroblasts are blocked by butyrate at a control point in G_1 several hours prior to the beginning of the S phase, and serum factors are required before and after the butyrate-sensitive step if cells are to progress into the S phase [8]. Low concentrations of cycloheximide have a similar effect on cell cycle progression and, interestingly, 3T6 or 3T3 fibroblasts are considerably more sensitive to these substances than are transformed cells [8–10]. These data were interpreted to indicate the requirement in mid G_1 of the synthesis of (a) labile, rapidly turning-over protein(s) which may become more stable (or more extensively expressed) in transformed cells [9,10]. One interesting candidate for such a protein is p 53, which was first detected in SV 40 transformed cells, where it was shown to form a tight complex with SV 40 large T antigen [11–13]. It is assumed that the formation of this complex leads to stabilisation of p 53 in the cell cycle of SV 40 transformed cells. Here we show that the synthesis of p 53 by 3T6 cells is strongly inhibited if sodium butyrate is added to the culture medium; in contrast, synthesis of p 53, like cell cycle progression, in SV 40 transformed mouse kidney cells, is remarkable resistant to the fatty acid.

2. MATERIALS AND METHODS

3T6 cells and SV 40 transformed mouse kidney cells (SVMK) were grown as in [7,8]. Sodium butyrate treatment was carried out for 24 h at a final drug concentration of 7 mM. For immunofluorescence, cells were washed twice with phosphate-buffered saline (PBS), fixed with cold acetone–methanol (7:3) and dried. Fixed cells were incubated with anti p 53 monoclonal antibody [14] (PAb 122 from ATCC) followed by fluorescein-labelled goat anti-mouse immunoglobulin, examined in a Zeiss fluorescence microscope and photographed.

Labelling of cells with [^{35}S]methionine was carried out by incubation for 3 h at 37°C with the radioactive amino acid (20–30 μCi/ml methionine-

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free medium containing 2% calf serum; 3 ml/10 cm dish). Cells were then washed twice with PBS, scraped into 0.5 ml/dish of lysis buffer (600 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% NP40, 5 mM EDTA, 0.2 mM/1 PMSF) and fully disrupted by 3 min sonication in a sonicator bath. After removing the debris by 10 min centrifugation in an Eppendorf centrifuge, the extracts were incubated for 30 min at 0°C with 10 μl of p 53 antibody (PAb 122) which was followed by 30 μl of a 1:1 suspension of protein A-Sepharose. Incubation at 4°C was continued overnight with gentle shaking. Protein A-Sepharose was then collected by centrifugation and washed 3 times with 500 μl each of 0.1 M Tris-HCl (pH 8.8), 1% 2-mercaptoethanol, 1% NP40, 0.5 M LiCl. The immunoprecipitates were finally eluted and dissociated with 500 μl of sample buffer for SDS gel electrophoresis [15] by heating for 10 min at 100°C. Protein A-Sepharose was removed by centrifugation and the supernatants were applied onto 12.5% SDS-polyacrylamide gels with 5% stacking gels. After electrophoresis the gels were fluorographed [16] and the autoradiogram scanned with a Helena Quick Scan.

3. RESULTS AND DISCUSSION

Using a monoclonal antibody (PAb 233) against mouse p 53 [14], the amount (determined by immunofluorescence) and rate of synthesis (determined by labelling with [^{35}S]methionine) of p 53 in 3T6 cells are strongly inhibited by sodium butyrate (fig.1,2). In SV 40 transformed mouse cells, in contrast, p 53 is only slightly effected by the substance (fig.1) in agreement with the much higher resistance to butyrate of the cell cycle progression of SV 40 transformed cells [8]. Following release of cells from the butyrate-induced G_1 block, 3T6 cells resume the synthesis of p 53 after a lag period of about 6 h (fig.2); cells enter the S phase a few hours later [8]. These results suggest that p 53 is synthesized in mid and late G_1 only a

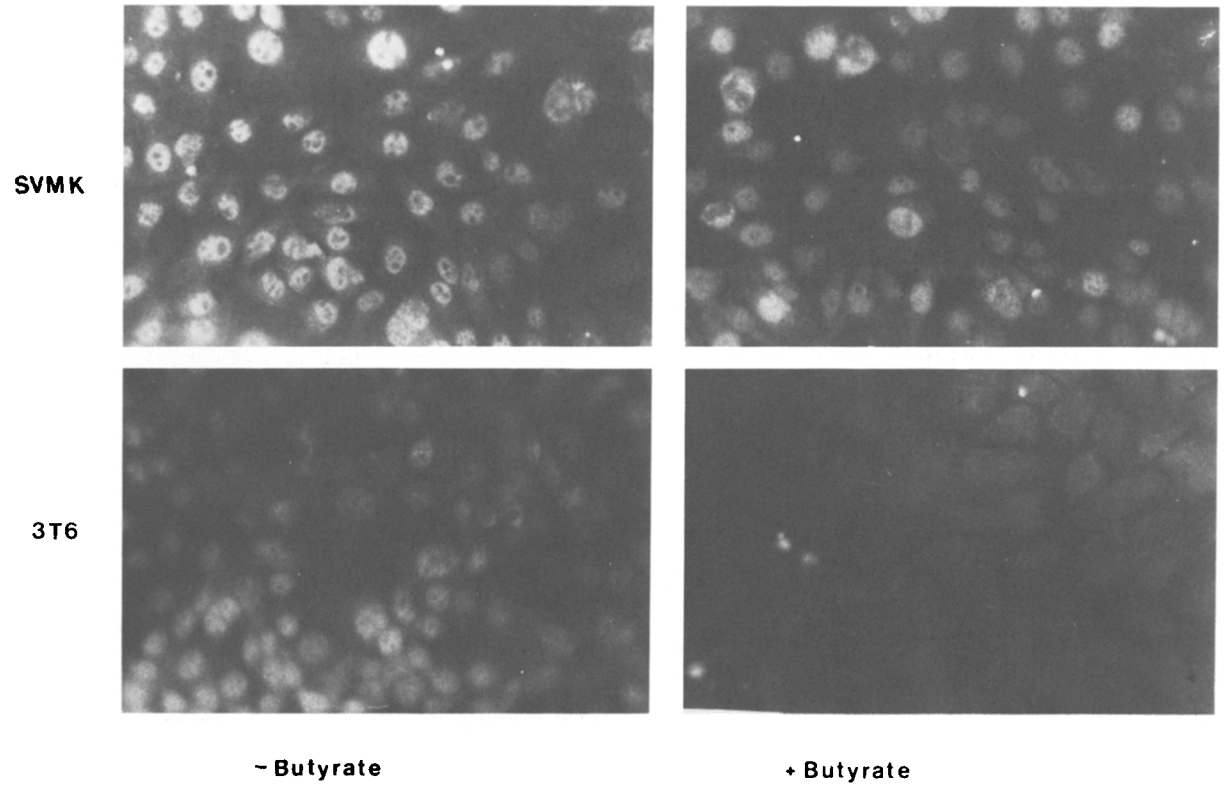

Fig.1. Immunofluorescence of p 53 in SV40 transformed cells (SVMK) and in 3T6 cells in the absence of butyrate or after 24 h treatment with 7 mM sodium butyrate.

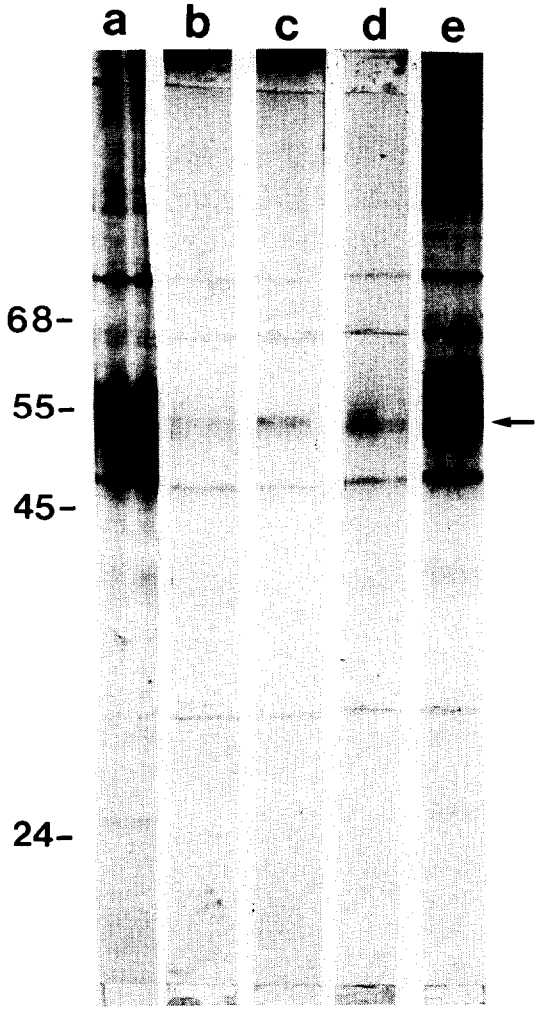

Fig.2. Immunoprecipitation and electrophoresis of ^{35}S-labelled p 53. Labelling was for 3 h in each case. (a) Normally grown 3T6 cells, (b) cells treated for 24 h and labelled in the presence of butyrate, (c) cells labelled from 0 to 3 h after release from the butyrate-induced block, (d) cells labelled from 3 to 6 h after butyrate removal, (e) cells labelled from 6 to 9 h after butyrate removal. The arrow indicates the position of p 53.

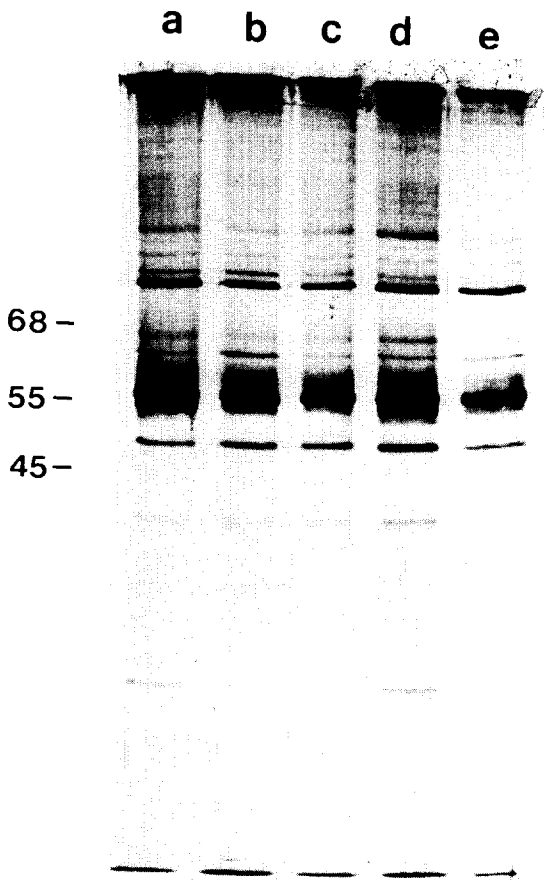

Fig.3. Immunoprecipitation and electrophoresis of ^{35}S-labelled p 53. (a) Normally grown 3T6 cells labelled for 3 h in the presence of 7 mM sodium butyrate (added at the same time as [^{35}S]methionine), (b) cells incubated in the presence of butyrate for 3 h followed by 3 h labelling, (c) cells incubated in the presence of butyrate for 6 h followed by 3 h labelling, (d) normally grown cells labelled for 3 h in the absence of butyrate, (e) same as (d) but followed by a 6 h chase with fresh, methionine-containing medium. For quantitation, gels were scanned and the peak areas of p 53 determined.

few hours before the S phase starts. Sodium butyrate may directly or indirectly interfere with p 53 synthesis. The lag of several hours after butyrate removal which passes before p 53 synthesis begins argues against direct interference of the fatty acid with p 53 expression. This is supported by the fact that 3 h pulse labelling carried out in the presence or absence of the fatty acid results in identical incorporation of [^{35}S]methionine into p 53 (fig.3, cf. lanes a and d). If the labelling is carried out from 3–6 h or from 6–9 h after addition of butyrate, radioactivity in p 53 is reduced to 77 and 50%, respectively (fig.3b,c). This result again indicates that the synthesis of p 53 starts a few hours after the butyrate-sensitive step in the G_1 phase of the cell cycle. It is

more likely therefore that sodium butyrate inhibits the production of other proteins in the cell cycle which then control the synthesis of p 53. This conclusion is in line with the idea of the existence of a series of consecutive events which have to be passed by cells progressing towards the S phase [17]. The role of p 53 in this series of events is not yet known but our results support the idea [18,19] that the protein is involved in cell cycle regulation.

Sodium butyrate is one of several substances known to cause terminal differentiation in murine erythroleukemia cells, a process which is accompanied by a cessation of cell growth. Interestingly, while this paper was in preparation two groups [20,21] reported that other inducers of differentiation, namely hexamethylenebisacetamide and dimethyl sulfoxide cause a decrease in p 53 synthesis in erythroleukemia cells concomitant with a commitment of these cells to terminal cell division. These results also implicate a role of p 53 in cell proliferation. Much evidence supporting this hypothesis came from studies which showed that a variety of transformed cells, including tumor cell lines induced by chemicals or DNA- and RNA-viruses, have elevated levels of this protein [11,22–25]. The half-life of p 53 varies dramatically in normal *vs* transformed cells: 20–60 min in 3T3 cells but longer than 20 h in SV 40 transformed 3T3 cells [26]. In pulse-chase experiments we found a half-life of about 6 h for p 53 in our 3T6 cell line (fig.3d,e); this is considerably longer than that of the protein in 3T3 cells implying that 3T6 cells in this aspect behave more like transformed ones which is in agreement with other properties of this cell line [9,10,27]. In confirmation of reports from other groups we also found that p 53 can hardly be detected in 3T3 cells by immunofluorescence (not shown) while it is readily visible in 3T6 cells and much better in SV 40 transformed mouse cells (fig.1). On the other hand, the growth rates of 3T3, 3T6 or SVMK cells are not nearly as variable as the concentration of immunologically reactive p 53 and studies in fact indicate that not all rapidly growing tumor cells exhibit high levels of the protein [28]. This discrepancy remains to be resolved before the role of p 53 in growth control can be understood.

Using serum stimulated 3T3 cells, authors in [19] showed that the microinjection of anti p 53 monoclonal antibody into these cells partly inhibited their transition into the S phase and furthermore that this inhibition was only effective if the antibody was injected up to 2 h after serum addition. Accordingly, p 53 was considered by these authors to be an early G_1 protein. Our experiments, on the contrary, suggest that the protein is synthesized in the second half of G_1. An explanation for this difference is not available as yet, however more direct information on the cell cycle events in which p 53 is involved could perhaps be obtained by injecting the isolated protein into serum-starved or butyrate-blocked cells and by determining whether such cells thereby gain the ability to enter the S phase. Such experiments are in progress.

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