Studies of the decrease of tyrosine-*O*-sulphated proteins in Rous sarcoma-virus-transformed rat embryo fibroblasts, line 3Y1

Examination of the sulphate activation and tyrosyl-protein sulphotransferase systems

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The sulphate activation and tyrosyl-protein sulphotransferase systems in normal 3Y1 rat embryo fibroblasts and the same cells transformed by Schmidt Ruppin subgroup-A-Rous sarcoma virus (SRA-3Y1) were examined. Employing metabolic [³⁵S]sulphate-labelling followed by PEI (polyethyleneimine)-cellulose thin-layer chromatography of the labelled cell lysates, it was found that the steady-state level of 'active' sulphate, adenosine 3'-phosphate 5'-phosphosulphate, was drastically lower in SRA-3Y1 cells compared with their normal counterparts. When the sulphate activating enzymes were tested, it appeared that the activities in 3Y1 homogenates were 2–2.5 times greater than those in SRA-3Y1 homogenates. An endogenous sulphation assay for tyrosyl-protein sulphotransferase revealed that activities in 3Y1 and SRA-3Y1 homogenates were comparable. Nearly identical patterns were observed with both sets of cells when [³⁵S]sulphated proteins generated in the endogenous assay were separated by two-dimensional gel electrophoresis. It therefore seems that the tyrosyl-protein sulphotransferase(s) are unimpaired in SRA-3Y1 cells. While the lower (approx. 8 times) sulphate uptake remains the major cause for the decrease of tyrosine-*O*-sulphated proteins in SRA-3Y1 cells [Liu & Lipmann, (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3695–3698], the 2–2.5-fold lower sulphate activating enzyme activities also contribute to some extent to the difference between the SRA-3Y1 and 3Y1 cells.

INTRODUCTION

Protein-tyrosine sulphation was first discovered in bovine fibrinogen in 1954 [1]. Free tyrosine-*O*-sulphate (TyrS), which is considered the primary degradation product of tyrosine-*O*-sulphated proteins, was subsequently reported to be present in human and other mammalian urines [2,3]. Fibrinogen alone, however, could not account fully for the amount of free TyrS excreted in the urine. It was therefore proposed that other proteins which are subjected to this type of covalent modification should exist. Nevertheless, no other tyrosine-*O*-sulphated proteins were identified in the 27 years following the initial discovery with the exception of a few small, biologically-active peptides [4–8]. By employing the [³⁵S]sulphate radioactive-labelling technique along with protein hydrolysis under alkaline conditions, it was shown in 1982 that modification by tyrosine sulphation indeed was widespread in proteins of diverse molecular masses [9]. Examples of the growing list of tyrosine-*O*-sulphated proteins identified include complement C4 [10], fibronectin [11], a hybridoma immunoglobulin G [12], α -fetoprotein [13], *Drosophila* yolk protein [14], secretogranins [15], collagen V [16] and a membrane protein of A 431 cells [17].

In our previous work on the comparison between normal and avian sarcoma virus-transformed rat embryo

fibroblasts, we detected a remarkable decrease of tyrosine-*O*-sulphated proteins in the transformed cells as compared to normal [18]. Following that finding, we were interested in investigating the reasons for the decrease. Since tyrosine sulphation is a post-translational modification and the sulphation requires 'active' sulphate, adenosine 3'-phosphate 5'-phosphosulphate (PAPS), as the sulphate donor [19], the steps involved in determining the level of tyrosine-*O*-sulphated proteins include sulphate transport through the cell membrane, activation of sulphate, the sulphotransferase-catalysed protein-tyrosine sulphation and biosynthesis of the proteins to be sulphated. In our previous study [18], we had detected a drastic lowering (approx. 8 times) in sulphate uptake by SRA-3Y1 cells as compared with the normal 3Y1 cells. We now report on the examination of the sulphate activation and tyrosyl-protein sulphotransferase systems in these two cell lines.

EXPERIMENTAL

Materials

ATP, adenosine 5'-phosphosulphate (APS), and PAPS were obtained from Sigma. Protein molecular-mass standards were purchased from Bethesda Research Laboratories. Pre-coated PEI (polyethyleneimine)-cel-

Abbreviations used: PAPS, adenosine 3'-phosphate 5'-phosphosulphate; APS, adenosine 5'-phosphosulphate; TyrS, tyrosine-*O*-sulphate; SRA-3Y1, Schmidt Ruppin subgroup-A-Rous-sarcoma-virus-transformed line 3Y1 rat embryo fibroblasts.

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lucose (CEL-300 PEI/u.v.²⁵⁴) t.l.c. plates were from Brinkmann. Carrier-free [³⁵S]sulphuric acid was purchased from New England Nuclear and PAP[³⁵S]S (306 Ci/mmol) was a generous gift from the same company. Tyrosine-*O*-sulphate standard was synthesized according to the method of Jevons [20]. Pronase was from Calbiochem-Behring. All other reagents were of the highest grade commercially available.

Cell culture

Rat embryo fibroblasts, lines 3Y1 and SRA-3Y1, were obtained from Dr. H. Hanafusa (The Rockefeller University). Cells were grown at 37 °C in Dulbecco's modified Eagle's medium containing 10% calf serum (Flow Laboratories).

Preparation of cell homogenates

Ten 100 mm dishes of 3Y1 or SRA-3Y1 cells were used for the preparation of cell homogenates. Confluent cells in each dish were rinsed twice with 10 ml portions of phosphate-buffered saline (0.137 M-NaCl/6.46 mM-sodium phosphate buffer, pH 7.4) and scraped off with a rubber policeman in the presence of 5 ml of phosphate-buffered saline. Cell suspensions from the 10 dishes were pooled together and centrifuged at 1000 *g* for 5 min. The supernatant was discarded. The cell pellet was resuspended in 5 ml of 50 mM-Hepes (pH 8.0) containing 5 mM-EDTA and transferred to a 15 ml Dounce tissue grinder. Cells were broken following 10 strokes using the tight plunger. The preparation was centrifuged for 5 min at 1000 *g* and the supernatant separated was designated as the 'crude homogenate'. Half of the crude homogenate was subjected to further centrifugation at 100 000 *g* for 60 min. The resulting supernatant containing the 'soluble' fraction and the pellet, resuspended and homogenized in the original volume of 50 mM-Hepes (pH 8.0) plus 5 mM-EDTA, was termed the 'particulate' fraction.

Analysis of steady-state level of PAPS in 3Y1 and SRA-3Y1 cells

For the analysis of the level of PAPS *in vivo*, radioactive-labelling using [³⁵S]sulphate was performed. Five 25 mm dishes of nearly confluent 3Y1 (or SRA-3Y1) cells were first rinsed three times with sulphate-free Dulbecco's modified Eagle's medium and incubated at 37 °C in 1.5 ml of Dulbecco's modified Eagle's medium containing 10% of the normal concentration of MgSO₄ plus [³⁵S]sulphate (0.1 mCi/ml; 1 Ci = 37 GBq). The five dishes of cells were radiolabelled for 1.5, 3, 6, 12 and 24 h, respectively. At the end of each labelling, the labelling medium was removed, followed by three rinses of the cells with phosphate-buffered saline to remove the residual free ³⁵S-labelled compounds. Cells in each dish were lysed in 0.25 ml of 0.1 M-NaOH, followed by neutralization with 0.25 ml of 0.1 M-HCl. The neutralized cell lysates were thereafter analysed for their PAP[³⁵S]S content using PEI-cellulose t.l.c. followed by autoradiography.

Standard assay for sulphate activation system

The assay method developed by Robbins [21] was used for the analysis of the sulphate activating enzymes of 3Y1 and SRA-3Y1 cells. The final volume (after the addition of either crude homogenate, soluble, or particulate fractions) of the reaction mixture was 50 μ l which contained, as final concentrations, 50 mM-Hepes

buffer (pH 7.5), 10 mM-K₂SO₄, 20 mM-MgCl₂, 10 mM neutralized ATP and [³⁵S]sulphate (0.83 mCi/ml). The reaction was carried out at 37 °C and was started by the addition of either crude homogenate, soluble, or particulate fractions (5 μ l). A time-course study was performed with the reaction proceeding for 1, 3, 10, 30 and 60 min, respectively. At the end of each reaction, the reaction mixture was heated at 100 °C for 1 min, followed by centrifugation in an Eppendorf microcentrifuge to remove the precipitate formed. Samples of the clear supernatants (5 μ l) thus obtained were spotted along with 1 μ g each of non-radioactive APS and PAPS on PEI-cellulose thin-layer plates impregnated with fluorescent dye. The plates were subjected to ascending chromatography using the solvent system described below. Upon the completion of the chromatography, the plates were air-dried and placed in a u.v. cabinet. Darkened spots observed under u.v. illumination were located. From comparison with the co-chromatographed standards, the positions of APS and PAPS were confirmed. The area corresponding to each of the spots was excised and placed in a 20 ml scintillation vial; 1 ml of 2 M-Tris/HCl (pH 8.9) was added to each vial, followed by constant shaking for 15 min to elute the AP[³⁵S]S or PAP[³⁵S]S. Finally, 10 ml of scintillation fluid (Hydrofluor; National Diagnostics, Somerville, NJ, U.S.A.) was added, the vial sealed and placed in a Packard model 3255 liquid-scintillation counter for the ³⁵S radioactivity determinations.

Endogenous assay for tyrosyl-protein sulphotransferase system

The method employed for the study of the tyrosyl-protein sulphotransferase system in 3Y1 and SRA-3Y1 cells was essentially that developed by Lee & Huttner [22]. The assay utilized PAP[³⁵S]S as the sulphate donor for the tyrosine sulphation of endogenous protein substrates present in the Golgi complex in the cell homogenate [23]. The reaction mixture (final volume 75 μ l) contained, as final concentrations, 40 mM-Hepes buffer (pH 7.4), 5 mM-2-mercaptoethanol, 10 mM-MgCl₂, 4 mM-MnCl₂, 5 mM-EDTA and PAP[³⁵S]S (0.3 μ Ci/75 μ l). The assay was started by the addition of 37.5 μ l of the sample (either crude homogenate, soluble, or particulate fractions) and was allowed to proceed at 37 °C for 1 h. At the end of the reaction, 75 μ l of 2 $\times$ Laemmli's SDS sample buffer was added and the mixture was heated for 1 min at 100 °C. Proteins solubilized in Laemmli's sample buffer were separated by SDS/polyacrylamide-gel electrophoresis [24] and on completion, the gel was stained with Coomassie Brilliant Blue, destained by a 25% methanol/7.5% acetic acid solution, and dried under vacuum. The tyrosine-*O*-[³⁵S]sulphate content of the [³⁵S]sulphated proteins fixed in the dried gel were analysed by a procedure previously developed which involves extensive Pronase hydrolysis, followed by a two-dimensional thin-layer separation of the resulting pronase hydrolysate [18].

Other analytical methods

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Laemmli [24] in 4.5%–16% polyacrylamide gels. The procedure of high-resolution two-dimensional gel electrophoresis was that of O'Farrell [25]. Protein determination was based on the method of Bradford [26]. PEI-cellulose t.l.c. was employed

for the separation of AP[³⁵S]S, PAP[³⁵S]S and free [³⁵S]sulphate. The developing solvent system used contained 0.75 M-Trizma base, 0.45 M-HCl and 0.5 M-LiCl [27].

RESULTS

Steady state level of PAPS in 3Y1 and SRA-3Y1 cells

The PAPS levels *in vivo* in the 3Y1 and SRA-3Y1 cells were examined using the [³⁵S]sulphate radioactive-

Fig. 1. Steady-state level of PAPS in 3Y1 and SRA-3Y1 cells

The Figure shows the autoradiograph of cell lysates of (a) 3Y1 and (b) SRA-3Y1 cells labelled for different lengths of time with [³⁵S]sulphate following PEI-cellulose t.l.c. Positions of PAPS, APS and the free sulphate are indicated by the arrows. Necessary dilutions were made so that the samples of cell lysates applied to the PEI-cellulose plate contained equal amounts (3 µg) of proteins.

labelling technique described in the Experimental section. Fig. 1 shows the autoradiograph of cell lysates, prepared from cells radio-labelled for different lengths of time, following PEI-cellulose t.l.c. PAP[³⁵S]S was found to be the only [³⁵S]sulphurylated adenylate present. AP[³⁵S]S which is an intermediate during the sulphate activation reaction, was probably present at a level too low to be detected in samples prepared throughout the time-course study. In the case of 3Y1 cells, judging from the intensity of the radioactivity of the PAPS spots, the steady-state level of PAP[³⁵S]S was reached soon after the labelling started, while for SRA-3Y1 cells, the time needed to reach the steady-state level of PAP[³⁵S]S was longer than 6 h. It is clear from Fig. 1 that the steady-state level of PAP[³⁵S]S in 3Y1 cells was considerably higher than that in SRA-3Y1 cells.

Assay of sulphate activating enzymes in 3Y1 and SRA-3Y1 cell homogenates

The crude homogenate and its two sub-fractions, soluble and particulate (see the Experimental section for their preparation), of either 3Y1 or SRA-3Y1 cells were tested for sulphate activation activity. Consistent with the results reported by others [28,29], the activity was found to reside in the soluble fraction. In this analysis (*in vitro*), AP[³⁵S]S appeared clearly as an intermediate during the formation of PAP[³⁵S]S. This is in contrast to the situation *in vivo* where little AP[³⁵S]S was detected (Fig. 1). Fig. 2 shows the comparison of the activities of sulphate activating enzymes in 3Y1 and SRA-3Y1 crude homogenates (see the Experimental section for method of determination). Amounts of AP[³⁵S]S and PAP[³⁵S]S formed during the time-course study were individually plotted. Throughout the 60 min period monitored, the level of AP[³⁵S]S present in 3Y1 samples was 2–2.5 times that present in SRA-3Y1 samples (Fig. 2a). The level of PAP[³⁵S]S measured concomitantly, for both 3Y1 and

Fig. 2. Comparison of sulphate activation activities between 3Y1 (●) and SRA-3Y1 (▲) crude homogenates

The amounts of AP[³⁵S]S formed during the 60 min time-course of sulphate activation assay are plotted in (a); while the amount of PAP[³⁵S]S formed is plotted in (b). Determination of AP[³⁵S]S and PAP[³⁵S]S was based on the amount of ³⁵S radioactivity present on the two sulphurylated adenylate spots resolved by PEI-cellulose t.l.c. shown in Fig. 1. Measurements were made at 1, 3, 15, 30 and 60 min, respectively. The radioactivity values correspond to those expected from 1 mg of protein in the 3Y1 or SRA-3Y1 crude homogenates used in the assay.

Fig. 3. Autoradiograph of [^{35}S]sulphated proteins generated during the endogenous sulphation assay following SDS/polyacrylamide-gel electrophoresis

Crude homogenate (C), soluble (S) and particulate (P) fractions prepared from 3Y1 cells (the three lanes on the left) and SRA-3Y1 cells (the three lanes on the right) were tested as described in the Experimental section.

Table 1. Comparison of tyrosyl-protein sulphotransferase activities in crude homogenate, soluble and particulate fractions prepared from 3Y1 and SRA-3Y1 cells

The values shown in the Table are radioactivities of Tyr[^{35}S]S released from 1 mg of protein (c.p.m./mg of protein; see the Experimental section for the analysis of Tyr[^{35}S]S) present in the crude homogenate, soluble or particulate fractions tested in the endogenous sulphation assays.

Fraction	Radioactivity of Tyr[^{35}S]S (c.p.m./mg of protein)	
	3Y1	SRA-3Y1
Crude homogenate	268	488
Soluble	50	70
Particulate	1242	1530

SRA-3Y1 cell homogenates, was found to be considerably lower than that of AP[^{35}S]S (Fig. 2b). In the case of 3Y1 homogenates, the amount of PAP[^{35}S]S continued to increase with time during the assay. For SRA-3Y1 samples, however, the increase in PAP[^{35}S]S proceeded only briefly, and after approx. 10 min the amount of PAP[^{35}S]S started levelling off and even showed a slight decrease upon prolonged incubation.

Fig. 4. Autoradiograph of [^{35}S]sulphated proteins generated during the endogenous sulphation assay following high-resolution two-dimensional gel electrophoresis

Crude homogenates prepared from (a) 3Y1 and (b) SRA-3Y1 cells were used in the assay. In the first dimension, isoelectric focusing (IEF) was performed, followed by SDS/polyacrylamide-gel electrophoresis in the second dimension.

This tendency was consistently observed in eight determinations using four different batches of SRA-3Y1 cells. It should be mentioned that the endogenous substrates for sulphation may be present in the crude homogenates which were used as the source of sulphate activating enzymes. The PAP[^{35}S]S formed during the assay could in part be drained away through such sulphation reactions. Also, some of the PAP[^{35}S]S formed could have been degraded by certain enzymes with nucleotide pyrophosphatase activities [30,31]. This latter possibility seems worth pursuing particularly in the case of SRA-3Y1 samples which showed this characteristic decrease of PAP[^{35}S]S upon prolonged incubation (Fig. 2b).

Assay of tyrosyl-protein sulphotransferase activity and analysis of endogenous protein substrates for tyrosine sulphation in 3Y1 and SRA-3Y1 cell homogenates

Tyrosyl-protein sulphotransferase was previously shown to be a Golgi enzyme [32]. This and the fact that there are endogenous protein substrates present in the Golgi complex make it possible to perform an endogenous assay (see the Experimental section for details).

Fig. 3 shows an autoradiograph of [^{35}S]sulphated proteins generated in the endogenous assay following SDS/polyacrylamide-gel electrophoresis. In contrast with sulphate activation reactions, the majority of sulphation reactions took place in the particulate fraction in which the Golgi complex is present. [^{35}S]Sulphated proteins fixed in the dried electrophoretic gel were eluted and analysed for Tyr[^{35}S]S [18]. In all fractions, Tyr[^{35}S]S accounted for more than 65% of the total ^{35}S radioactivity, the rest probably being carbohydrate-bound [^{35}S]sulphate. Results compiled in Table 1 show that the particulate fractions contained an overwhelmingly higher amount of protein-bound Tyr[^{35}S]S than the soluble fractions. Interestingly, SRA-3Y1 samples exhibited a higher content of Tyr[^{35}S]S than their 3Y1 counterparts. This phenomenon could be due to the under-sulphation of the endogenous protein substrates in SRA-3Y1 cells which results from the lower sulphate uptake [18] and the lower sulphate activation activities in these cells as shown in the Experimental section. The electrophoretic patterns of [^{35}S]sulphated proteins in the homogenates of the two cell types were surprisingly similar as shown in Fig. 3. Indeed, nearly identical patterns of [^{35}S]sulphated proteins were observed on the autoradiograph when two-dimensional gel electrophoresis was performed (Fig. 4). These results seem to suggest that both the activity of tyrosyl-protein sulphotransferase and the biosynthesis of protein substrates for sulphation were mostly unimpaired in the transformed SRA-3Y1 cells as compared to the normal 3Y1 cells.

DISCUSSION

The present study is an attempt to follow through our previous finding of a decrease in tyrosine-*O*-sulphated proteins in avian sarcoma virus-transformed rat embryo fibroblasts [18]. Here, we examined, as part of an effort to unravel the mechanisms involved in that decrease, the sulphate activation and tyrosyl-protein sulphotransferase systems in 3Y1 and SRA-3Y1 rat embryo fibroblasts.

Previous studies [31,32,33] had revealed changes in the cellular level of 'active' sulphate (PAPS) during developmental processes or in certain diseases. For example, during embryonic development, PAPS had been shown to be increasingly available in corneal cells [32], whereas in cultured skin fibroblasts from a patient with Lowe's syndrome, the level of PAPS was found to be lower than normal [31]. In our metabolic [^{35}S]sulphate-labelling experiments, we detected a dramatic difference in the steady-state level of PAPS between 3Y1 and SRA-3Y1 cells (Fig. 1). In view of the lower (approx. 8 times) sulphate-uptake capability of SRA-3Y1 cells as compared with the 3Y1 cells [18], the finding was not unexpected. Analyses *in vitro* further indicated that the sulphate-activation activities of 3Y1 homogenates were considerably higher than those of SRA-3Y1 homogenates. An approx. two-and-a-half fold difference was detected in the generation of APS and even bigger differences were found for PAPS formation during the 60 min time course. In the sulphate activation assays *in vitro*, APS appeared clearly as an intermediate, being present at a concentration approx. three times that of the final product, PAPS. However, *in vivo*, APS seemed to be present at a very low steady-state level and only

PAP [^{35}S]S was discernible in the metabolic [^{35}S]sulphate-labelling experiments (Fig. 1). The difference between the results *in vivo* and *in vitro* could be due to the spatial arrangements of the two sulphate activating enzymes which are disrupted upon breakage of the cells, even though previous reports [28,29] as well as our present study indicate that the two enzymes are located in the cytosolic fraction. Conrad & Woo [32] had also reported that APS appeared as an intermediate during their sulphate activation assays *in vitro*. In their case, however, the level of APS during the reaction was low compared to the final product, PAPS. It is not clear whether the higher level of APS found in our study is due to the difference in assay conditions or to other reasons. In the case of SRA-3Y1 homogenates, the prolonged incubation of the reaction mixture resulted in a drop in the amount of PAPS. Although it could be proposed that some of the PAPS formed was drained away through the sulphation of certain endogenous substrates present in the SRA-3Y1 homogenates, the 3Y1 homogenates, under identical experimental conditions, did not show the same tendency. A feasible possibility is that, in SRA-3Y1 samples, the PAPS formed was subjected to a greater degree of degradation by certain enzymes with nucleotide pyrophosphatase activities [30,33]. Such enzymes are currently under investigation in the two kinds of cells.

Tyrosyl protein sulphotransferase had been shown to be located in the Golgi complex [23]. Our data indicated that the majority of the enzymic activity for protein-tyrosine sulphation was present in the particulate fraction (Table 1). Furthermore, it was shown that 3Y1 and SRA-3Y1 homogenates had comparable tyrosyl-protein sulphotransferase activities. The higher value in SRA-3Y1 than in 3Y1 samples could be due to the fact that, in 3Y1 cells, the endogenous proteins were already partially sulphated, while in SRA-3Y1 cells, the sulphation level of the endogenous proteins was considerably lower due to the decreased sulphate uptake [18] and sulphate activation activities (present study). The cell homogenates prepared from SRA-3Y1 cells, therefore, contain a higher concentration of sites available for sulphation as compared with the samples prepared from 3Y1 cells. Both one-dimensional (Fig. 3) and two-dimensional (Fig. 4) gel-electrophoretic patterns of [^{35}S]sulphated proteins generated in the endogenous assays were very similar for 3Y1 and SRA-3Y1 homogenates. These findings further suggested that the biosynthesis of proteins to be sulphated was not significantly affected in SRA-3Y1 cells. With a sufficient supply of 'active' sulphate, PAPS, these proteins could be sulphated to the same extent as in 3Y1 cells.

In summary, in the comparison of the sulphate activating enzymes in 3Y1 and SRA-3Y1 homogenates, we found a lowering (2–2.5 times) of their activities in the latter. This lowering should contribute, in addition to the decrease in sulphate uptake [18], to the dramatic reduction of tyrosine-*O*-sulphated proteins in the transformed SRA-3Y1 cells. Tyrosyl protein sulphotransferase and the biosynthesis of protein substrates to be sulphated, on the other hand, appeared largely unimpaired.

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