Decrease of tyrosine-O-sulfate-containing proteins found in rat fibroblasts infected with Rous sarcoma virus or Fujinami sarcoma virus

(protein sulfation/sulfate uptake/change upon retrovirus infection)

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ABSTRACT Our interest was aroused by the recent report by Huttnert [Huttnert, W. B. (1982) Nature (London) 299, 273–276] on general sulfation of tyrosine residues of proteins in normal and malignant transformed tissues. Here we report on the reduction of sulfation in embryonic rat fibroblasts, line 3Y1, infected with Rous sarcoma virus or Fujinami sarcoma virus. In view of the instability of tyrosine O-sulfate in strong acid, the protein sulfation was tested for after incubation with [35S]sulfate and exhaustive Pronase hydrolysis. We found in general a reduction of sulfation in transformed tissue. It was greatest in the fibroblasts permanently transformed with Rous sarcoma virus. When fibroblasts transformed by the temperature-sensitive Fujinami sarcoma virus, line ts225-3Y1, were used for comparison of sulfation at nonpermissive and permissive temperatures, the latter showed a strong reduction. Furthermore, we tested these cells for the uptake of inorganic [35S]sulfate. Uptake appeared highly reduced in the permanently infected fibroblasts, but ts225-3Y1 grown at permissive and nonpermissive temperatures exhibited no difference. Uptake at both temperatures was comparable to uptake by normal 3Y1 cells. A recently much investigated cell surface protein, fibronectin, was reported to be lost on malignant transformation and to contain sulfate in an undetermined location. We found that ts225-3Y1 cells grown at permissive temperature released fibronectin that contained tyrosine O-sulfate.

The recent study of sulfation of tyrosine in proteins, in both normal and tumor cells, reported by Huttnert (1) showed this to be much more common than expected from previous studies.

In the late 1950s, when testing a number of biosynthetic systems for their dependence on ATP, Lipmann and coworkers analyzed enzymatic sulfate activation and transfer to various acceptors, among them phenol (2). Robbins and Lipmann (3) found in eukaryotic cells that activation of sulfate required two ATPs (which can be abbreviated PPPAdo):

\[ \text{SO}_4^2- + \text{PPPAdo} \rightarrow \text{O}_3\text{S}^-\text{O}^-\text{PAdo} + \text{PP} \]

\[ \text{O}_3\text{S}^-\text{O}^-\text{PAdo} + \text{PPPAdo} \rightarrow \text{O}_3\text{S}^-\text{O}^-\text{PAdoP} + \text{PPAdo} \]

The product, 3'-phosphoadenosine 5'-phosphosulfate, was called PAPS, an acronym that is generally used for the "active" sulfate. (The IUPAC-IUB has recommended PAdoPS.) It has become commercially available from Sigma and in 35S form from New England Nuclear.

In a recent publication by Lee and Hubtnter (4) on the extracts of a rat pheochromocytoma cell line found to contain protein-bound sulfate, they showed that PAPS could be used for in vitro sulfation of these proteins. This implies that the extracts produce PAPS by the described eukaryote enzyme system.

We decided to study tyrosine sulfation in relation to the transformation of rat fibroblasts by avian sarcoma viruses. With these viruses, the transcript of the transforming gene, the src gene, had been discovered by Erikson et al. (5) to be a protein kinase. Hunter and Sefton (6) observed it to be tyrosine specific and to cause increased tyrosine phosphorylation.

It will appear from the results reported here that sometimes rather drastic reduction in tyrosine-O-sulfated proteins parallels infection with sarcoma virus, in contrast with the increase of tyrosine O-phosphate in a number of malignantly transformed cells.

EXPERIMENTAL PROCEDURES

Materials. Protein molecular weight standards were obtained from Bethesda Research Laboratories. Precoated TLC cellulose plates were purchased from Brinkmann. Carrier-free [35S]sulfuric acid was from New England Nuclear and Pronase was from Calbiochem–Behring.

Cell Cultures. Rat embryo fibroblast line 3Y1, this cell line permanently infected with Schmidt–Ruppin A Rous sarcoma virus (SRA-3Y1), and the same line infected by a temperature-sensitive mutant of Fujinami sarcoma virus (ts225-3Y1) were all obtained from H. Hanafusa (Rockefeller University). The Fujinami sarcoma virus is similar to the Rous sarcoma virus. All cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum (Flow Laboratories). For the cells infected with the temperature-sensitive mutant in the temperature-shift experiments the permissive temperature was 34.5°C and the nonpermissive temperature was 40.5°C.

Radioactive Labeling. Subconfluent cells grown in 35-mm dishes were labeled with [35S]sulfate (0.3 mCi/ml; 1 Ci = 37 GBq) in Dulbecco’s modified Eagle’s medium without unlabeled sulfate. Labeling was for 20 hr. The medium was then removed and the cells were washed and solubilized in Laemmli sample buffer (7) for further workup. Matching nonlabeled dishes were similarly prepared for the determination of protein content.

Determination of Sulfate Uptake. Cells were grown in 100-mm plastic dishes in Dulbecco’s modified Eagle’s medium containing 10% calf serum. When the cells reached confluence, the dishes were drained and rinsed with three 10-ml

Abbreviations: Tyr(SO3), tyrosine O-sulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

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portions of Hanks' balanced salt solution. After rinsing, 4 ml of Hanks' balanced salt solution containing 4 µCi of [35S]sulfate was added to each dish. Dishes of cells were then incubated at their respective growth temperatures for 15 min. The radioactive solution was removed and dishes were rinsed with three 10-ml portions of Hanks' solution; the cells were solubilized by 5 ml of 0.1 M NaOH. Aliquots of the solubilized cell samples were used for determination of radioactivity and protein content.

**Temperature-Shift Experiment.** Temperature shifts from 40.5°C (nonpermissive temperature) to 34.5°C (permissive temperature) and from 34.5°C to 40.5°C were performed with ts225-3Y1 cells. Dishes of subconfluent cells were labeled for 10 hr with [35S]sulfate (0.3 mCi/ml) in sulfate-free Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum and then shifted to the other temperature at various time intervals. Cells were harvested to 30 hr after temperature shifts. The cells were solubilized and analyzed for tyrosine O-sulfate ([Tyr(SO3)] after exhaustive Pronase hydrolysis as described below.

**Miscellaneous Methods.** Tyr(SO3) standard was prepared according to the procedure developed by Jevons (8). The modified biuret method of Zamenhof and Chargaff (9) was used for protein determination, with bovine serum albumin as the standard.

**Tyr(SO3) Analysis.** To determine the sulfate content of proteins we followed the example of Hutner (1) to determine the content of Tyr(SO3) by enzymatic hydrolysis at pH 7.8 with Pronase in view of the great instability of Tyr(SO3) in acid. We used exhaustive hydrolysis instead of autoclaving with barium hydroxide, which appeared to destroy some Tyr(SO3). Hutner mentioned that he had found Pronase hydrolysis alone also effective. The cell lysates were subjected to electrophoresis in a sodium dodecyl sulfate/5.5-16% polyacrylamide gradient gel (7). After electrophoresis, the gel was stained with Coomassie brilliant blue in 50% methanol/10% acetic acid (vol/vol) and destained with 25% methanol/7.5% acetic acid solution. The gel was dried under reduced pressure at room temperature. Each lane of the dried gel was cut from the top to the dye front at the positions of the molecular weight standards used: cytochrome c (Mw, 12,300), β-lactoglobulin (Mw, 18,400), α-chymotrypsinogen (Mw, 25,700), ovalbumin (Mw, 43,000), bovine serum albumin (Mw, 68,000), phosphorylase b (Mw, 92,500), and myosin H chain (Mw, 200,000). To improve digestibility the gel pieces were further sliced into small pieces and placed in a test tube. Two milliliters of 50 mM ammonium bicarbonate solution containing Pronase at 150 µg/ml was then added. The preparation was incubated at 37°C with shaking to allow proteins in the gel to be hydrolyzed and eluted. After 24 hr, 0.2 ml of Pronase at 1.5 mg/ml in 50 mM ammonium bicarbonate was added and digestion was continued for an additional 24 hr. The samples at intermediate times after addition of the second portion of Pronase solution showed 24 hr to be more than sufficient for complete hydrolysis.

For quantification, the resulting gel eluate was separated by centrifugation and 1.1 ml was lyophilized. To the residue, 50 µl of nonradioactive Tyr(SO3) standard solution (1 mg/ml) was added, and the residue was carefully redissolved. Ten microliters of this solution was spotted on a TLC cellulose plate (20 × 20 cm). The plate was then subjected to high-voltage electrophoresis (500 V, 90 min) in 5% (vol/vol) acetic acid/0.5% pyridine, pH 3.5 (10). After electrophoresis, the plate was air dried and subjected to the second dimensions to ascending chromatography in 1-butanol/formic acid/2-propanol/water (3:1:1:1, vol/vol) (11). Upon completion of the chromatography, the plate was dried and sprayed with ninhydrin solution (0.5% in acetone). The ninhydrin spot of the Tyr(SO3) was then scraped off for radioactivity determination.

**RESULTS**

Identification of Tyr(35SO3) Present in Proteins. Fig. 1 is an autoradiograph showing the separation of Tyr(35SO3) by ascending chromatography from the massive radioactive streak representing the nonprotein 35S radioactivity. As an example, the normal 3Y1 rat fibroblast protein giving the high peak in Fig. 2A was used for this analysis. The ninhydrin-stained spot was scraped off and its radioactivity was measured for quantitation of the protein-bound Tyr(SO3).

Comparison of Tyr(SO3) Content of Normal and Transformed Rat Embryo Fibroblasts. Proteins of various molecular weight ranges were analyzed for Tyr(35SO3) as described above.

As shown in Fig. 2A, permanently transformed cells, grown for several generations, exhibited a strongly decreased level of Tyr(SO3) throughout the whole molecular weight range as compared to their normal counterparts.

When cells infected by temperature-sensitive virus were incubated for several weeks at the permissive temperature of 34.5°C their proteins contained 1/2 to 1/3 the Tyr(SO3) compared to the untransformed cells grown at 40.5°C. Note that this comparison between normal and transformed cells is based on 1 mg of protein in the cell lysate used for sodium dodecyl sulfate gel electrophoresis.

In summary, in Fig. 2A, normal cells are compared with permanently transformed ones, which show an extremely low content of Tyr(SO3). In Fig. 2B, cells infected by a temperature-sensitive virus and grown at the permissive temperature for several weeks also show a lower content of Tyr(SO3) compared to the cells grown at the nonpermissive temperature, but the difference is less pronounced than in Fig. 2A.

**Uptake of Sulfate by the Normal and Transformed Cells.** Uptake of sulfate was measured and the results are given in Table 1. It appears that only in the permanently transformed cells was the sulfate uptake drastically reduced; in cells infected with the temperature-sensitive virus strain the sulfate uptake remained normal at the permissive temperature, although the Tyr(SO3) content (Fig. 2B) was lowered.

Change of Tyr(SO3) Content of ts225-3Y1 Cells After Temperature Shifts. In the experiments shown in Fig. 3, we used ts225-3Y1, the same line of rat fibroblasts infected with the...
temperature-sensitive mutant of Fujinami virus used in the
experiment shown in Fig. 2B. However, in Fig. 3A we follow
closely the transition from normal to transformed states and
in Fig. 3B the reversal from the transformed to normal.

Fig. 3A shows the results of shifting from 40.5°C (nonpermissive) to 34.5°C (permissive). Dishes of subconfluent cells were labeled with [35S]sulfate (0.3 mCi/ml) in Dulbecco’s modified Eagle’s medium containing 10% dialyzed calf serum and incubated at 40.5°C for 10 hr. Then, individual dishes were shifted at different time intervals, as indicated, to permissive temperature and harvested. Cell lysates were prepared and analyzed for Tyr(SO3). The number of hours indicated for each line represents the length of time cells have been incubated at the new temperature after the shift.

For the cells in Fig. 3A, shifting from nonpermissive to permissive temperature, the Tyr(SO3) content remained unchanged for 15 hr; only between 15 and 30 hr did it start to show a gradual reduction. Yet, within 15 hr the cell morphology had already converted to that of the malignant type. Glucose transport had also changed to abnormal at 15 hr.

In the reverse experiment (Fig. 3B), change from permissive temperature, Tyr(SO3) started at 28% of nonpermissive and stayed there for 6 hr; at 15 hr it had increased to 56% and morphology was becoming normal; at 30 hr it had changed to 74% of normal. Thus, the increase of Tyr(SO3) parallels morphological change.

This contrast of Fig. 3 A and B shows that the decrease of Tyr(SO3) may be slow compared to the conversion to other parameters of transformation.

**DISCUSSION**

In view of the great acid lability of Tyr(SO3), instead of isolating the sulfated protein we used, like Huttner (1), Pronase hydrolysis at pH 7.8 to isolate the Tyr(SO3). However, the Ba(OH)2-linked hydrolysis at high temperature used by Huttner was omitted because of losses found in Tyr(SO3). We replaced it with prolonged enzymatic hydrolysis, testing for completeness.

The Tyr(SO3) content in transformed cells as compared to their normal counterparts (Fig. 2) seems to be time-dependent with respect to the state of transformation. This suggestion is based on the finding that permanently transformed SRA-3Y1 cells exhibited the most drastic reduction in their Tyr(SO3) content (Fig. 2A), while the temperature sensitive virus-infected ts225-3Y1 cells grown at permissive temperature for several weeks retained a relatively higher level of Tyr(SO3) (Fig. 2B). Quite indicative were the measurements of sulfate uptake in Table 1, which showed its drastic reduction only with the permanently transformed SRA-3Y1 cells. In contrast, the ts225-3Y1 level of sulfate uptake was independent of whether growth was at permissive or nonpermissive temperature and was similar to that in normal 3Y1 shown in the first line of the table.

The results shown in Fig. 3A of ts225-3Y1 cells shifting from nonpermissive to permissive temperature support the proposition of a time lag for sulfated protein release. The change in Tyr(SO3) content was not yet apparent after 15 hr, when morphological change and increase of glucose transport had already developed, and appeared only after 30 hr. In Fig. 3B, with change from permissive to nonpermissive temperature, the increase in Tyr(SO3) is more gradual and parallels disappearance of malignant morphology.

This work indicates that decrease in Tyr(SO3) content in sarcoma tissue appears to be due to the increased secretion of Tyr(SO3)-containing compounds. Thus, our attention was drawn to the reported loss of fibronectin from the membrane coincident with malignant transformation (12–14). Fibronectin also had been shown to contain sulfate (15); the location, however, of the sulfate remained unknown. We have begun work on the nature of sulfate in fibronectin. Fibronectin has been isolated from the supernatant fraction of the transformed ts225-3Y1 rat fibroblasts (Fig. 2B) by gelatin-Sepharose chromatography and elution with urea. After Pronase proteolysis, Tyr(SO3) appeared to be present in sizable amounts.

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