Vol. 112, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1983 Pages 198-205

SYNTHESIS AND MATURATION OF CROSS-REACTIVE GLYCOPROTEIN IN FIBROBLASTS DEFICIENT IN ARYLSULFATASE A ACTIVITY

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Received February 24, 1983

SUMMARY The biosynthesis of arylsulfatase A was studied in cultured fibroblasts by pulse-chase labeling with [2-^{3}H]mannose; the enzyme was isolated by immunoprecipitation and denaturing polyacrylamide gel electrophoresis. In normal fibroblasts, and in fibroblasts from a patient with multiple sulfatase deficiency, the enzyme was synthesized as a glycoprotein of apparent molecular weight of 59,000; half of it was processed over a period of 4 days to M_r= 57,000. The precursor chain of M_r= 59,000 was secreted in the presence of 10 mM NH_4Cl. An immunoprecipitable glycoprotein of normal size was synthesized by fibroblasts from two unrelated patients with metachromatic leukodystrophy, but this material disappeared within twenty hours. In fibroblasts from an individual with pseudodeficiency of arylsulfatase A, the immunoprecipitable precursor glycoprotein was smaller (M_r= 56,000). The synthesis of cross-reactive proteins with altered properties supports the concept of allelic mutations as the genetic basis of metachromatic leukodystrophy and of arylsulfatase A pseudodeficiency.

Lysosomal enzymes may be studied as metabolites that are radiolabeled biosynthetically in intact cells, isolated and analyzed by gel electrophoresis. This approach has shown that lysosomal enzymes are made as precursors which undergo processing of protein and carbohydrate moieties (reviewed in 1). It has also been invaluable for focusing on the biochemical defect in several genetic disorders of lysosomal function, including I-cell disease (2-4), combined β-galactosidase/neuraminidase deficiency (5), GM_2 gangliosidosis (6) and multiple sulfatase deficiency (7).

We have used this approach for deficiency states of the lysosomal enzyme, arylsulfatase A, of which the normal synthesis and processing were recently

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0006-291X/83 $1.50

described (4). The best known deficiency state is metachromatic leukodystrophy, a fatal autosomal recessive disorder, in which the lack of arylsulfatase A activity results in accumulation of cerebroside sulfate, primarily in the brain (8). In multiple sulfatase deficiency, another serious disorder inherited in autosomal recessive manner, arylsulfatase A is but one of several sulfatases with decreased activity. Since the degree of deficiency can be altered with culture conditions (9) this disease has been attributed to a problem in regulation; a recent study has implicated both reduced synthesis and increased turnover of arylsulfatase A in the patients' fibroblasts (7). A less known but interesting condition is that of "pseudodeficiency," which has been reported among healthy relatives of patients with metachromatic leukodystrophy (reviewed in 10,11). Cell homogenates from such individuals have a marked deficiency of arylsulfatase A activity, although intact cells can metabolize exogenous cerebroside sulfate (12) On the basis of pedigree analysis and studies of catalytic activity (10) it has been proposed that the condition arises from the heterozygous combination of an allele for metachromatic leukodystrophy with a relatively common allele that encodes an arylsulfatase A of low activity *in vitro*.

MATERIALS AND METHODS

Cell culture and radioactive labeling - Normal and pseudodeficient human skin fibroblasts were derived from samples submitted for diagnosis; a family study of the pseudodeficient individual has been presented[1] (10). Other fibroblast cultures were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.J. (GM 197 and 905, infantile metachromatic leukodystrophy; GM 196, heterozygote for metachromatic leukodystrophy; GM 4681, multiple sulfatase deficiency). The cultures were maintained as previously described (2). For each experiment, the cells were grown in 75 cm^2 tissue culture flasks for at least 14 days before labeling with 0.5 mCi of [4,5-^{3}H]leucine, or 1 mCi of [2-^{3}H]mannose (Amersham). Labeling conditions were as previously described (2,3) except for the use of flasks instead of plates, an increase in medium volume to 8 ml and a decrease of glucose concentration to 25 µg/ml when labeling with [2-^{3}H]mannose. After labeling or chase, the medium was removed, the cells were washed twice with 0.9%

[1] The relevant pedigree is shown in Schaap et al, Fig. 2 (10); however, this pedigree had been incorrectly assigned to family 1. The correct assignment is family 2, the Israeli Arab family. The individual whose cells we have studied is the mother of the metachromatic leukodystrophy patient.

NaCl and extracted for 5 min, while still attached to the dish, with 1 ml of 1% Nonidet P-40 (Bethesda Research Laboratories) in 10 mM Tris-HCl, pH 7.4, containing 10 mg/ml bovine serum albumin (Calbiochem) (6). Protein was precipitated from medium with 80% saturated $(NH_4)_2SO_4$. Each cell extract and medium concentrate was treated with 10 µl of 3% protamine sulfate prior to immunoprecipitation.

Preparation of antiserum - Highly purified human liver arylsulfatase A, provided by Drs. J. Edmond and G. Lawrence, was used to immunize a goat, using twice the amount of protein specified previously (13). Following the fifth boost, a liter of plasma was obtained by plasmapheresis. Serum was prepared by clotting the fibrin for 20 hr at 4°C with 10 mM $CaCl_2$ adjusted to pH 7.4. The serum was clarified by filtration, dialyzed against 0.15 M NaCl containing 10 mM Na phosphate buffer pH 7.0, and stored at -20°C.

Immunoprecipitation, electrophoresis and fluorography - Arylsulfatase A was immunoprecipitated with the aid of protein A-bearing *Staphylococcus aureus* (Immunoprecipitin, 10% w/v suspension, Bethesda Research Laboratories). To remove fibronectin and other unidentified components that bind non-specifically to the bacteria, each sample of cell extract or medium concentrate equivalent to one flask, was preabsorbed with 5 µl of goat anti-human fibronectin (Cappel). After 30 min on ice, 150 µl of the *S. aureus* suspension was added and the mixture was kept on ice an additional 15 min. The precipitate was removed by centrifugation and the supernatant absorbed two more times, with 50 µl of the bacterial suspension each time. Immunoprecipitation of arylsulfatase A was then carried out by addition of 1 µl of antiserum, incubation for at least 16 h at 4°C, addition of 15 µl of *S. aureus* suspension for 30 min. Under these conditions, 0.3 ng of arylsulfatase A (calculated from the specific activity [14]) was precipitated. The immunoprecipitates were washed and then solubilized and reduced in two steps (15). The samples were subjected to polyacrylamide gel electrophoresis by the method of Laemmli (16) with minor modifications (2) and the radioactive bands were visualized by fluorography (17,18).

Other procedures - Arylsulfatase A activity was determined using p-nitrocatechol sulfate as substrate (19). Protein was measured by the procedure of Lowry *et al* (20). The procedure of Kudoh et al (21) was used for administration of cerebroside sulfate labeled with [^{14}C]stearic acid (supplied by Dr. D. Wenger) to cultured fibroblasts, extraction of labeled lipids and separation by thin layer chromatography; radioactive bands were visualized by fluorography.

RESULTS

Activity of arylsulfatase A in the fibroblast strains studied is shown in Table I. In cell homogenates, the activity of metachromatic leukodystrophy cells was 10% of normal, that of the pseudodeficient cells, 15%, and of multiple sulfatase deficiency cells, 47% of the normal control. That last value could be halved by lowering the pH (not shown). Incubations in the presence of 10 mM NH_4Cl showed that the cell cultures could secrete in 20 h an amount of arylsulfatase A equivalent to 1-2% of normal cell extract activity.

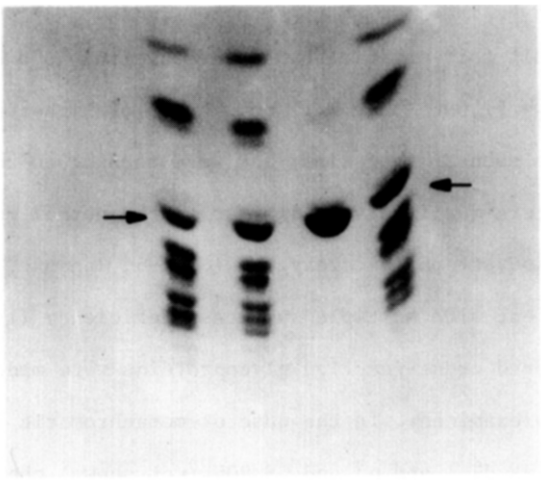
Despite the low arylsulfatase A in cell extracts, intact pseudodeficient cells could degrade exogenous cerebroside sulfate, in contrast to the cells from

TABLE I. ACTIVITY OF ARYLSULFATASE A IN CULTURES OF DIFFERENT GENOTYPE

Culture	*Activity in* Cell Homogenates	NH_3-induced secretions
Normal	592	10.5
Pseudodeficient	91	12.5
Metachromatic leukodystrophy GM 197	56	7.8
Metachromatic leukodystrophy heterozygote, GM 196	380	6.8
Multiple sulfatase deficiency GM 4681	280	4.6

Activity is expressed as nmol p-nitrocatechol sulfate hydrolyzed /h/mg cell protein. Values in the two columns are derived from separate experiments; medium containing 10 mM NH_4Cl was in contact with cells for 20 h before collection.

the metachromatic leukodystrophy patient (Fig. 1). This is in agreement with previous reports (12,21). Fibroblasts from the patient with multiple sulfatase deficiency likewise degraded cerebroside sulfate.



a b c d

Fig. 1. Utilization of exogenous cerebroside sulfate. The cells were incubated 4 days in the presence of radioactive cerebroside sulfate (60 nmol, 200,000 cpm) per flask. Lipids were separated by thin layer chromatography and visualized after fluorographic exposure for 6 days. a, normal control; b, pseudodeficient; c, metachromatic leukodystrophy; d, multiple sulfatase deficiency. Arrows mark unused cerebroside sulfate. Other bands are lipids into which [^{14}C]stearic acid, derived from metabolized cerebroside sulfate, had been incorporated.

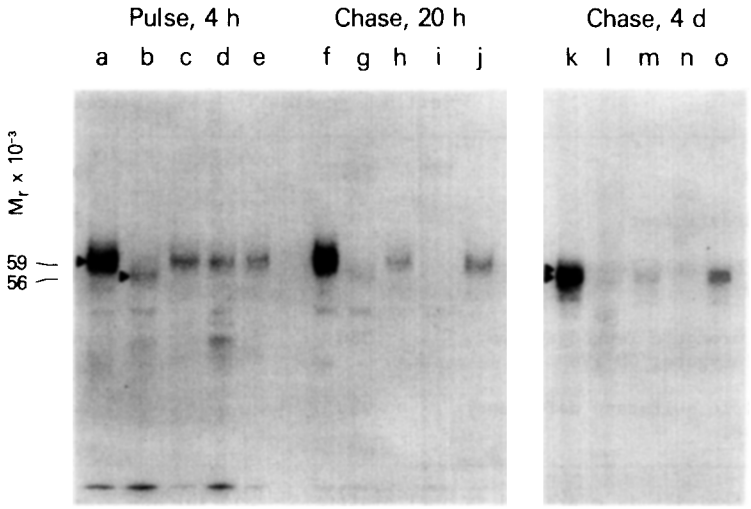


Fig. 2. Pulse-chase labeling of arylsulfatase A. Each lane represents one culture labeled with 1 mCi [^{3}H]mannose for 4 h (pulse, lanes a-e), at which time 40 mg of glucose was added to begin the chase for 20 h (f-j) or 4 days (k-o). For each time point, the samples are presented in the following order: normal, pseudodeficient, metachromatic leukodystrophy heterozygote, metachromatic leukodystrophy, multiple sulfatase deficiency. The X-ray film was exposed to the gel for 3 weeks for lanes a-j, a month for lanes k-o.

Normal fibroblasts labeled with [2-^{3}H]mannose for 4 h contained one immunoprecipitable glycoprotein of apparent M_r= 59,000 (Fig. 2, lane a). After a chase of 4 days half this material had been converted to a somewhat smaller glycoprotein of M_r= 57,000 (lane k). Some bands of higher electrophoretic mobility were also seen at that time, and were thought to represent degradation products. A pattern qualitatively similar to the normal was seen for cells of a metachromatic leukodystrophy heterozygote (GM 196, lanes c, h and m) and of GM 4081, a patient with multiple sulfatase deficiency (lanes e, j and o).

However, altered cross-reactive glycoproteins were made by the other fibroblast strains examined. In the case of metachromatic leukodystrophy, GM 197 (Fig. 2 lanes d, i and n) and GM 905 (not shown) the precursor was of normal size but disappeared within 20 h. The pseudodeficient fibroblasts (lanes b,g and l) synthesized a smaller precursor, of apparent M_r= 56,000; this material did not seem to be changed in size during the chase, although the amount appeared much reduced. Use of [^{3}H]leucine instead of [^{3}H]mannose yielded similar results but a greater number of spurious bands.

Incubation of fibroblasts in the presence of 10 mM NH_4Cl prevents the transfer of newly synthesized hydrolases into lysosomes and causes the enzymes to be secreted (2,22). The arylsulfatase A glycoproteins immunoprecipitated from ammonia-induced secretions had the same apparent molecular weight as the precursor extracted from the corresponding cells (Fig. 3).

DISCUSSION

Arylsulfatase A is a protein present in low abundance; assuming that the specific activity of the fibroblast enzyme is similar to that of arylsulfatase A purified from liver (14), we calculate that arylsulfatase A accounts for 1/6000 of the fibroblast protein. The enzyme has been reported to have an unusually long half-life of two months (7). These properties may account for the low level of labeling observed in our experiments. We do not know why the labeling was less than that reported in the extensive studies of Waheed et al (4,7).

It has been suggested that the pseudodeficient phenotype is the result of compound heterozygosity for two different mutant alleles at the aryl-

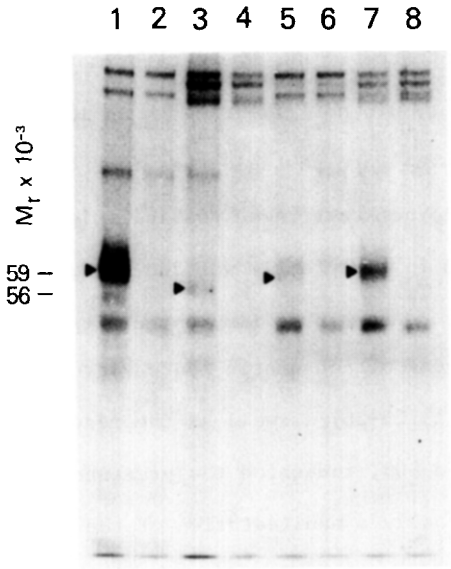


Fig. 3. Secretion of arylsulfatase A in the presence of NH_4Cl. Each pair of lanes represents the secretions of one flask, collected after 20 h in the presence of 10 mM NH_4Cl and 1 mCi [^{3}H]mannose. The usual procedure for immunoprecipitation was followed in the odd-numbered member of each pair, whereas excess antigen (10 µg of non-radioactive arylsulfatase A) was added to the even-numbered one. Only the bands displaced by excess antigen are identifiable as arylsulfatase A glycoprotein. Samples are in the following order: normal, pseudodeficient, metachromatic leukodystrophy, and multiple sulfatase deficiency.

sulfatase A locus (10,11). One of these would be the rare gene encoding an inactive arylsulfatase A (the metachromatic leukodystrophy gene). The second, (the pseudodeficiency gene) would encode an arylsulfatase A of low activity *in vitro*, but nevertheless able to degrade cerebroside sulfate in intact cells. In order to account for the frequent occurrence of low arylsulfatase A activity among healthy relatives of metachromatic leukodystrophy patients, the second allele would have to be relatively common in the general population. Hertz and Bach have suggested that a fifth of the population of Israel may be heterozygous for the pseudodeficiency gene (manuscript in preparation). Waheed et al (4) have suggested that the two arylsulfatase A polypeptides co-purified from placenta (major, M_r= 60,500; minor, M_r= 57,000) were products of allelic genes.

Thus the glycoprotein of apparent M_r= 56,000 synthesized by the pseudodeficient cells is a good candidate for the product of the postulated pseudodeficiency gene. The presence of this glycoprotein in ammonia-induced secretior rules out its being a lysosomal degradation product. However, to reconcile our results to the hypothesis of compound heterozygosity, we assume that in the pseudodeficient cells tested by us, the metachromatic leukodystrophy allele either was silent (i.e., did not give rise to an immunoprecipitable protein) or encoded a protein even more unstable than that of GM 197 and GM 905. Unfortunately, we could not test these possibilities because the metachromatic leukodystrophy proband in that family was deceased; we hope to identify similar families in the future. Absence of immunoprecipitable protein has been reported for cells of one metachromatic leukodystrophy patient (4), even though other reports (11,23-26) as well as the results reported here with cell strains GM 197 and 905, indicated the presence of cross-reactive material. This is probably a manifestation of the genetic heterogeneity that must be expected in any rare disorder.

While this manuscript was in preparation, we learned that Fluharty *et al* (27), using different methodology, had found arylsulfatase A reduced both in size and in amount in fibroblasts from four individuals with pseudodeficiency, including the one studied here.

ACKNOWLEDGEMENT

We thank Drs. Gary Lawrence and John Edmond of the Department of Biochemistry, University of California, Los Angeles, CA, for their gift of purified human liver arylsulfatase A, and Dr. David Wenger, Department of Pediatrics, University of Colorado, Denver, CO, for the gift of radioactive cerebroside sulfate.

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