Sorting and Secretion of Adrenocorticotropin in a Pituitary Tumor Cell Line after Perturbation of the Level of a Secretory Granule-specific Proteoglycan

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ABSTRACT A mouse anterior pituitary tumor cell line (AtT-20) that secretes adrenocorticotropin and β endorphin sorts the proteins it transports to the surface into two exocytotic pathways. AtT-20 cells also synthesize a secretory granule-specific sulfated molecule and secrete it on stimulation (Moore, H.-P., B. Gumbiner, and R. B. Kelly, 1983, *J. Cell Biol.*, 97:810–817). We show here that this molecule is sensitive to proteolysis and that the residual sulfated material co-migrates with a chondroitin sulfate standard on thin-layer electrophoresis. Furthermore, this sulfated molecule is completely sensitive to chondroitinase ABC digestion. Thus the secretory granule-specific sulfated molecule is a proteoglycan with chondroitin sulfate side chains. We examined the role of proteoglycans in the sorting and secretion of adrenocorticotropin in AtT-20 cells by severely decreasing the amount of this vesicle-specific proteoglycan in two ways. First, a xyloside was used to inhibit proteoglycan biosynthesis; second, a variant of the AtT-20 cell line was isolated that synthesized little of the sulfated proteoglycan. In neither case was the sorting or secretion of adrenocorticotropin detectably altered, suggesting that the proteoglycan is not required for these processes.

Although it has been more than ten years since proteoglycans were first proposed to play a role in packaging of secretory proteins into secretory granules (1), the hypothesis has still only indirect support. Sulfated proteoglycans and sulfated glycoproteins have been found in many secretory granules including zymogen granules (2), pituitary granules (3–5), chromaffin granules (6), and synaptic vesicles (7, 8). Proteoglycans and sulfated glycoproteins appear to interact with the contents of secretory granules in several cell types. Sulfated glycosaminoglycan chains can cause aggregation of zymogen granule contents in vitro (9), mast cell granule contents stay aggregated after exocytosis (10), and prolactin granule contents remain aggregated even after membrane removal (3, 4, 11). Such interaction between proteoglycans and vesicle contents could permit them to carry secretory proteins from the Golgi complex to the condensing vacuoles, or to condense the contents of newly formed secretory organelles.

The role of proteoglycans in sorting and packaging of secretory proteins into secretory granules can be tested by using a secretory cell line, AtT-20, derived from a tumor of the mouse pituitary (12). In AtT-20 cells there are apparently two pathways of secretion (13). A viral membrane protein encoded by an endogenous leukemia virus and the precursor of adrenocorticotropin (ACTH)[1] are externalized constitutively, that is, rapidly without storage or regulation. These cells also have a regulated pathway in which the ACTH precursor, proopiomelanocortin (POMC), is processed to mature ACTH, stored in secretory granules, and specifically released in response to secretagogues. The sorting mechanism appears to be universal for peptide hormones since, when the proinsulin gene is expressed in AtT-20 cells, the proinsulin product is also stored and processed in a manner comparable to POMC (14). The nature of the recognition signal on the prohormones responsible for sorting into the two pathways is unknown at present. It does not seem to involve asparagine-linked sugars (15).

We have tried to assess the importance of proteoglycans in the regulated secretory pathway of AtT-20 cells. We find that a major sulfated component of secretory granules is a chon-

[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin.

droitin-sulfate proteoglycan, which is sorted preferentially into the regulated pathway. To determine whether this proteoglycan might be involved in sorting or packaging of ACTH, we isolated a variant that made greatly reduced levels of that proteoglycan and used drugs that perturbed proteoglycan biosynthesis. In neither case was the sorting, storage, or release of ACTH significantly affected. The data presented here suggest that sulfated glycosaminoglycan side chains are not required for storage and release of ACTH.

MATERIALS AND METHODS

Materials: [^{35}S]Methionine and [^{35}S]sulfate were from Amersham Corp. (Arlington Heights, IL). 8-Br-cAMP, iodoacetamide, phenylmethylsulfonyl fluoride, poly-D-lysine, 4-methyl umbelliferyl-β-D-xyloside, chondroitinase ABC, and Type VI bacterial protease (Pronase E) were from Sigma Chemical Co. (St. Louis, MO). Reagents for SDS polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA). En^{3}Hance was from New England Nuclear (Boston, MA) and dialysis tubing was from Spectrum Medical (Los Angeles, CA). 0.45-μm pore membrane filters, type HA, were from Millipore Corp. (Bedford, MA) and thin-layer cellulose strips were from Gelman Sciences, Inc., (Ann Arbor, MI). Chondroitin sulfate (4- and 6-sulfates) and heparan sulfate were gifts from Dr. David Simpson (Laboratory of Biomedical and Environmental Sciences, University of California at Los Angeles). Antisera and other reagents for the ACTH radioimmunoassay and immunoprecipitation have been described (16).

Cell Culture and Metabolic Labeling with [^{35}S]Methionine and [^{35}S]Sulfate: AtT-20/D-16V cells were cultured as described (16), except that cells were grown on poly-D-lysine–treated tissue culture dishes to minimize cell loss. [^{35}S]Methionine pulse labeling was carried out after a 20-min starvation in methionine-free medium. Cells were labeled at 37°C with 150 μCi [^{35}S]methionine/ml for 20 min. Two consecutive 1-h chases were carried out in medium without serum. Secretion was stimulated by the addition of 5 mM 8-Br-cAMP to the chase medium. Media and cell extracts were routinely treated with 0.3 mg/ml phenylmethylsulfonyl fluoride and iodoacetamide. [^{35}S]Sulfate labeling was carried out in a similar fashion, except that the labeling period and amount of label varied with each experiment. Details are given in the figure legends. 4-Methyl umbelliferyl-β-D-xyloside was used at 0.5–1.0 mM concentrations to inhibit proteoglycan synthesis. Cells were pretreated at least 30 min before radiolabeling and throughout each experiment.

SDS Gel Electrophoresis and Fluorography: 10–18% exponential gradient SDS polyacrylamide gels were performed according to the method of Laemmli (17). Gels were impregnated with En^{3}Hance, dried, and exposed to Kodak XAR5 film at −80°C. Fluorograms were scanned with an LKB soft laser scanning densitomer (LKB Instruments, Inc., Gaithersburg, MD) for quantitation.

Gel Purification of Radiolabeled Material: After electrophoresis, SDS polyacrylamide gels were stained with Coomassie Brilliant Blue, and the molecular weight standards were used as markers to cut desired regions from the wet gel. Proteins were eluted exactly as described by Hager and Burgess (18). Acetone precipitates were dried and resuspended in the desired buffer.

Enzymatic Digestions: Proteolysis was carried out on gel purified, radiolabeled samples by incubation with Pronase E at 3 mg/ml, 37°C for 18 h in 50 mM ethanolamine, pH 7.2.

Chondroitinase digestions were carried out on radiolabeled medium samples after dialysis and resuspension in 50 mM Tris, pH 7.5, 100 mM sodium acetate, and 0.2 mg/ml BSA. 0.05 U of chondroitinase ABC was added and the reaction was incubated at 37°C for 1.25 h.

Thin-layer Electrophoresis: Protease-digested samples in 0.2 M calcium acetate were spotted onto mylar-supported cellulose electrophoresis strips. Electrophoresis was carried out in 0.2 M calcium acetate at 5 mA for 1.5 h. Standard glycosaminoglycans were visualized by staining with 0.1% Alcian blue 8GN (Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH) in 10% acetic acid.

Screening for Stimulated Sulfate Release Variants of AtT-20 Cells: AtT-20 cells were plated at low density so that individual colonies could be picked and cloned. After expanding the cloned cultures, 2-cm^2 wells were seeded and grown to confluence. The cells were then starved and labeled with [^{35}S]sulfate at 20–50 μCi/well for 3 h. After 1 h of chase, fresh medium was added to duplicate wells, with or without the addition of 8-Br-cAMP. After an additional hour of chase this medium was collected and filtered onto a Millipore membrane filter. After extensive washing with 100 mM HEPES, pH 7.4, and 100 mM potassium sulfate, the filter was dried and exposed to x-ray film. The intensity of spots from stimulated and unstimulated samples were compared to identify clones that did not exhibit enhanced release of sulfated material in the presence of the secretagogue 8-Br-cAMP.

RESULTS

AtT-20 Cells Synthesize, Store, and Secrete a Sulfated Proteoglycan

It has been suggested that the secretory granules of AtT-20 cells, like those of many exocrine and endocrine cells, contain a proteoglycan (16). The suggestion came from the observation that purified secretory granules contained a sulfated macromolecule with a characteristic mobility on SDS PAGE very similar to that of the glycosaminoglycan oligosaccharides described by Hampson and Gallagher (19). Storage of the sulfated material in the cell paralleled that of mature ACTH and it was coordinately secreted with mature ACTH when the cells were stimulated to secrete by the addition of 8-Br-cAMP to the medium. We have used enzymatic and physical methods to show that the sulfated material has indeed the properties of a proteoglycan.

The unusual electrophoretic mobility of the granule-specific sulfated macromolecule is shown in Fig. 1. Cells were labeled with [^{35}S]sulfate for 3.5 h and chased for 1.5 h. These conditions allow most of the sulfated material to be secreted from the cell, except that stored in secretory granules (16). The

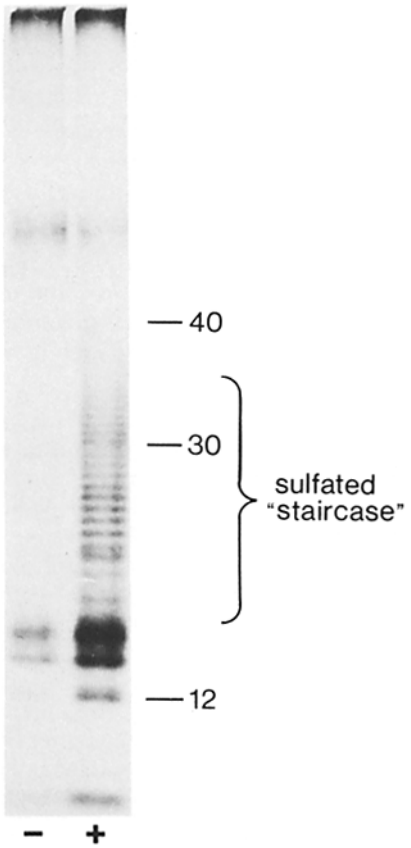

FIGURE 1 AtT-20 cells secrete a sulfated "staircase" upon stimulation with cAMP. Cells were labeled for 3.5 h with [^{35}S]sulfate at 250 μCi/ml, followed by a 1.5-h chase. Chase medium with (+) or without (−) 5 mM 8-Br-cAMP was collected from an equal number of cells after an additional 1.5 h chase and concentrated by trichloroacetic acid precipitation. Samples were run on a 10–18% gradient SDS polyacrylamide gel. The fluorogram was exposed for 2 wk.

sulfated material released from the cells in the next 1.5 h, either in the absence of stimulation or stimulating with 8-Br-cAMP, was collected, concentrated and analyzed by SDS PAGE. It is clear that secretagogue stimulation enhances the release of sulfated material, including bands in an unusual "staircase" arrangement with M_r in the range of 25,000–35,000.

To determine whether this "staircase" material contained protein, we eluted it from the gel and digested it extensively with protease. When protease-digested material was compared with the undigested material by SDS PAGE, the patterns shown in Fig. 2 were obtained. Exposure to protease did not destroy the sulfate-labeled material, but increased its electrophoretic mobility. Note that the characteristic pattern of bands is preserved in the protease-digested material. This result shows that the characteristic set of sulfate-labeled bands contain a protein component and also that the heterogeneity is not due to the protein core.

The banded structure disappears, however, after treatment with chondroitinase. Sulfated material released into the medium during stimulation with 8-Br-AMP was treated with chondroitinase ABC (or AC) and analyzed by SDS PAGE (Fig. 3 *a*). Whereas other sulfated components were unaffected by chondroitinase digestion, the characteristic staircase pattern disappeared. As further evidence that the sulfate label was associated with glycosaminoglycans of the chondroitin sulfate type, secreted material was treated with protease as in Fig. 2, and analyzed by thin-layer electrophoresis (Fig. 3 *b*). After protease digestion a single spot was obtained that migrated close to a chondroitin sulfate standard.

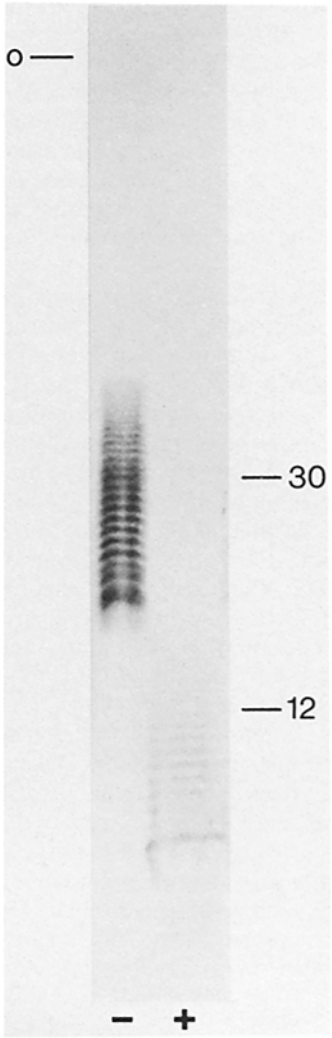

FIGURE 2 Pronase digestion of the sulfated staircase increases its electrophoretic mobility but does not change the banded pattern. The sulfated staircase from a [^{35}S]sulfate-labeled medium sample was gel-purified and digested with protease (see Materials and Methods). Both the digested (+) and undigested (−) samples were lyophilized, resuspended in SDS sample buffer, and rerun side by side on a second 10–18% SDS polyacrylamide gel. The fluorogram was exposed for ~1 mo. (O) Denotes the beginning of the separating gel.

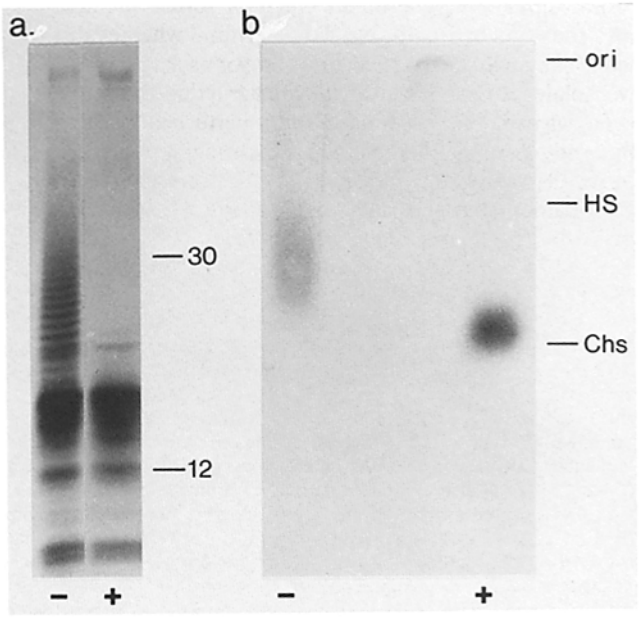

FIGURE 3 The sulfated staircase is a chondroitin sulfate proteoglycan. (a) The sulfated staircase is sensitive to chondroitinase ABC digestion. Cells labeled for 4.5 h with [^{35}S]sulfate at 400 μCi/ml were stimulated with 8-Br-cAMP for 1 h. Medium was collected and one half of the sample was digested with chondroitinase ABC. Both samples were analyzed on the same 10–18% SDS polyacrylamide gel. The fluorogram was exposed for 15 h. (−) Without and (+) with chondroitinase ABC added. (*b*) Pronase-treated staircase material co-migrates with chondroitin sulfate on thin-layer cellulose acetate electrophoresis. Gel-purified [^{35}S]sulfate-labeled staircase material was prepared and pronase-digested as described in Materials and Methods. The pronase-treated (+) and -untreated (−) samples as well as standard glycosaminoglycans were electrophoresed on the same thin-layer cellulose strip. [^{35}S]sulfate-labeled material was detected by autoradiography. A 10-d exposure is presented. *Chs*, chondroitin sulfate; *HS*, heparin sulfate; *ori*, the origin of electrophoresis.

Thus, this sulfated macromolecule is sensitive to digestion with protease and chondroitinase, and the residue remaining after proteolysis has the same electrophoretic mobility as a chondroitin sulfate standard. We conclude that the granule-specific sulfated staircase has the properties of a proteoglycan with chondroitin sulfate side chains.

The Granule-specific Proteoglycan Has a High Molecular Weight Precursor

If this proteoglycan is involved in sorting or regulated secretion, it would be expected to partition into the regulated pathway at least as well as ACTH. Although we find little of the characteristic chondroitin sulfate proteoglycan released constitutively this is insufficient evidence to claim selective sorting. If for example, the staircase form has a high molecular weight precursor, the apparent selectivity could result from a proteolytic activity that is restricted to the regulated route to the surface. This is not unreasonable since mature ACTH in secretory granules is generated from a large precursor, POMC, by a series of proteolytic events that are restricted to the regulated pathway (13, 20). We find from pulse-labeling ex-

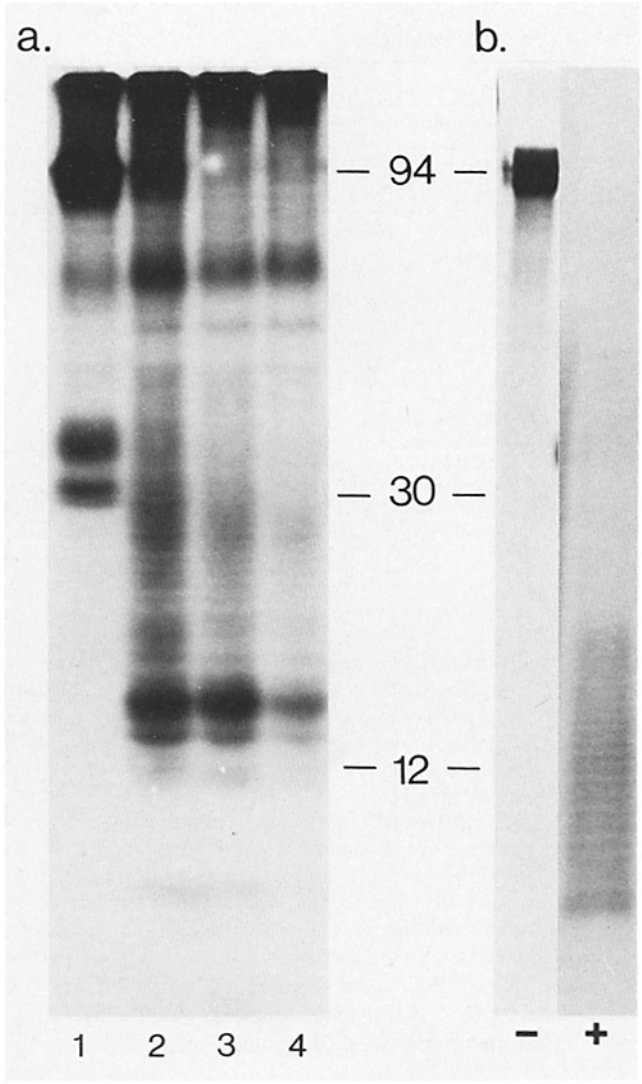

FIGURE 4 The vesicle-specific proteoglycan has a high molecular weight precursor. (*a*) The 25,000–35,000-mol-wt vesicle-specific proteoglycan is not present in a pulse-labeled cell extract and appears only after 1 h of chase. AtT-20 cells were pulse-labeled for 15 min with [^{35}S]sulfate at 1.35 mCi/ml. Cell extracts were made from identical dishes of cells immediately (lane *1*), after 1 h of chase (lane *2*), or after 2 h of chase, either minus (lane *3*) or plus (lane *4*) 8-Br-cAMP stimulation. Cell extracts made by trichloroacetic acid precipitation of a postnuclear supernatant were analyzed on a 10–18% SDS polyacrylamide gel. The fluorogram was exposed for 13 d. (*b*) Pronase digestion of the 95,000-mol-wt band present in pulse cell extracts generates a low molecular weight ladder indistinguishable from that produced by pronase digestion of the 25,000–35,000-mol-wt vesicle-specific proteoglycan. The 95,000-mol-wt region of a gel identical to that shown in lane *1* of *a* was eluted and digested with pronase as described in Materials and Methods. (–) Undigested and (+) digested samples were rerun on the same 10–18% SDS polyacrylamide gel. Exposure was for 1 mo.

periments that the proteoglycan in secretory granules may have a large precursor. When cells were pulse-labeled with [^{35}S]sulfate for 15 min, none of the characteristic proteoglycan was observed in the cell extract (Fig. 4*a*, lane *1*). Only after a 1-hour chase period did the staircase material appear. To identify the region of the gel that might contain a pulse-labeled precursor, we eluted bands of sulfated material from the gel and subjected them to protease digestion as before. Only material migrating with an M_r of 95,000 gave rise to the characteristic banded pattern (Fig. 4*b*). This proteolytic product was indistinguishable from that generated by proteolysis of the isolated staircase material (compare with Fig. 2). If we assume that the material at an M_r of 95,000 is indeed a proteoglycan precursor, then some precursor is secreted constitutively (Table I). The final "sorting ratio" is however comparable to that found in similar experiments for ACTH and its precursor. The proteoglycan is sorted into secretory vesicles at least as well as ACTH.

Perturbation of Proteoglycan Biosynthesis by a Xyloside Inhibitor

We have obtained further evidence that the sulfated material is a proteoglycan and that it is selectively sorted into secretory granules by using an inhibitor of proteoglycan biosynthesis. The addition of glycosaminoglycans to the protein cores of some proteoglycans can be inhibited by xylose analogues, since the first sugar added to the serine or threonine of the polypeptide backbone is a xylose (21, 22). AtT-20 cells were exposed to the xylose analogue 4-methyl umbelliferyl-β-D-xyloside (0.5 mM) for 30 min before labeling and throughout the experiment. After 3 h of growth in [^{35}S]sulfate the cells were collected and the sulfated material in the extract was analyzed by SDS PAGE (Fig. 5). Under these conditions the proteoglycan is not detected by autoradiography. However, the characteristic set of bands again appeared in the presence of the xyloside but with a higher electrophoretic mobility. The number, spacing, and mobility of the bands were indistinguishable from those of the sugar chains that remained after protease treatment of the proteoglycan (compare Fig. 5 with Figs. 2 and 4*b*). These results are consistent with the mode of action of this class of inhibitors. They act as chain initiators for glycosaminoglycan biosynthesis and can effectively block proteoglycan glycosylation by competing with the protein core (23).

It has been reported that free glycosaminoglycan chains, induced by treatment with a *p*-nitrophenyl-β-D-xyloside inhibitor, may be directed into the secretory granules of mast cells (24). If Golgi-synthesized glycosaminoglycans are selectively sorted into secretory granules, this would imply that there is a recognition system for glycosaminoglycan chains, unattached to protein. To test if free glycosaminoglycan chains are sorted in our system, we pulse-labeled AtT-20 cells with [^{35}S]sulfate for 15 min in the presence or absence of the proteoglycan synthesis inhibitor 4-methyl umbelliferyl-β-D-xyloside as described above. (This inhibitor may be preferable to the nitrophenyl xyloside inhibitor since the nitrophenyl group could resemble the peptide core [24].) To estimate the amount of sulfated material released constitutively, first- and second-hour chase media were collected, concentrated, and analyzed by SDS PAGE. We examined regulated release by having 8-Br-cAMP present during the second hour. The autoradiograms were quantified by densitometry (Table I). Compared with proteoglycan sorting, only a minor fraction of the newly synthesized glycosaminoglycan chains entered the AtT-20 granules. The free glycosaminoglycan chains appear in the regulated pathway of secretion with an efficiency about 20-fold less than the vesicle-specific proteoglycan. This supports the claim that the proteoglycan is preferentially sorted into secretory granules and suggests that the peptide backbone may be involved.

TABLE I

Sorting of the Vesicle-specific Proteoglycan and the Free Glycosaminoglycan Chain

	Without xyloside					With xyloside		
	Homogenate		Medium					
	Proteoglycan	Precursor	Proteoglycan	Precursor	Total	Homogenate GAG	Medium GAG	Total
0–1 h	72.1	1.8	ND	32.8	106.7	104	587	621
1–2 h control	71.5	ND	ND	ND	71.5	69	83	152
1–2 h stimulated	33.3	ND	51.1	ND	84.4	44	120	164
Amount released (0–2 h) control (A)			ND	32.8	32.8		670	670
Increment released (1–2 h) on stimulation (B)			51.1	ND	51.1		37	37
Sorting ratio (B/A)					1.6			0.06

Following published procedures (16), AtT-20 cells were pulse-labeled for 15 min with [^{35}S]sulfate, either in the presence or the absence of 4-methyl umbelliferyl-β-D-xyloside, and then chased for 1 h. An aliquot of cells was homogenized at the end of 1 h of chase and the sulfated proteins in the homogenate were analyzed by PAGE (as in Fig. 4*a*). The amounts of the vesicle-specific proteoglycan or the free glycosaminoglycan (*GAG*) chains in the presence of the xyloside inhibitor were quantified by densitometry of the autoradiographs. The sulfated proteins released by the cells into the medium during the same period were analyzed identically. Another two aliquots of cells were chased for a further 1-h period. The stimulated aliquot had 5 mM 8-Br-cAMP in the bathing medium; the control had none. At the end of the labeling period the sulfated material released during the second hour of chase, and that remaining in the cell homogenates, were analyzed as before. To remain in the linear range of the photographic film, we exposed gels for different lengths of time and normalized them to a 2-d exposure. Recovery of the pulse-label was never <80%. *ND*, no detectable amount of the species in question. To measure sorting it is necessary to look at the sum of precursor and mature forms. Since the half-time for constitutive release in these cells is ~40 min (13, 16), the amount released in 2 h in the absence of stimulation is a reasonable measure of the amount leaving by the constitutive pathway. Stimulated release is the increment over the control cells in the presence of 8-Br-cAMP. The ratio of stimulated release to constitutive release, B/A, is a convenient measure of sorting.

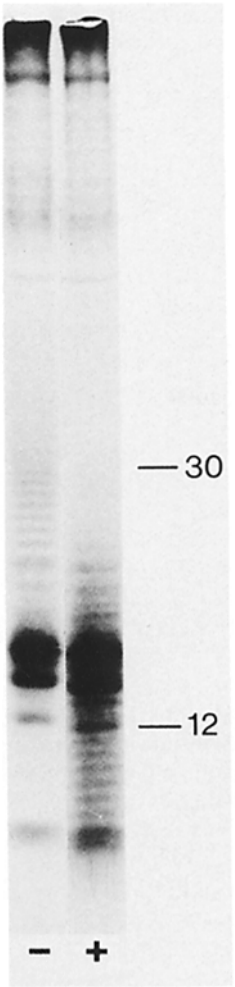

FIGURE 5 The synthesis of the vesicle-specific proteoglycan is inhibited by 4-methyl umbelliferyl-β-D-xyloside. Cell extracts were prepared after a 3-h labeling with [^{35}S]sulfate at 300 μCi/ml by boiling cells in 2% SDS. Only a low molecular weight ladder is detected in cells treated (+) with 4-methyl umbelliferyl-β-D-xyloside for 30 min before and during the labeling period (compare with untreated lane [−]). The fluorogram was exposed for 1 wk.

A Variant AtT-20 Clone Lacks the Granule-specific Proteoglycan

To establish the function of the granule-specific proteoglycan, we isolated an AtT-20 clone that apparently lacks it. Clones from nonmutagenized AtT-20 stocks were grown, labeled with [^{35}S]sulfate, and screened for the lack of an enhanced release of [^{35}S]sulfate when secretion was stimulated with 8-Br-cAMP. One clone discovered in this way, AtT-20/CNB, did not release the proteoglycan on stimulation (Fig. 6). Cells of the CNB clone and AtT-20 cells were labeled for 3.5 h with [^{35}S]sulfate, chased for 1.5 h, and then exposed to 8-Br-cAMP. While other sulfated proteins could be recognized in the medium upon stimulation, indicating that exocytosis was not blocked, the sulfated proteoglycan was absent (Fig. 6, lane *4*). The lack of release cannot be explained by retention within the cell, since the proteoglycan is not observed even in extracts of the CNB clone (Fig. 6, lanes *7* and *8*). This variant clone apparently can still make some sulfated secretory products but not the granule-specific proteoglycan.

Secretion of ACTH Is Not Affected by the Absence of the Vesicle-specific Proteoglycan

Since we could lower the concentration of granule-specific proteoglycan in two ways, by using either the inhibitor of its biosynthesis or the variant clone AtT-20/CNB, we asked if the proteoglycan was required for the storage and release of ACTH. Both wild-type and CNB cells were pretreated with 1 mM xyloside inhibitor for 5 h before labeling and throughout the experiment. Inhibited and control cells were pulse-labeled for 20 min with [^{35}S]methionine followed by a 1-h chase. As before, cells to be stimulated were exposed to 8-Br-cAMP for 1 h after the chase. To detect secreted hormone, we immunoprecipitated the labeled ACTH from the medium using affinity-purified antibodies, analyzed it by SDS PAGE, and quantified it by densitometry. The absence of detectable amounts of the proteoglycan, either in the clone CNB or induced by the xyloside inhibitor, did not prevent or reduce the stimulated release of mature ACTH (Fig. 7*a*). Since the amount of ACTH released did not decrease when the level of the proteoglycan was drastically reduced, it appears that sorting occurs normally even when the proteoglycan is absent from the secretory granules.

To confirm these results by an independent method, we analyzed secretion of total ACTH by using a radioimmunoassay. Cells were treated with xyloside as described above

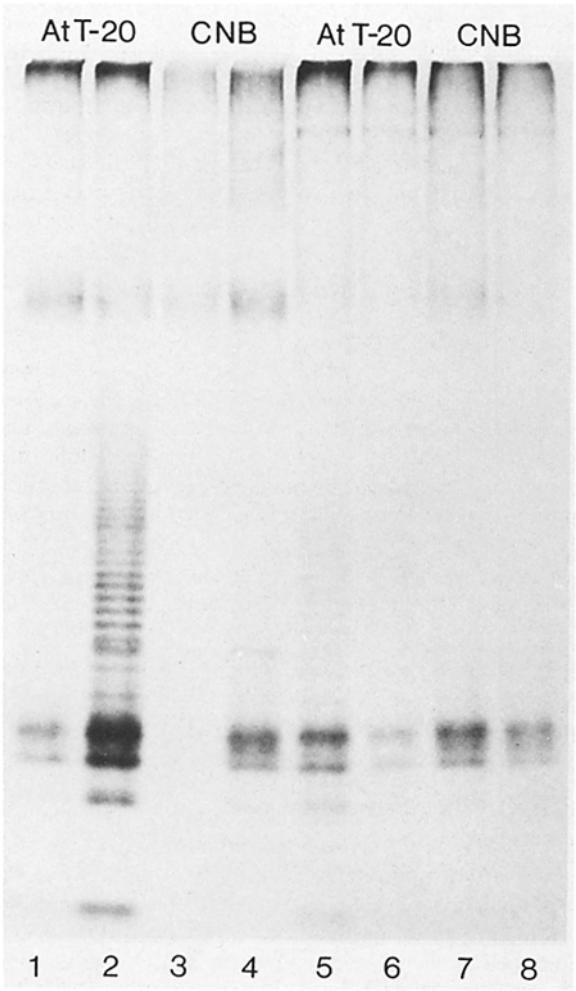

FIGURE 6 A variant AtT-20 clone CNB lacks the vesicle-specific proteoglycan. AtT-20 cells or variant CNB cells were labeled with [^{35}S]sulfate at 250 μCi/ml for 3 h and chased for 1 h in unlabeled medium. Material released into the medium in the next hour period, either with (lanes *2* and *4*) or without (lanes *1* and *3*) stimulation was analyzed by SDS PAGE. Cell extracts were prepared after the chases by boiling cells in 2% SDS. Lanes *6* and *8* were from stimulated cells, and lanes *5* and *7* from unstimulated controls. Lanes *3*, *4*, *7*, and *8* were from the variant CNB cell line; the others were from normal AtT-20 cells. The bands visible in the CNB variant (*M*, 16,000–18,000) are probably sulfated forms of the N-terminal peptides of POMC, peptides known to have asparagine-linked oligosaccharides.

and media with and without cAMP from duplicate dishes of cells were assayed for ACTH. As can be seen in Fig. 7*b*, the secretion of total ACTH was unaffected in variant CNB and in cells treated with xyloside. Furthermore, when secretory granules were purified from either CNB cells or xyloside-treated AtT-20 cells, they contained normal levels of ACTH by radioimmunoassay and were recovered at the same density (data not presented). Taken together these data strongly suggest that although the AtT-20 cells make a specific chondroitin sulfate proteoglycan and segregate it into secretory granules, it is not required for sorting or release of ACTH. If, as suggested earlier (25), the high density of the secretory vesicles is due to their ACTH content, we have no evidence for a reduction in storage.

DISCUSSION

The secretory granules of AtT-20 cells, like those of other endocrine and exocrine cells, contain high concentrations of secretory products. There are an estimated 16,000 molecules of ACTH per secretory vesicle of 200 nm diam in AtT-20 cells (25). A molecular mechanism that might simplify the sorting and packaging of ACTH would be a carrier molecule that could recognize and bind many hormone peptides simultaneously, and transport them out of the Golgi apparatus. Attractive candidates for such carrier proteins are the sulfated proteoglycans and sulfated glycoproteins that are found in many secretory granules. We have identified and characterized a secretory granule-specific component in AtT-20 cells that appears to be a chondroitin sulfate proteoglycan. We have tried to determine the role of this proteoglycan by reducing its amount in AtT-20 cells but have found no detectable effect on sorting, storage, or secretion of ACTH.

There are several explanations for this negative result. One is that only a small amount of the vesicle-specific staircase proteoglycan is required for sorting and packaging of ACTH. If this is the case, the amount must be very small. In xyloside-treated cells we cannot detect the proteoglycan by autoradiography. In variant CNB, it is detectable although at <5% of the normal amount. To reduce the level further we treated the variant CNB with the xyloside inhibitor. Although a slight reduction in the regulated secretion of ACTH was observed

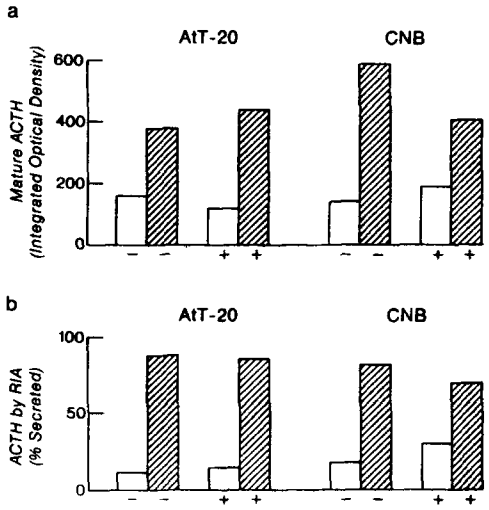

FIGURE 7 The secretion of ACTH is not altered by the absence of the granule-specific proteoglycan. (*a*) Mature ACTH detected by immunoprecipitation is unaltered by xyloside treatment or in variant CNB. Cells treated (+) or untreated (–) with 0.5 mM 4-methyl umbelliferyl-β-D-xyloside were pulse-labeled with [^{35}S]methionine and chased in unlabeled medium for 1 h; the culture medium was then collected in the absence (white bars) or presence (striped bars) of 8-Br-cAMP for 1 h. Labeled ACTH was immunoprecipitated with affinity-purified anti-ACTH antibodies and analyzed by SDS PAGE. After fluorography, the film was analyzed by densitometric scanning and the amounts of mature ACTH (13,000- and 4,500-mol-wt forms) were determined by peak area integration. (*b*) Total ACTH detected by radioimmunoassay is not altered by xyloside or in variant CNB. A radioimmunoassay was used to determine the total ACTH secreted by AtT-20 and CNB cells in the presence (+) or absence (–) of the proteoglycan synthesis inhibitor 4-methyl umbelliferyl-β-D-xyloside, in the presence or absence of the secretagogue, 8-Br-cAMP. The values presented are normalized to total cellular protein and represent the average of two to four experiments.

(Fig. 7), this change was not reproducible. Interpretation of the xyloside experiments is complicated by the ability of the xylosides to stimulate free glycosaminoglycan chain synthesis in AtT-20 cells (Table I). Thus, although free glycosaminoglycan chains are inefficiently packaged into secretory vesicles, the absolute amount of glycosaminoglycan chains present in granules is not altered by a very large amount owing to the stimulation of glycosaminoglycan synthesis. It does seem clear, however, from the data in Table I and Fig. 7, that ACTH does not partition with the free glycosaminoglycan chains. It is thus unlikely that ACTH associates with the sugar chains of the proteoglycan in either a sorting or condensing step. ACTH may partition with the protein core of the proteoglycan. However, there is currently no compelling reason to implicate this protein over any other intravesicular protein (16). Finally, it is possible that some other minor proteoglycan that we have not detected is responsible for the sorting and packaging of ACTH. Sulfated material is sometimes seen at the top of running gels (e.g., Fig. 1) with an electrophoretic mobility similar to that observed for proteoglycans (e.g., reference 7). This material is apparently released constitutively, however, since its externalization is not dependent on 8-Br-cAMP (Fig. 1). A proteoglycan that is not sulfated or is insensitive to 4-methyl umbelliferyl-β-D-xyloside could play a role in sorting.

There is no evidence at present that posttranslational sulfation of POMC is necessary for sorting. We have found that inhibition of N-linked glycosylation of POMC by tunicamycin has no effect on sorting (reference 15 and A. Katzen and H.-P. Moore, unpublished observations). Although POMC and some of its products are sulfated in AtT-20 cells as in the intermediate lobe of the rat pituitary (26), the sulfation is no longer detectable in cells grown in tunicamycin (H.-P. Moore, unpublished observations), presumably because the sulfation is occurring on N-linked oligosaccharides (27). Since sorting is not altered in the presence of tunicamycin, sulfation on N-linked sugars of ACTH or other glycoproteins is apparently not required for ACTH sorting in AtT-20 cells. Indeed, several vesicle-specific proteins have been identified that are not sulfated (16).

Qualitatively similar results to those shown in Table I have been obtained using xyloside inhibitors to perturb proteoglycan biosynthesis in mast cells (24, 28). The material released into the medium without stimulation is relatively enriched in free glycosaminoglycan chains. Some glycosaminoglycan chains do arrive in the secretory vesicle and can be released from them by exocytosis. Quantitatively, however, a much larger fraction of the free glycosaminoglycan chains enter mast cell granules than enter ACTH-containing granules. Several explanations for why free glycosaminoglycan chains might be directed to secretory vesicles in mast cells have already been proposed (24). An additional possibility may be that constitutive secretion is a minor route of secretion in mast cells, and the distribution of free glycosaminoglycan chains perhaps reflects the relative internal volume of each pathway to the surface.

The phenotype of the CNB variant is poorly understood at present. The rationale behind screening for secretory mutants was that whereas the constitutive pathway may be essential for cell growth, the regulated secretory pathway is not (13). Variants lacking that pathway might therefore be predicted to occur with high probability in a population of AtT-20 cells. In fact, in a screen of about 200 clones we found none that had lost the regulated secretory pathway for ACTH (H.-P. Moore, unpublished observations). In contrast, two variants that failed to secrete the sulfated material upon stimulation were found in a screen of only 15 clones. The expression of the vesicle-specific sulfated proteoglycan thus appears to be an unstable characteristic of this cell line. The defect is probably not in sulfation; other secretory proteins are sulfated normally (see Fig. 6). Morever, when CNB cells are treated with xyloside, they do have the ability to synthesize and sulfate a low molecular weight staircase that is indistinguishable from that made by AtT-20 cells, although less material is detected in CNB cells. Therefore, it is unlikely that reduction in 3′-phosphoadenosine-5′-phosphosulfate causes the variant phenotype. It is also unlikely that the proteoglycan is turning over more slowly and so is present in normal amounts, but is undetectable by sulfate labeling. The turnover time of this proteoglycan is regulated by its rate of secretion from the cell and there is no evidence that it is secreted more slowly in the CNB variant. An intriguing parallel may be the action of monensin on oligosaccharide synthesis. At low monensin concentrations, sulfation of glycosaminoglycan chains stops but sulfation of N-linked oligosaccharides continues (29). Lacking an antibody to the proteoglycan, we are unable to distinguish whether the polypeptide chain is absent in the variant, or present but either not sulfated or not proteolytically processed correctly.

If the vesicle-specific proteoglycans are not involved in transport or packaging of peptide hormones, an alternative function must be sought. The regulated pathway is clearly not the exclusive pathway of proteoglycan secretion since other cells that lack the pathway secrete proteoglycans. A possible role for granule-specific proteoglycans is based on the observation that cholinergic synaptic vesicles contain an antigenically unique proteoglycan-like material (7) that is also found extracellularly in the synaptic junctional region (30). Since the sites of exocytotic transmitter release in neurons are confined to specialized domains of the plasma membrane, extracellular matrix components externalized via synaptic vesicles could modify the extra-cellular matrix at the release sites. It is known that a unique region of extracellular matrix is associated with the exocytosis sites at neuromuscular junctions (31), where it plays a role in bringing the regenerating nerve terminal in register with the postsynaptic receptors. Perhaps unique proteoglycans in the secretory vesicles of endocrine cells play a similar role in associating release sites with specialized extracellular domains.

We thank members of the Kelly laboratory, especially Steve Carlson and Hsiao-Ping Moore, for advice and stimulating discussion and Leslie Spector for help with preparing the manuscript.

This research was supported by National Institutes of Health (NIH) grants NS15927 and NS09878 to R. B. Kelly. T. Burgess was supported by a University of California at San Francisco Regents Fellowship, and NIH/National Cancer Institute Institutional Training grant T32 CA09270.

Received for publication 29 May 1984, and in revised form 9 July 1984.

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