

The Major Tyrosine-sulfated Protein of the Bovine Anterior Pituitary is a Secretory Protein Present in Gonadotrophs, Thyrotrophs, Mammotrophs, and Corticotrophs

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ABSTRACT The anterior pituitary is a complex secretory tissue known to contain several sulfated macromolecules. In the present study, we identified the major tyrosine-sulfated protein of the bovine anterior pituitary and investigated its cellular and subcellular localization. This protein consisted of two tyrosine-sulfated polypeptides of molecular weight 86,000 and 84,000 that were highly homologous to each other. In agreement with previous biochemical studies, the tyrosine-sulfated protein of M_r 86,000/84,000 was found to be secretory, as it was observed in the matrix of secretory granules by immunoelectron microscopy. Immunofluorescence studies indicated that the tyrosine-sulfated, secretory protein of M_r 86,000/84,000, referred to as TSP 86/84, was present in all endocrine cells except for some somatotrophic cells. Higher levels of immunoreactivity for TSP 86/84 were observed in gonadotrophic and thyrotrophic than in mammotrophic and corticotrophic cells. This appeared to result from the occurrence of TSP 86/84 in all secretory granules of the former cells and in only some secretory granules of the latter cells. We discuss the possibility that TSP 86/84 may have a role in the packaging of several distinct peptide hormones into secretory granules. One, though not the only, possible function of tyrosine sulfation may concern the sorting of this protein in the Golgi complex.

Secretory proteins are often post-translationally modified before their secretion (for reviews see references 1–4). One post-translational modification that has been frequently observed in secretory proteins is sulfation. Sulfation of unglycosylated proteins occurs on tyrosine residues, whereas sulfation of glycosylated proteins may occur on tyrosine residues carbohydrate residues, or both (see references 5 and 6). One function that has been suggested for carbohydrate sulfation of glycoproteins and proteoglycans present inside secretory granules is a role in the packaging of secretory products (for reviews see references 2 and 7).

The functional role of tyrosine sulfation is not yet known. Previous and current investigations (listed in reference 6) have shown that all tyrosine-sulfated proteins studied until now, though functionally diverse, share the property of being secretory proteins. It has therefore been suggested (8, 9) that tyrosine sulfation, which is catalyzed by tyrosylprotein sulfotransferase (10) in the Golgi complex (11a), may have some role in the process of secretion of certain proteins.

Since the anterior pituitary is a well-characterized secretory tissue containing at least five classes of endocrine cells, we were interested in studying tyrosine sulfation of adenohypophyseal proteins and in identifying the major tyrosine-sulfated protein in this gland. Sulfated macromolecules of the anterior pituitary have been the subject of several earlier studies (11–14). Recently, an acidic sulfated protein of the anterior pituitary was identified and characterized (15–17). Some properties of this protein suggested that it was a secretory protein: it accumulated in the chase medium of anterior pituitary slices in pulse-chase experiments (15, 16), and it was found in subcellular fractions highly enriched in prolactin (PRL)¹ granules (16). However, the release of this sulfated protein into the chase medium, in contrast to that of PRL, was not significantly inhibited by dopamine (16). One possible

¹ *Abbreviations used in this paper:* GH, growth hormone; PRL, prolactin; TSP 86/84, tyrosine-sulfated secretory protein of M_r 86,000/84,000.

explanation for these findings is that this sulfated protein occurred not only in PRL granules but also in secretory granules of other cell types of the anterior pituitary, and that its concentration in the latter cells was greater than in the PRL cell. In the present study, we show that this protein is present in secretory granules, that it occurs in four of the five classes of endocrine cells, and that it is the major tyrosine-sulfated protein of the bovine anterior pituitary.

MATERIALS AND METHODS

Materials

We obtained ampholytes from LKB, trypsin-TPCK from Worthington Biochemical Corp. (Freehold, NJ), and pronase from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany [FRG]). Carrier-free [³⁵S]sulfate and ¹²⁵I-protein A were obtained from Amersham Corp. (Braunschweig, FRG). Anti-ovine PRL antiserum was the kind gift of Dr. C. H. Li, Hormone Research Laboratory, University of California, San Francisco. Dr. V. Locatelli, Department of Pharmacology, University of Milan, kindly supplied anti-human adrenocorticotropin (ACTH) antiserum. Antisera raised against the β -subunits of human luteinizing hormone, human follicle-stimulating hormone, or human thyroid-stimulating hormone were kindly provided by Dr. A. F. Parlow, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, Harbor UCLA Medical Center, Torrance, CA. Rhodamine-conjugated goat IgG anti-rabbit IgG was obtained from Cappel Laboratories (Cochranville, PA) and protein A from Sigma Chemical Co. (St. Louis, MO). We synthesized tyrosine sulfate, threonine sulfate, and serine sulfate as described (18).

[³⁵S]Sulfate Labeling, Partial Purification, and Gel Electrophoresis of Tyrosine-sulfated Secretory Protein of *M_r* 86,000/84,000 (TSP 86/84)

Cow anterior pituitary slices were prepared and labeled with carrier-free [³⁵S]sulfate (0.2 mCi/ml) as described (11). Labeled slices were homogenized 1:10 (wt/vol) in a SDS-containing stop solution (10), boiled immediately for 3 min, and analyzed by SDS PAGE (19). In the experiments designed to obtain partially purified TSP 86/84, 200 mg of the [³⁵S]sulfate-labeled slices was homogenized (950 rpm, 12 strokes) in 2 ml of homogenization buffer (20 mM Tris/HCl, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM phenylmethyl sulfonyl-fluoride). The homogenate was centrifuged at 140,000 *g* for 1 h, and TSP 86/84 was partially purified from the resulting supernatant by a heat treatment as described elsewhere (20).² Partially purified TSP 86/84 was analyzed by two-dimensional (2D) PAGE (21), by the use of 2.5% (vol/vol) ampholytes, pH 3.5–5; 2.5% (vol/vol) ampholytes, pH 5–7; and 2% (vol/vol) ampholytes, pH 3.5–10 in the first dimension, and 7.5% gels in the second dimension. After SDS PAGE or 2D PAGE, gels were fixed, stained, destained, dried, and autoradiographed or fluorographed as described (10).

Tryptic Fingerprinting of Radioiodinated TSP 86 and TSP 84

Gel pieces containing TSP 86 and gel pieces containing TSP 84 were cut separately from a dried 2D gel, swollen in 30% methanol/10% acetic acid, and individually subjected to the radioiodination procedure described by Elder et al. (22), by the use of 100 μ Ci of carrier-free ¹²⁵I per gel piece. Exhaustive digestion of the radioiodinated proteins with trypsin and 2D separation of the resulting peptides by thin-layer electrophoresis and chromatography were performed with minor modifications as described (22). The thin-layer sheets were autoradiographed at -70°C by the use of intensifying screens.

Tyrosine Sulfate Analysis of TSP 86/84

Two lanes of a dried SDS gel containing aliquots of [³⁵S]sulfate-labeled total protein of cow anterior pituitary were swollen in 30% methanol/10% acetic acid. We then subjected one gel lane to a short acid treatment (1 M HCl, 5 min, 90°C) as described in detail elsewhere (6), while we kept the other gel lane

in 30% methanol/10% acetic acid as a control. Both gel lanes were washed extensively in 30% methanol/10% acetic acid, prepared for fluorography, dried, and fluorographed (10).

[³⁵S]Sulfate-labeled proteins contained in individual dried gel pieces were hydrolyzed with barium hydroxide and analyzed for the presence of tyrosine [³⁵S]sulfate by one-dimensional thin-layer electrophoresis as described (6). Alternatively, the [³⁵S]sulfate-labeled proteins were subjected to extensive pronase digestion, and then sulfated fragments were analyzed by one-dimensional thin-layer electrophoresis (6).

Antibodies

ANTI-TSP 86/84 ANTIBODIES. Because of the great homology of TSP 86 to TSP 84 (see Fig. 2), TSP 86 and TSP 84, purified to apparent homogeneity, were used together for the preparation of a rabbit anti-TSP 86/84 antiserum (see reference 17). Anti-TSP 86/84 antibodies were affinity-purified by use of partially purified TSP 86/84 (17) coupled to Sepharose 4B.

ANTI-GH ANTIBODIES. Rabbit anti-bovine GH (NIH-GH-B 18) antibodies were raised and purified by affinity chromatography as described (15).

Radioimmunolabeling of TSP 86/84 on Nitrocellulose Filters

We rapidly dissected the anterior lobe from a freshly obtained cow pituitary gland and froze it in liquid N₂. The frozen tissue was crushed on dry ice to a fine powder of which 100 mg was homogenized in 2 ml of the SDS stop solution and then immediately boiled for 3 min. Total tissue protein was subjected to SDS PAGE on 10% gels and transferred (0.3 A, 5.5 h) to nitrocellulose filter paper (pore size, 0.45 μm) as described by Burnette (23), except that the transfer buffer contained 0.1% SDS. Filters were sequentially incubated for 1 h in phosphate-buffered saline (PBS) containing 0.1% glutaraldehyde, for 1 h in 0.1 M glycine-Tris, pH 7.4, overnight in PBS containing 8% bovine hemoglobin and 0.05% NaN₃ (blocking medium), and then for 12 h in blocking medium containing 15 $\mu\text{g}/\text{ml}$ of either anti-TSP 86/84 antibodies or preimmune immunoglobulins (obtained by ammonium sulfate precipitation followed by dialysis against PBS). Filters were washed extensively in PBS containing 0.1% (vol/vol) Nonidet P-40, incubated for 2 h in blocking medium containing 0.1 μCi ¹²⁵I-protein A (70–100 $\mu\text{Ci}/\mu\text{g}$) per ml, washed extensively, dried, and autoradiographed at -70°C by use of intensifying screens. Complementary to the radioimmunolabeling, other filters were stained after transfer with amido black (23).

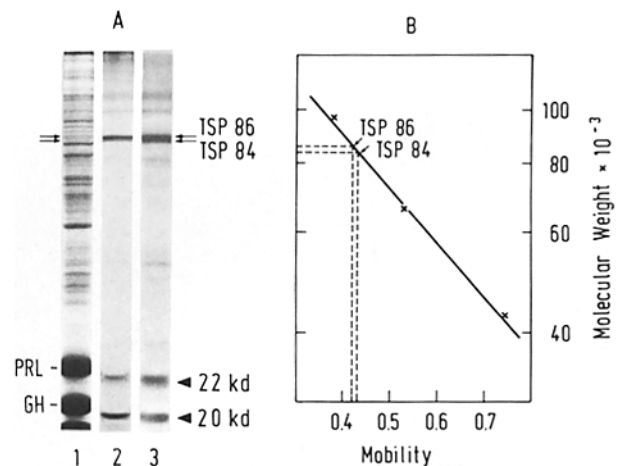


FIGURE 1 (A) Sulfated proteins of the cow anterior pituitary separated by SDS PAGE. (Lane 1) Coomassie Blue staining of total protein separated in a 10% polyacrylamide gel; (lane 2) autoradiography and (lane 3) fluorography of the gel showing the [³⁵S]sulfate-labeled proteins. The arrows indicate the positions of TSP 86 and TSP 84, which appear as two distinct bands in the autoradiogram but not in the fluorogram. The arrowheads indicate two major sulfated bands of 22,000 and 20,000 mol wt found just below the PRL and GH bands, respectively. (B) Determination of the apparent molecular weight of TSP 86 and TSP 84 after SDS PAGE in a 7.5% gel, using phosphorylase b (97,000), bovine serum albumin (67,000), and ovalbumin (43,000) as molecular weight standards.

² Hille, A., R. W. H. Lee, and W. B. Huttner. Manuscript in preparation.

Immunofluorescence

We dissected cow anterior pituitaries immediately after slaughter, immersed them into ice-cold 4% formaldehyde (dissolved in 0.12 M cacodylate buffer, pH 7.4), and cut them into 1–2 mm thick slices. The slices were fixed for 1 hr in the same ice-cold fixative, postfixed for 1 h in an ice-cold solution containing 1% OsO₄ in cacodylate buffer, dehydrated, and embedded in Epon as described (24). Cutting of 1- μ m-thin serial sections, removal of Epon (25), and indirect immunofluorescence were performed as described by De Camilli et al. (26). For each glass slide, 150 μ l of Triton buffer (20 mM phosphate buffer, pH 7.4, 450 mM NaCl, 0.3% [vol/vol] Triton X-100, and 15% normal goat serum), containing either 10 μ g of anti-TSP 86/84 antibodies or the indicated concentrations of anti-hormone antibodies/antisera or preimmune serum, were used.

In some experiments, anti-TSP 86/84 antibodies (40 μ g in 600 μ l of Triton buffer) were preabsorbed by incubation for 16 h at 4°C with purified TSP 86/84 (30 μ g, eluted from a preparative SDS gel and precipitated by acetone [17]), and a subsequent centrifugation at 10,000 g for 5 min. We used the resulting supernatant for immunostaining (150 μ l/glass slide).

Immunoelectron Microscopy

Thin sections, obtained from the same blocks that were used for the immunofluorescence studies, were collected on Formvar-carbon coated nickel grids. Immunolocalization of TSP 86/84 was performed according to Bendayan

et al. (27). In brief, the sections were pretreated with a saturated aqueous solution of sodium metaperiodate, washed thoroughly in distilled water and then incubated in a solution containing 90 μ g/ml anti-TSP 86/84 antibodies, 20 mM phosphate buffer, pH 7.3, 450 mM NaCl, and 0.5% bovine serum albumin. After they were washed in PBS, the thin sections were labeled with protein A-colloidal gold complexes which had been prepared by reduction of chloroauric acid with phosphorus and successive binding of protein A to colloidal gold as described (28). We then stained the sections with uranyl acetate and lead nitrate, and examined them under a Philips 400 EM electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

RESULTS

Identification of the Major Tyrosine-sulfated Protein of the Anterior Pituitary

Slices of cow anterior pituitary glands were labeled with inorganic [³⁵S]sulfate and proteins were analyzed by SDS PAGE (Fig. 1). In addition to the sulfated macromolecules at the top of the gel, major sulfated bands in the 86,000–84,000-mol-wt region, at 22,000 mol wt directly below the PRL band, and at 20,000 mol wt directly below the growth hormone

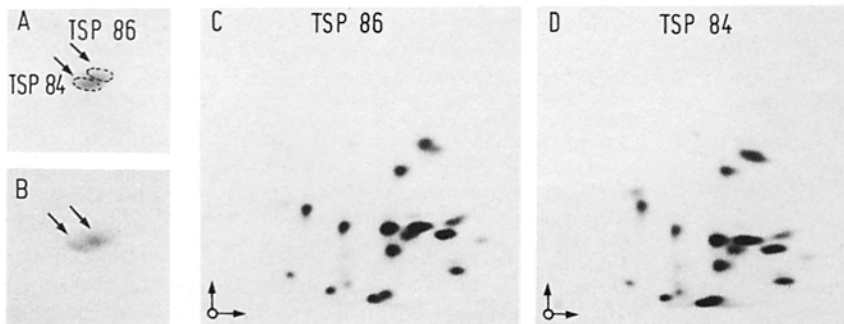


FIGURE 2 TSP 86 and TSP 84 are two homologous polypeptides. Partially purified [³⁵S]sulfate-labeled TSP 86 and TSP 84 were separated by 2D PAGE followed by staining with Coomassie Blue and autoradiography. The portion of the Coomassie Blue-stained gel and of the corresponding autoradiogram showing TSP 86 and TSP 84 are shown in A and B, respectively. TSP 86 and TSP 84 were individually cut from the gel, as indicated in A by the dotted lines, radioiodinated, and subjected to tryptic fingerprinting. C and D show autoradiograms of the radio-

iodinated peptides of TSP 86 and TSP 84, respectively, after 2D separation on cellulose thin-layer sheets (origin, lower left corner; horizontal dimension, electrophoresis, anode left, cathode right; vertical dimension, ascending chromatography).

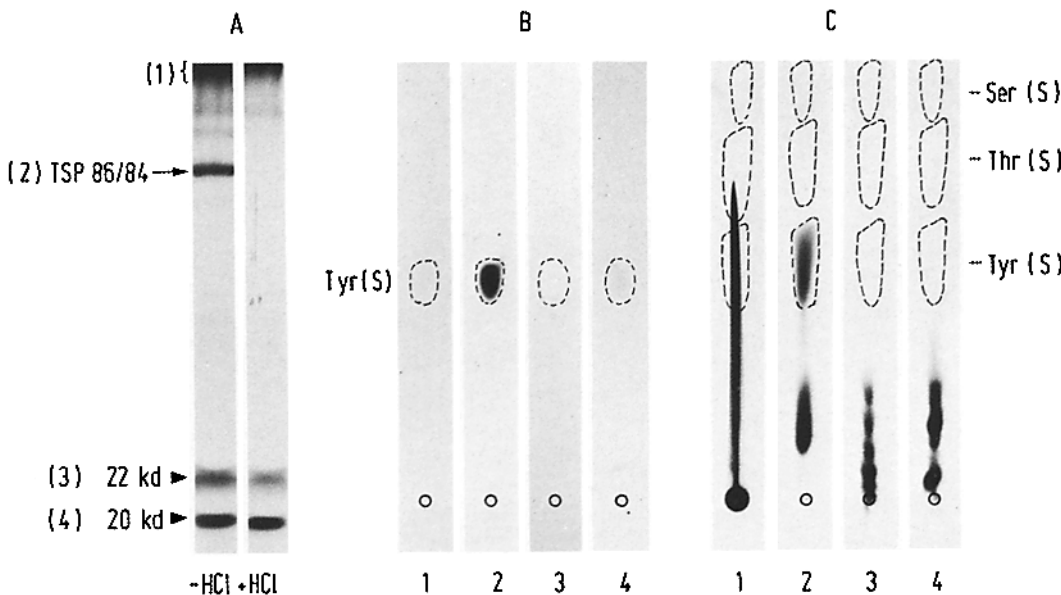


FIGURE 3 TSP 86/84 is the major tyrosine-sulfated protein of the anterior pituitary. (A) Fluorograms showing the effect of the acid treatment of SDS polyacrylamide gels (10%) containing [³⁵S]sulfate-labeled macromolecules of the anterior pituitary. (Left lane) control (-HCl), 8-d exposure; (right lane) acid treatment (+HCl), 10-d exposure. The positions of sulfated macromolecules found at the top of the gel (1), of TSP 86/84 (2), and of the sulfated 22,000 mol wt (3) and 20,000 mol wt (4) bands are indicated on the left. These four groups of sulfated macromolecules

were cut from a non-acid-treated control gel and subjected individually to either alkaline hydrolysis with barium hydroxide (B) or extensive pronase digestion (C). The alkaline hydrolyzate and the pronase digests were analyzed by thin-layer electrophoresis, followed by autoradiography of the cellulose thin-layer sheets as shown. Numbers 1–4 in B and C correspond to those in A, indicating the four groups of sulfated macromolecules analyzed. The dashed lines indicate the positions of sulfated amino acid standards, as visualized by ninhydrin staining. Tyr (S), tyrosine sulfate; Thr (S), threonine sulfate; Ser (S), serine sulfate.

(GH) band were observed by fluorography. By autoradiography, we could distinguish two closely adjacent sulfated bands of M_r 86,000 and 84,000. These two sulfated polypeptides corresponded to the acidic sulfated secretory protein described previously (16, 17) with an apparent molecular weight of $\sim 70,000$ in a different gel system (15). Based on the results of the present study, the sulfated polypeptides of M_r 86,000 and 84,000 are referred to as TSP 86 and TSP 84, respectively.

We analyzed the relationship between TSP 86 and TSP 84, separated from each other by 2D PAGE (Fig. 2, A and B), by radioiodination and subsequent tryptic fingerprinting. The

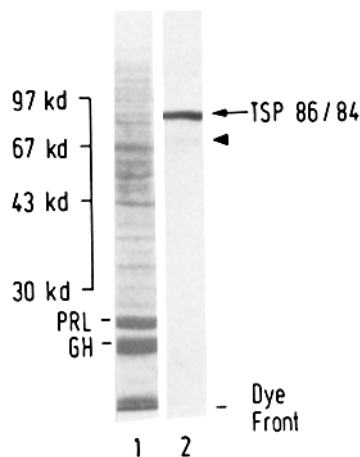


FIGURE 4 Specificity of affinity-purified antibodies directed against TSP 86/84. Total protein of anterior pituitary was separated on a 10% SDS polyacrylamide gel (120 μ g per lane), transferred to nitrocellulose filters, and either stained with amido black (lane 1) or subjected to radioimmunolabeling by use of anti-TSP 86/84 antibodies and 125 I-labeled protein A followed by fluorography (lane 2). The arrow indicates the position of TSP 86/84. The arrowhead indicates the position of a weakly immunostained band of 67,000 mol wt that is related to TSP 86/84, as indicated by tryptic fingerprinting (not shown).

pattern of 125 I-labeled peptides obtained from TSP 86 (Fig. 2C) was almost identical to that obtained from TSP 84 (Fig. 2D), an observation confirmed by mixing experiments (data not shown). In view of the apparent high homology of TSP 86 with TSP 84, these two polypeptides, collectively referred to as TSP 86/84, were used together for further analysis.

The sulfated residue(s) of TSP 86/84 were determined and compared with those of the other major sulfated bands present at the top of the gel, at 22,000 and 20,000 mol wt. The radioactive sulfate incorporated into TSP 86/84 (which made up $\sim 18\%$ of the total radioactive sulfate incorporated into the major sulfated proteins) was virtually quantitatively released from the protein fixed in the gel by a short acid treatment (6) known to hydrolyze the ester bond of tyrosine sulfate (Fig. 3A). Alkaline hydrolysis of TSP 86/84 with barium hydroxide followed by thin-layer electrophoresis of the alkali-stable material indicated that $\sim 80\%$ of the incorporated radioactive sulfate could be recovered as tyrosine sulfate (Fig. 3B). In contrast to the result with TSP 86/84, the short acid treatment of the gel released only small amounts of radioactive sulfate from the material at the top of the gel, at 22,000 and at 20,000 mol wt (Fig. 3A). Little ($\leq 8\%$) of the radioactive sulfate of these bands was recovered as tyrosine sulfate after alkaline hydrolysis (Fig. 3B), since the vast majority of the sulfate was alkali labile and removed from the samples as insoluble barium sulfate. Mild hydrolysis of TSP 86/84 by pronase digestion liberated about half of the tyrosine sulfate, and the rest was recovered in the form of a peptide that was found between the origin and the tyrosine sulfate spot (Fig. 3C). Pronase digestion did not liberate significant amounts of tyrosine sulfate from the sulfated 22,000 and 20,000-mol-wt material but yielded a series of sulfated fragments, presumably sulfated glycopeptides, that were found between the origin and the position of the tyrosine sulfate standard. The pronase-

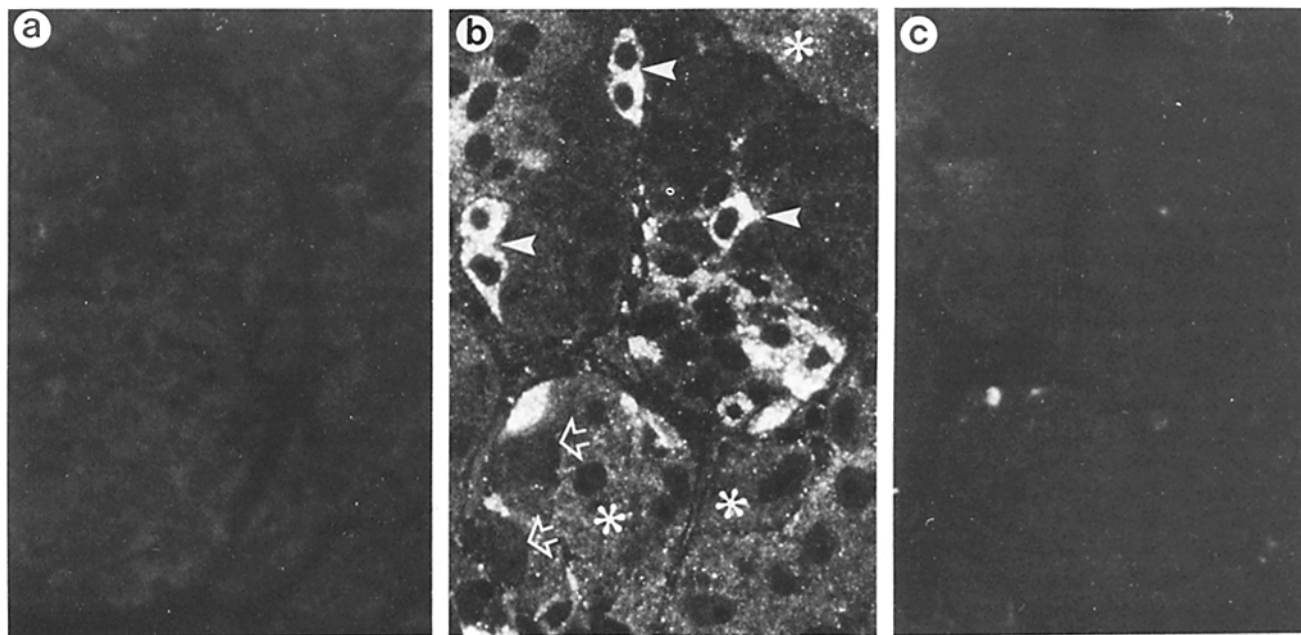


FIGURE 5 Micrographs showing the distribution of TSP 86/84 immunoreactivity in cow anterior pituitary, as revealed by immunofluorescence in 1 μ m-thick plastic sections. (a) Preimmune serum (1:30 dilution). (b) Anti-TSP 86/84 antibodies. Intense, dotted fluorescence that spares the nuclei is present in some cells (indicated by arrowheads), which are localized predominantly at the periphery of the lobules. Moderate, dotted fluorescence is present in most of the other cells (indicated by asterisks). Some cells, indicated by the open arrows, do not immunostain for TSP 86/84. (c) Anti-TSP 86/84 antibodies preabsorbed with purified TSP 86/84; serial section to that shown in b. (a-c) $\times 700$.

digested, sulfated material from the top of the gel yielded a smear of radioactive material. No serine sulfate or threonine sulfate was obtained after the pronase digestion of any of the sulfated material (Fig. 3C). The results shown in Fig. 3, A–C complemented each other and indicated that of the four major sets of sulfated macromolecules of the anterior pituitary, TSP 86/84 was the major tyrosine-sulfated protein.

Cellular Localization of TSP 86/84 by Immunofluorescence

Antibodies were raised against purified TSP 86/84 and purified by affinity chromatography. We determined the specificity of these antibodies, to be used for the immunocytochemical localization, by immunoblotting after SDS PAGE of total anterior pituitary protein (Fig. 4). The anti-TSP 86/84 antibodies specifically recognized the TSP 86/84 doublet (Fig. 4, lane 2). No immunolabeling was seen with preimmune immunoglobulins (data not shown). Specific binding of anti-TSP 86/84 antibodies to TSP 86/84 was also seen in immunoblots obtained after 2D PAGE of total anterior pituitary protein (data not shown).

The cellular localization of TSP 86/84 in the bovine anterior pituitary was studied by immunofluorescence. In order to maximize the level of resolution, we prepared 1 μm -thick sections from Epon-embedded tissue and used them for immunofluorescence after removal of the Epon. Whereas only autofluorescence was observed with preimmune serum (Fig. 5a), most cells of the anterior pituitary were immunostained with the anti-TSP 86/84 antibodies (Fig. 5b). Three levels of immunofluorescence were observed (Fig. 5b). First, intense fluorescence was observed in some cells that were scattered throughout the gland and located mainly at the periphery of the lobules and around the vessels. Second, moderate fluorescence was present in cells that were more abundant than the brightly fluorescent cells and constituted the majority of cells of most lobules. Third, some cells did not appear to be immunostained under the present experimental conditions. The fluorescence in the immunostained cells had a dotted appearance and was not observed in the nucleus. Both intense and moderate immunofluorescence appeared to be specific for the presence of TSP 86/84 in the cells since neither was observed when the anti-TSP 86/84 antibodies had been preabsorbed with purified TSP 86/84 (Fig. 5c).

To correlate the occurrence of TSP 86/84 with that of the

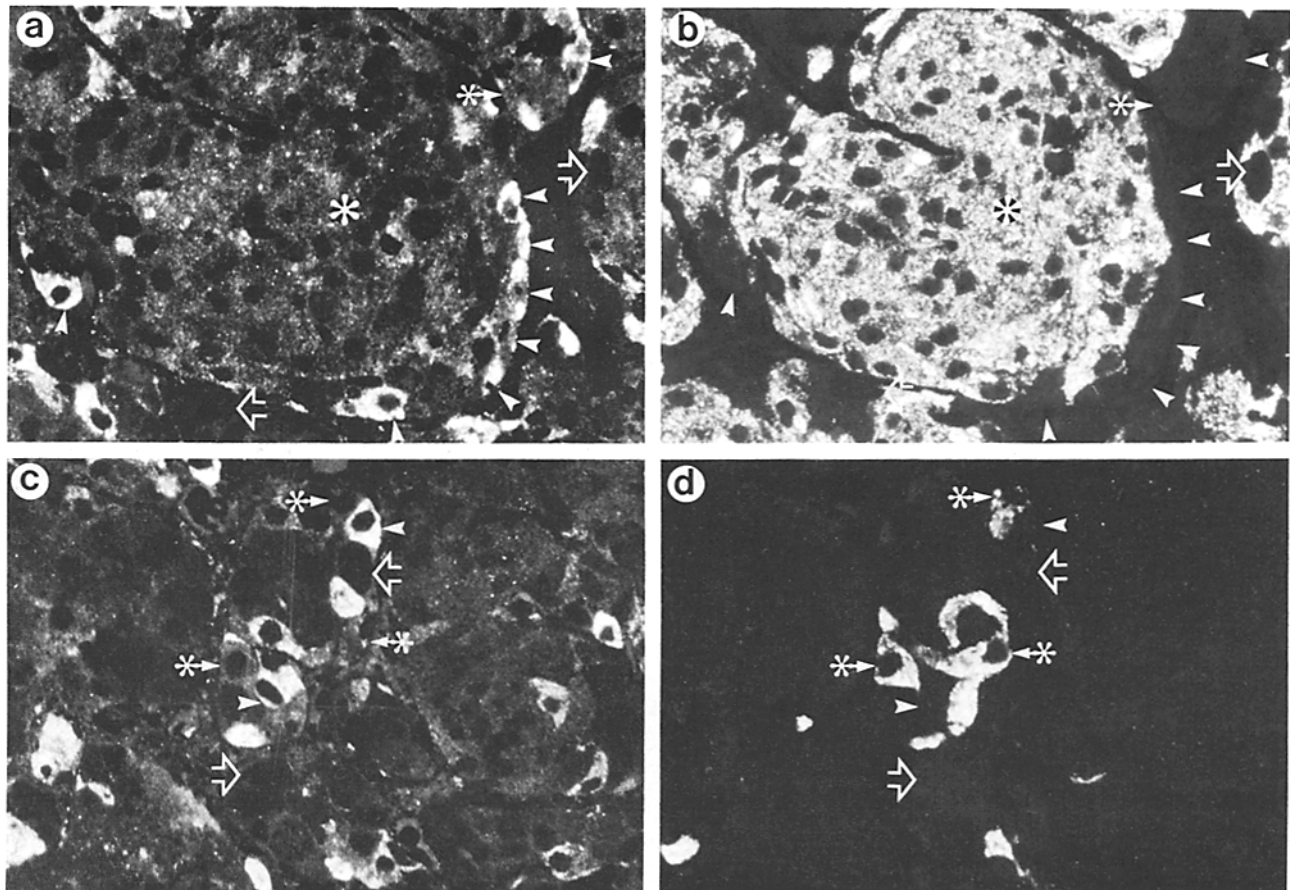


FIGURE 6 Fluorescence micrographs showing that the mammotrophic and corticotrophic cells of the anterior pituitary immunostain moderately for TSP 86/84. (a and b) 1 μm -thick serial sections immunostained with anti-TSP 86/84 antibodies (a) and PRL antiserum (1:50 dilution) (b). Bright dotted fluorescence for PRL is observed in most of the cells (asterisk, b). These cells are moderately stained for TSP 86/84 (a). A minor proportion of cells that are moderately stained for TSP 86/84 (pointed asterisk, a) do not stain for PRL (b). Intensely TSP 86/84-positive cells (arrowheads, a) and TSP 86/84-negative cells (open arrows, a) do not immunostain for PRL (b). (c and d) 1 μm -thick serial sections immunostained with anti-TSP 86/84 antibodies (c) and ACTH antiserum (1:50 dilution) (d). Bright dotted fluorescence for ACTH is observed in a few cells (pointed asterisks, d) which constitute a minor proportion of the cells that stain moderately for TSP 86/84 (c). Intensely TSP 86/84 positive cells (arrowheads, c) and TSP 86/84-negative cells (open arrows, c) do not immunostain for ACTH (d). a–d, $\times 500$.

various adenohypophyseal hormones, serial sections were immunostained either with anti-TSP 86/84 antibodies or with antisera directed against one of the major anterior pituitary hormones. Both PRL-positive cells (Fig. 6, *a* and *b*) and ACTH-positive cells (Fig. 6, *c* and *d*) were moderately immunostained for TSP 86/84. Both luteinizing hormone- and follicle-stimulating hormone-positive cells (Fig. 7, *a-c*) and thyroid-stimulating hormone-positive cells (Fig. 7, *d* and *e*) were intensely immunostained for TSP 86/84. The immunofluorescence observed with anti-GH antibodies showed two different patterns: (*a*) several strongly stained cells and (*b*) other cells with a more diffusely scattered, dot-like staining (Fig. 8*b*). The strongly GH-positive cells did not appear to immunostain for TSP 86/84 (Fig. 8*a*). The second type of GH-positive cells seemed to correspond to some moderately

TSP 86/84-positive cells that also immunostained for PRL, as shown in Fig. 6.

Subcellular Localization of TSP 86/84 by Immunoelectron Microscopy

The dot-like immunofluorescent staining for TSP 86/84, together with previous biochemical data (15, 16), suggested a localization of this protein in secretory granules. To confirm this possible localization, we performed immunoelectron microscopy on thin plastic sections by the use of anti-TSP 86/84 antibodies and protein A-colloidal gold complexes. As shown in Figs. 9 and 10, immunoreactivity was restricted mainly to the matrix of secretory granules. In the endoplasmic reticulum and the Golgi complex, no significant immuno-

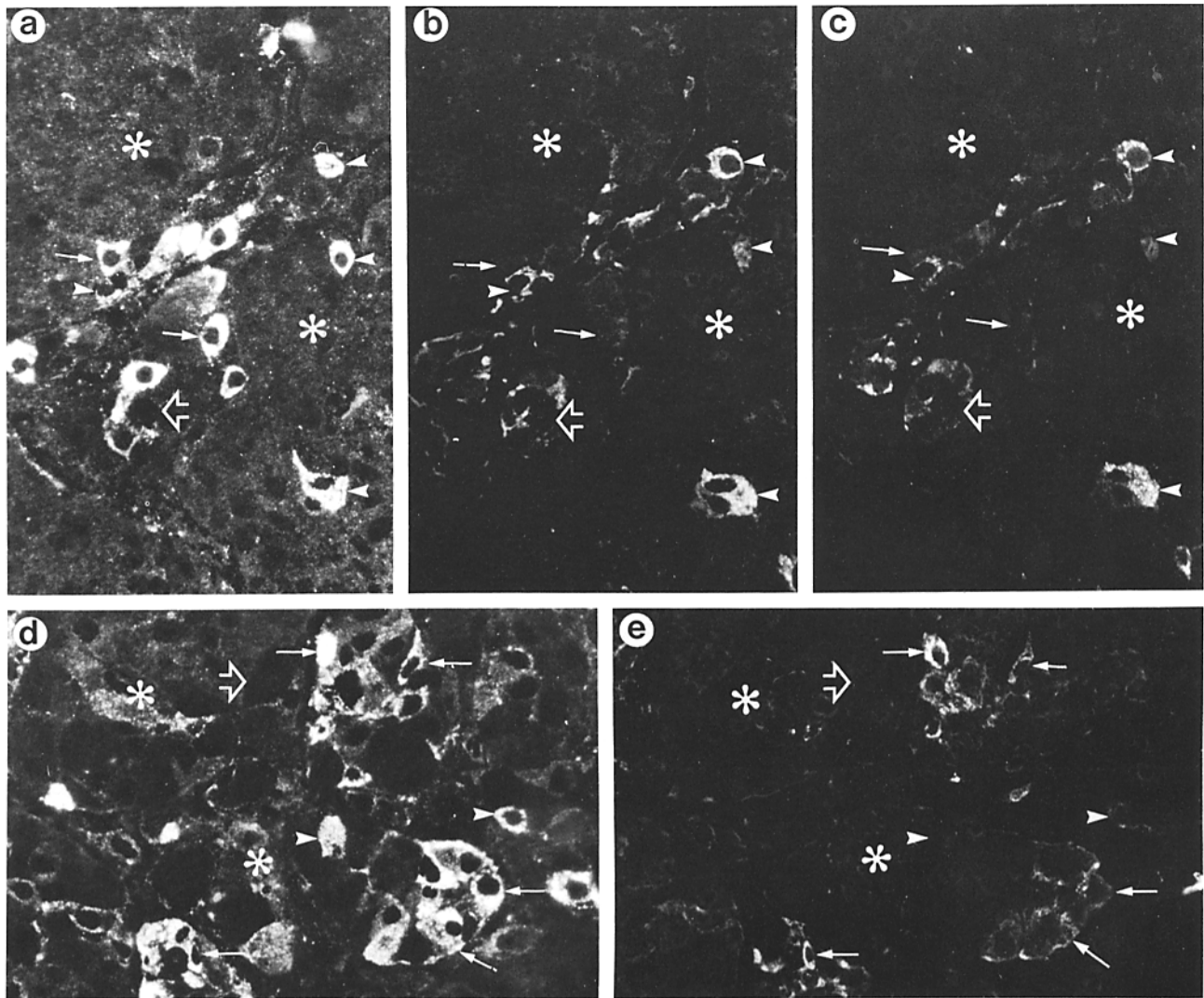


FIGURE 7 Fluorescence micrographs showing that the gonadotrophic and thyrotrophic cells of the anterior pituitary are intensely immunostained for TSP 86/84. (*a-c*) 1 μm -thick serial sections immunostained with anti-TSP 86/84 antibodies (*a*), luteinizing hormone (LH) antiserum (1:2 dilution) (*b*), and antiserum (1:2 dilution) (*c*). A few cells (arrowheads) show bright dotted fluorescence for both LH (*b*) and follicle-stimulating hormone (*c*). These cells are some, but not all (see arrows), of the cells that are intensely stained for TSP 86/84 (arrowheads, *a*). Moderately TSP 86/84-positive cells (asterisks, *a*) and TSP 86/84-negative cells (open arrow, *a*) do not immunostain for LH (*b*) and FSH (*c*). (*d* and *e*) 1 μm -thick serial sections immunostained with anti-TSP 86/84 antibodies (*d*) and thyroid-stimulating hormone antiserum (1:2 dilution) (*e*). A few cells show dotted fluorescence for thyroid-stimulating hormone (arrows, *e*). These cells are some, but not all (see arrowheads), of the cells that are intensely stained for TSP 86/84 (arrows, *d*). Moderately TSP 86/84-positive cells (asterisks, *d*) and TSP 86/84-negative cells (open arrows, *d*) do not immunostain for thyroid-stimulating hormone (*e*). (*a-e*) $\times 500$.

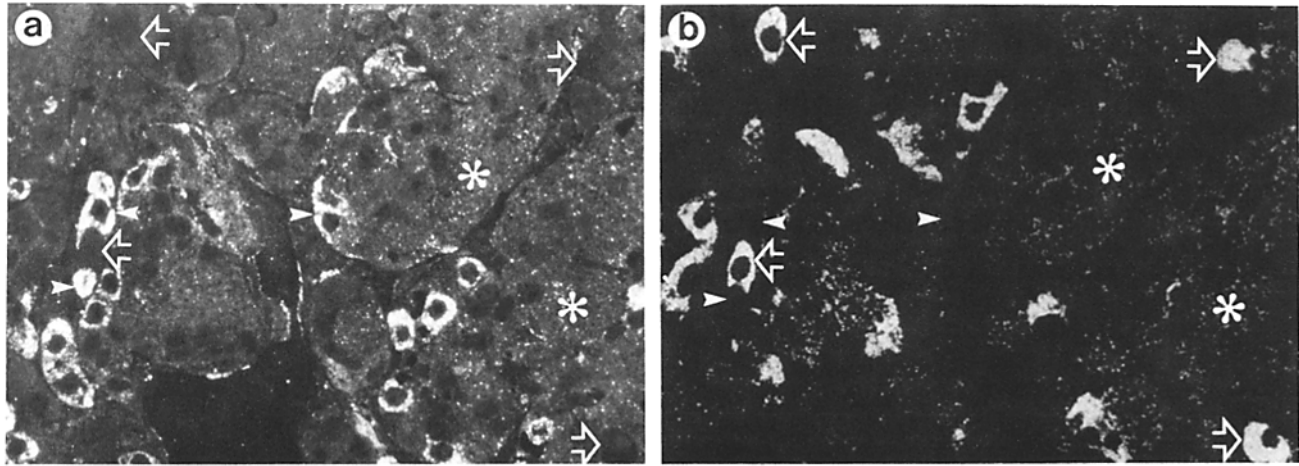


FIGURE 8 Fluorescence micrographs showing that those somatotrophic cells that are highly immunostained for GH do not immunostain for TSP 86/84. (a and b) 1 μm -thick serial sections immunostained with anti-TSP 86/84 antibodies (a) and anti-GH antibodies (4.5 $\mu\text{g}/150 \mu\text{l}$) (b). Several cells show bright dotted fluorescence for GH (open arrows, b). These cells do not stain for TSP 86/84 (open arrows, a). Sparse dotted fluorescence for GH is observed in other cells that are moderately stained for TSP 86/84 (asterisks). No staining for GH is observed in the intensely TSP 86/84-positive cells (arrowheads). (a and b) $\times 500$.

reactivity could be detected with the present methodology. Other subcellular structures did not immunostain for TSP 86/84.

Three levels of immunoreactivity could be distinguished. In some cells, all secretory granules were uniformly immunolabeled for TSP 86/84 (Fig. 9). These cells usually occurred at the periphery of the lobules and contained relatively small secretory granules. These cells were probably gonadotrophs or thyrotrophs, shown in Fig. 7 to be intensely immunofluorescent for TSP 86/84. In other cells, which were the most abundant cells of the lobules, only a small subpopulation of secretory granules was immunolabeled for TSP 86/84 (Fig. 10). In these cells, the TSP 86/84-positive granules were usually smaller and less electron dense than the TSP 86/84-negative ones. These cells probably were mammotrophs, shown in Fig. 6 to be moderately immunofluorescent for TSP 86/84. In addition, some cells showed no immunoreactivity for TSP 86/84 (Fig. 9). The secretory granules of these cells (presumably the strongly GH-positive cells shown in Fig. 8) were usually larger than the TSP 86/84-positive granules seen in the other cells.

Gold grains were counted in 120 profiles of cells with secretory granules immunoreacting for TSP 86/84. In any given experiment, gold grain density over TSP 86/84-positive granule profiles varied minimally. Among different sets of experiments, the gold grain density over TSP 86/84-positive granules ranged from 350 to 950/ μm^2 . Background values were usually <20 grains/ μm^2 .

DISCUSSION

The present study demonstrates that the major tyrosine-sulfated protein of the anterior pituitary, TSP 86/84, is found in most adenohypophyseal cells. We confirmed the secretory nature of TSP 86/84, suggested by previous biochemical studies (15, 16), by the immunoelectron microscopical localization of this protein in the matrix of secretory granules. The different levels of immunofluorescence for TSP 86/84, i.e., intense and moderate staining, could result from: (a) differences in the amount of granules per cell; (b) differences in the average concentration of TSP 86/84 per granule; or (c) the

selective occurrence of TSP 86/84 in only a subpopulation of granules of the moderately stained cells. The results of immunoelectron microscopy do not support the first two possibilities but are consistent with the third. We cannot exclude the possibility that the observed differences in the level of immunoreactivity for TSP 86/84 resulted from differences in the accessibility of TSP 86/84 to the antibodies. We do not, however, favor this possibility since one would expect to find more TSP 86/84 in the anterior pituitary than is observed biochemically if this protein were present in similar concentrations in the immunocytochemically negative granules and in the positive ones. In addition, the observed immunoreactivity for TSP 86/84 in only a minority of granules of the mammotrophic cells is consistent with the previous result (16) that inhibition of exocytosis of PRL granules by dopamine did not lead to a significant reduction of TSP 86/84 secretion from the anterior pituitary.

The cellular localization of TSP 86/84 in gonadotrophs, thyrotrophs, mammotrophs, and corticotrophs, and the molecular weight and isoelectric point of this protein differ from those of any known hormone precursor produced in the anterior pituitary. However, the possibility cannot be excluded that TSP 86/84 is a new large hormone or hormone precursor. Alternatively, TSP 86/84 may not be a hormone itself but may have some role in the processing, sorting, or packaging of certain peptide hormones. TSP 86/84 is an acidic protein (10, 15–17), with $\sim 20\%$ of the amino acids as glutamic acid.³ By means of this large number of negative charges, TSP 86/84 may function as a helper protein in the packaging of various hormones: it may bind either to positively charged domains of hormones by direct electrostatic interaction or to negatively charged domains indirectly through mediation by divalent cations.

We have recently found that TSP 86/84, together with p113/105 (10) and chromogranin A (29), constitute a family of proteins for which we propose the name secretogranins.³ These three distinct proteins share certain biochemical prop-

³ Rosa, P., A. Hille, A. Zanini, and W. B. Huttner. Manuscript in preparation.

erties and occur in secretory granules of several endocrine cells³ (see also references 30–32 for chromogranin A). It is interesting to note that chromogranin A has been found to occur in somatotrophic cells of the bovine anterior pituitary (31), which do not appear to contain TSP 86/84. It is therefore possible that TSP 86/84 and the other members of the secretogranin family may to a certain extent complement each other in their occurrence within secretory granules of various endocrine cells.

The function of tyrosine sulfation of TSP 86/84 is not yet known. It seems likely that tyrosine sulfation has a more

specific role than simply providing TSP 86/84 with additional negative charges. TSP 86/84 contains at most 4 mol of sulfate per molecule,³ which would contribute very little to the sum of negatively charged groups on this glutamic acid-rich protein. Tyrosine sulfation may affect a possible hormonal activity or a proteolytic processing of TSP 86/84. However, taking into consideration the results obtained with other tyrosine-sulfated proteins (listed in reference 6; see also references 8 and 9), we favor the possibility that tyrosine sulfation is involved in the process of protein secretion. The occurrence of TSP 86/84 in a subpopulation of secretory granules of the

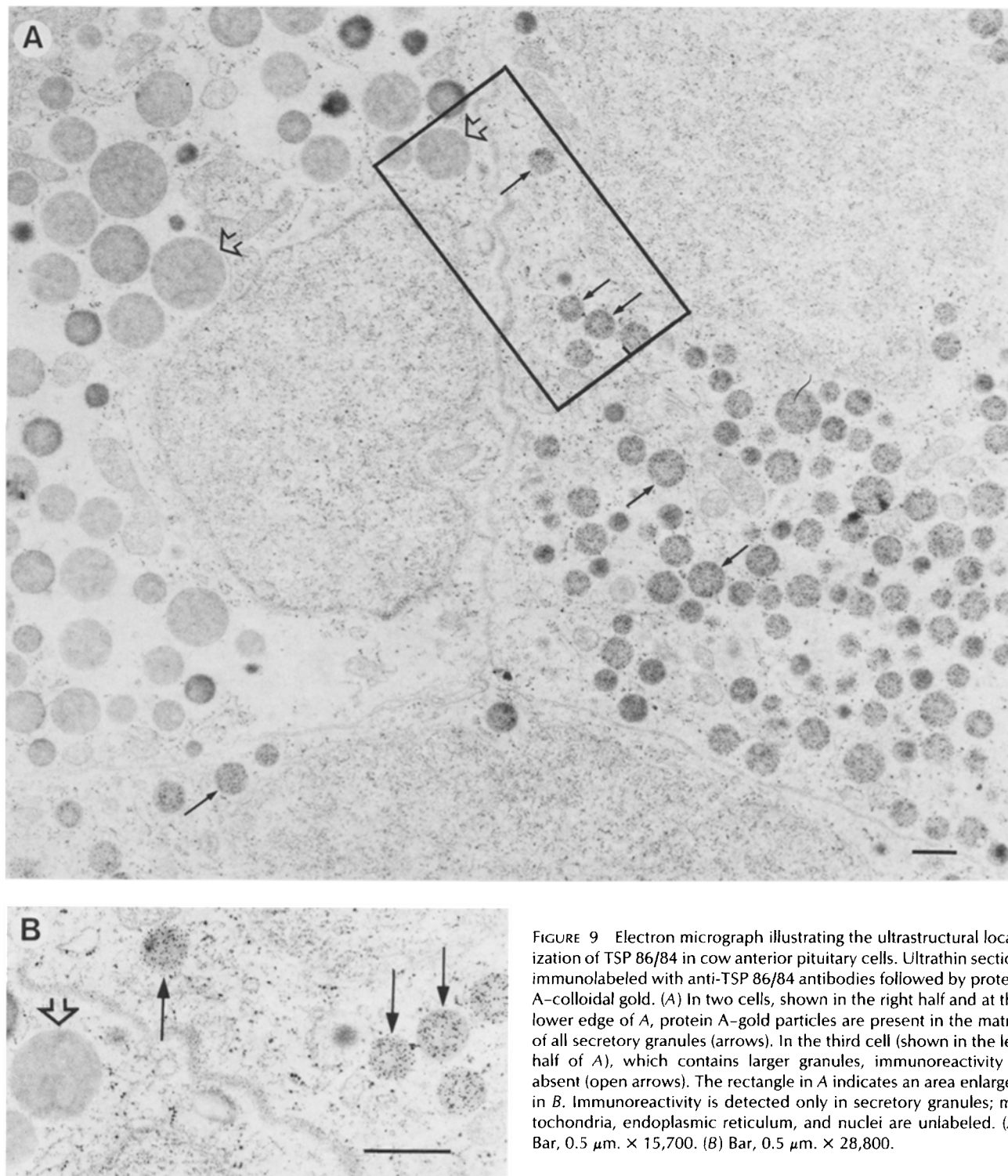


FIGURE 9 Electron micrograph illustrating the ultrastructural localization of TSP 86/84 in cow anterior pituitary cells. Ultrathin section immunolabeled with anti-TSP 86/84 antibodies followed by protein A-colloidal gold. (A) In two cells, shown in the right half and at the lower edge of A, protein A-gold particles are present in the matrix of all secretory granules (arrows). In the third cell (shown in the left half of A), which contains larger granules, immunoreactivity is absent (open arrows). The rectangle in A indicates an area enlarged in B. Immunoreactivity is detected only in secretory granules; mitochondria, endoplasmic reticulum, and nuclei are unlabeled. (A) Bar, 0.5 μm . $\times 15,700$. (B) Bar, 0.5 μm . $\times 28,800$.

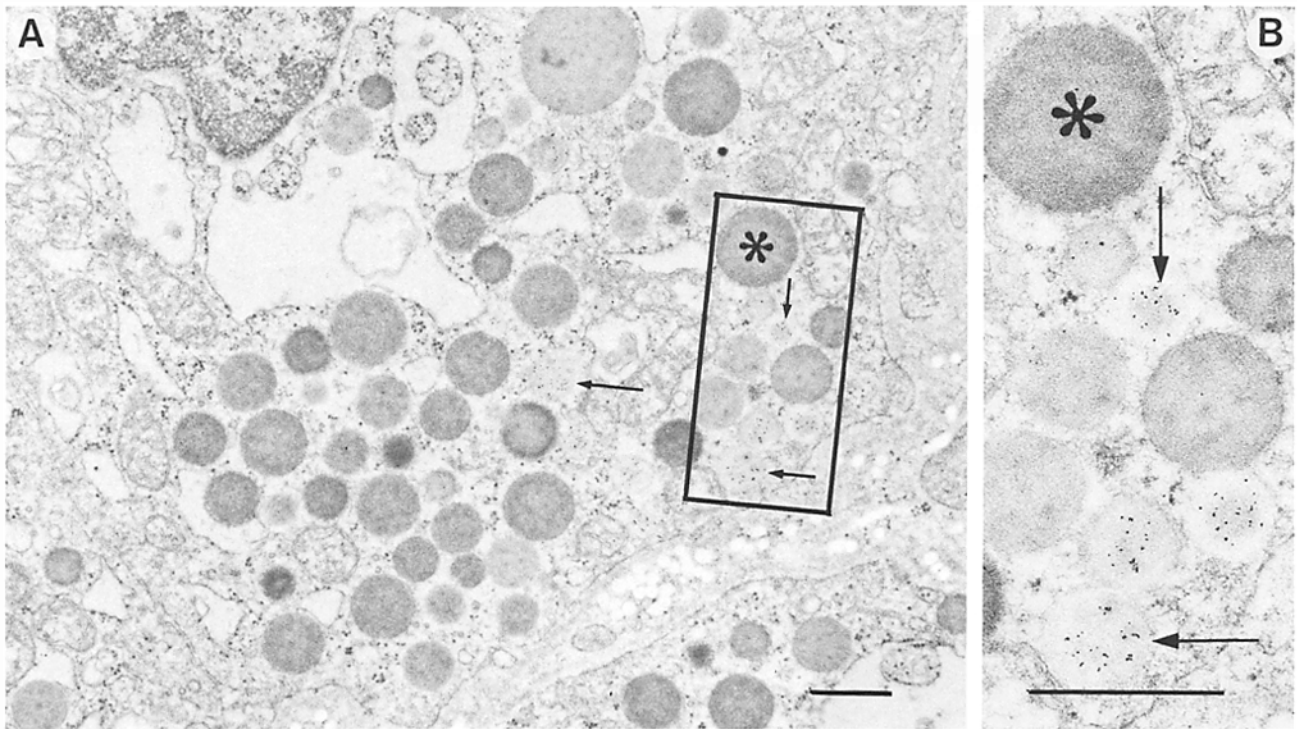


FIGURE 10 Ultrastructural immunolocalization of TSP 86/84 by the protein A-gold technique. (A) In the cell that occupies most of A, immunoreactivity is detectable in the matrix of only a few secretory granules (arrows). These granules are less electron dense and smaller than the unlabeled ones (asterisks). The rectangle in A shows an area enlarged in B. (A) Bar, 0.5 μm . $\times 22,000$. (B) Bar, 0.5 μm . $\times 53,000$.

mammotrophic cells suggests a specific sorting mechanism for this protein. It will be interesting to determine whether tyrosine sulfation is somehow involved in such a sorting mechanism. Sorting of TSP 86/84 would indirectly cause the sorting of proteins with an affinity for TSP 86/84 and could thereby provide a common mechanism for the specific routing of several different peptide hormones to secretory granules.

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