29. T. Sasaki and M. A. Findeis, unpublished results.
30. M. Ikehara *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4695 (1986); S. Nishikawa *et al.*, *Biochem. Biophys. Res. Commun.* **138**, 789 (1986); S. Nishikawa *et al.*, *Biochemistry* **26**, 8620 (1987).
31. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E. Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Research Articles

Molecular Sorting in the Secretory Pathway

KOONG-NAH CHUNG, PETER WALTER, GREGORY W. APONTE, HSIAO-PING H. MOORE

Proteins can be secreted from animal cells by either a constitutive or a regulated pathway; those destined for regulated secretion are actively sorted into dense-core secretory granules. Although sorting is generally assumed to be accomplished by specific carriers, the nature of these carriers remains elusive. In this study, peptide hormones were used as affinity ligands to purify a set of 25-kilodalton proteins from canine pancreatic tissue. Their ligand specificities and patterns of expression have the characteristics of sorting carriers.

IN MULTICELLULAR ORGANISMS, PROPER COMMUNICATION between cells requires precise temporal and spatial control of secretion. Many animal cells segregate their secretory products into distinct types of secretory vesicles. These vesicles differ in their proteolytic processing activities, in the sites at which they fuse with the plasma membrane, and in their dependence on external stimuli for release (*1–4*). Most epithelial cells manufacture two types of vesicles so that proteins can be selectively shuttled to either the apical or the basolateral surface (*5, 6*). Specialized secretory cells also segregate their secretory products into two types of vesicles, for constitutive and regulated release. For example, the mouse pituitary cell line AtT-20 packages adrenocorticotropic hormone (ACTH) in storage granules and releases it when the cell receives an external stimulus (*7, 8*). By contrast, the cell secretes several viral proteins and laminin by a constitutive pathway in which Golgi-derived vesicles fuse continuously with the plasma membrane (*9–11*). Segregation of proteins into hormone-regulated and nonregulated pathways has also been reported in the pituitary cell line GH_3 (*12*), PC-12 cells (*13*), and pancreatic beta cells (*14*). The observation that two secretory pathways can coexist in the same cell suggests that there is a selective mechanism to sort secretory proteins into either the

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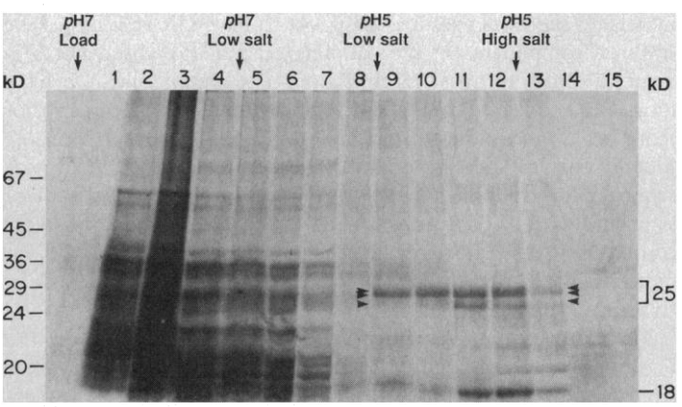

Fig. 1. Identification of proteins that bind to prolactin-Sepharose. Detergent-solubilized Golgi lysates were chromatographed on a prolactin-Sepharose column, and fractions were analyzed by 10 to 18 percent SDS-PAGE. (Lanes 1 to 4) Flow-through fractions from column during loading of Golgi lysates at neutral *p*H. (Lanes 5 to 8) Fractions collected during subsequent washings with neutral *p*H-loading buffer. (Lanes 9 to 12) Fractions collected on eluting the column with 50 m*M* potassium acetate (KOAc), *p*H 5.0, buffer. (Lanes 13 to 15) Fractions collected upon eluting column with 1*M* KOAc, *p*H 5.0, buffer. The positions of molecular standards (kilodaltons) are indicated on the left. A set of 25-kD proteins, which bound to the prolactin column, is indicated by the bracket on the right. Crude dog pancreas Golgi preparations, recovered from the interphase of a 1.3*M* sucrose cushion (*23*), were centrifuged for 1 hour at 100,000*g* and resuspended in 50 m*M* Hepes, *p*H 7.4, 400 m*M* KOAc, 0.5 m*M* EDTA (10 ml final volume for 250 g of wet tissue). Nikkol (Nikkol BL-8SY, Nikko Chemicals Co., Ltd., Tokyo, Japan) and phenylmethylsulfonylfluoride (PMSF) were added to a final concentration of 2 percent and 0.5 m*M*, respectively, and the mixture was incubated on ice for 30 minutes. Insoluble materials were cleared by centrifugation at 100,000*g* for 30 minutes, and the composition of the supernatant was adjusted to 50 m*M* KOAc, 0.5 percent Nikkol, 15 mM Hepes, *p*H 7.4. Four 200-μl samples of the Golgi lysates were applied to a 200-μl affinity resin, prepared by coupling sheep prolactin (Sigma) to CNBr-activated Sepharose (Pharmacia) at 10 mg/ml. The column was washed with four column volumes of each of the following buffers: loading buffer (12.5 m*M* Hepes, *p*H 7.4, 50 m*M* KOAc, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol); elution buffer I (50 m*M* KOAc, *p*H 5.0, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol); and elution buffer II (1*M* KOAc, *p*H 5.0, 0.5 m*M* EDTA, 0.5 m*M* PMSF, 0.5 percent Nikkol). All procedures were carried out at 4°C. Proteins from each fraction were precipitated with 80 percent acetone at −20°C for 60 minutes before SDS gel electrophoresis.

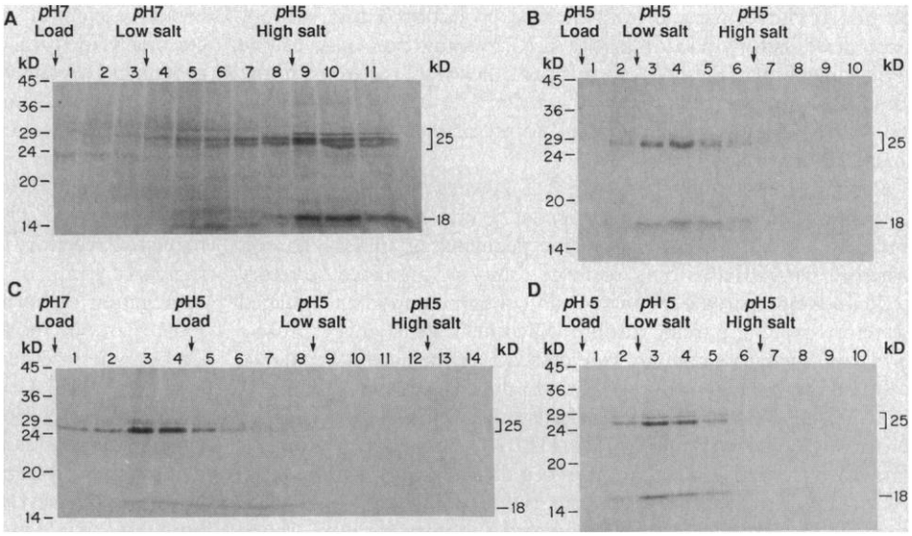

Fig. 2. Rebinding of the HBP25's to prolactin and BSA columns. (A and B) Materials eluted at low *p*H from a prolactin column (fractions 9 to 12 from Fig. 1) were neutralized, and passed over a second column containing either prolactin-Sepharose (A) or BSA-Sepharose (B). (**A**) (Lanes 1 to 3) Flow-through; (lanes 4 to 8) washing fractions with neutral *p*H-loading buffer; (lanes 9 to 11) elution with 1*M* KOAc, *p*H 5.0 (elution buffer II). (**B**) (Lanes 1 to 4) Flow-through; (lanes 5 to 8) washing fractions with neutral *p*H-loading buffer; (lanes 9 to 12) elution with 50 m*M* KOAc, *p*H 5.0 (elution buffer I); (lanes 13 and 14) elution with 1*M* KOAc, *p*H 5.0 (elution buffer II). At neutral *p*H, the HBP25's were retained by the prolactin but not the BSA column. (**C** and **D**): Same as in (A) and (B) except that the eluates from the first prolactin column were not neutralized before being placed on the second columns. (**C**) Loading of eluates from a prolactin column onto a second prolactin column at *p*H 5.0. (**D**) Loading of eluates from a prolactin column onto a BSA column at *p*H 5.0; (lanes 1 and 2) flow-through; (lanes 3 to 6) washing fractions with 50 m*M* KOAc, *p*H 5.0 (elution buffer I); (lanes 7 to 10) elution with 1*M* KOAc, *p*H 5.0 (elution buffer II). All fractions were 200 μl in volume. The HBP25's were not retained by either column at *p*H 5.0. Golgi lysates were chromatographed on a prolactin column as described in Fig. 1. For (A) and (B), fractions eluted with elution buffer I were immediately neutralized with an equal volume of 30 m*M* KOH in 12.5 m*M* Hepes, 0.5 m*M* EDTA, 0.5 percent Nikkol. The pooled material was placed on a second column and chromatographed as indicated.

regulated or the constitutive pathway.

In the AtT-20 cell system, there is mounting evidence that proteins destined for constitutive vesicles move by some "bulk flow" mechanism, while those destined for regulated granules are targeted by specific recognition molecules. Several molecules that are not expected to contain any sorting signal enter the constitutive vesicles and are exported efficiently. For example, Burgess and Kelly (*15*) showed that, in cells blocked in their synthesis of proteoglycans with 4-methylumbelliferyl-β-D-xyloside, the free glycosaminoglycan chains were secreted preferentially by way of constitutive vesicles. Furthermore, if the cytoplasmic protein globin is introduced into the lumen of the endoplasmic reticulum (ER) by fusion to the signal peptide of β-lactamase (*16*), it is also constitutively secreted (*3*). In contrast to the relatively nonspecific transport to the constitutive pathway, efficient targeting of proteins to regulated secretory granules appears to require specific signals. Gene fusion studies show that a protein normally secreted by the constitutive pathway can be diverted to the regulated pathway by fusion to sequences encoding a regulated secretory protein (*17*). These observations suggest that sorting may be mediated by cellular carriers that recognize regulated but not constitutive proteins. Such recognition molecules have not yet been identified, although morphological examination of beta cells suggests that proinsulin is associated with the membranes of Golgi cisternae (*18, 19*). Here we describe the isolation of proteins that specifically bind to regulated secreted hormones.

Identification of intracellular hormone-binding proteins. To find transport carriers, we used affinity chromatography to isolate proteins that bind regulated secretory proteins. We selected canine pancreas which, like other pancreatic tissues, contains predominantly exocrine acini cells and a few hormone-secreting endocrine cells; both of these cell types are enriched in regulated secretory granules. Previous DNA transfection studies have shown that the sorting machinery is conserved among endocrine and exocrine cells of diverse tissue and species origins (*20*). For example, the machinery for packaging mouse ACTH into dense-core secretory granules in AtT-20 cells can sort accurately human growth hormone (*21*), rat and human proinsulin (*14, 22*), and rat trypsinogen (*11*). Thus, a sorting machinery similar to the one in AtT-20 cells should also be present in endocrine and exocrine cells of the pancreas. Because regulated secretory proteins are sorted from constitutive proteins at the "trans-most" cisternae of the Golgi apparatus (*14*), a crude Golgi membrane fraction was used as starting material.

Crude canine pancreas Golgi membranes, isolated by differential centrifugation (*23*), were suspended in a Hepes buffer at *p*H 7.4, solubilized with 2 percent of the nonionic detergent Nikkol, and centrifuged at 100,000*g* for 30 minutes. The supernatant was adjusted to 0.5 percent Nikkol and chromatographed on Sepharose-4B resin that had been coupled to a regulated secretory protein, sheep prolactin (10 mg per milliliter of resin; Sigma). After being loaded with the Golgi lysate, the column was washed and eluted with a buffer at *p*H 5.0 (Fig. 1). A set of proteins with sizes in the 25-kD range, which we call hormone-binding proteins (HBP25's) was eluted from the column with *p*H 5.0, 50 m*M* potassium acetate buffer (fractions 9 to 12). Further elution of the column with 1*M* potassium acetate buffer at *p*H 5.0 did not elute any more proteins (fractions 13 to 15). In some experiments, we also observed a smaller band (at about 18 kD) that eluted at low *p*H (fractions 9 to 12). However, the lower band was not seen reproducibly and, unlike the 25-kD proteins, it showed some affinity for a nonspecific column containing bovine serum albumin (BSA) (Fig. 2). For these reasons, we did not pursue this protein further at this time.

Binding properties of the HBP25's. Binding of HBP25's to prolactin column is reversible. When eluted material at *p*H 5.0 was neutralized and passed over the same column, the binding profile was similar (Fig. 2A); most of the HBP25's were retained by the prolactin column and were not eluted until the *p*H was lowered. However, the neutralization procedure did appear to partially inactivate some of the HBP25's, since a fraction of the neutralized HBP25's bound to the column with lower affinity and became eluted when the column was washed at neutral *p*H (lanes 7 and 8). To eliminate the possibility that binding of the HBP25's to the column might be due to nonspecific ionic interactions, the neutralized material was passed over a BSA-Sepharose control column. BSA was chosen because it is a constitutively secreted protein and its isoelectric point (*p*I = 5.5) is close to that of sheep prolactin (*p*I = 5.0). The HBP25's did not bind to the BSA-Sepharose column and were recovered in flow-through fractions (Fig. 2B). The binding of HBP25's to the prolactin column was highly dependent

on *p*H. If eluted material from the first prolactin column was not neutralized before reloading, the HBP25's did not bind to the prolactin column (Fig. 2C). In this case, they were recovered in the same flow-through fractions as from the BSA column (Fig. 2D). Thus, the HBP25's specifically bind to prolactin at neutral *p*H and dissociate from it at low *p*H.

Previous transfection studies on AtT-20 cells established that the sorting machinery is able to recognize several different hormones and segregate them into the same regulated granules. To test whether the HBP25's bind to more than one regulated secretory protein, we prepared Sepharose resin containing another regulated secretory protein, porcine insulin. When neutralized eluates from a prolactin-Sepharose column were passed over the insulin-Sepharose column, the HBP25's bound tightly to the column and were eluted in a *p*H-dependent manner similar to their elution pattern from prolactin-Sepharose (Fig. 3). The HBP25's also bound to human growth hormone (hGH), another regulated secretory protein. In contrast, under identical conditions the HBP25's did not bind to proteins that do not enter the regulated secretory pathway. When neutralized eluates were passed over hemoglobin- (Hb), myoglobin- (Myo), and BSA-Sepharose columns, the HBP25's were found in the flow-through fractions (Fig. 3A). Recently, Matsuuchi *et al.* showed that immunoglobulin (Ig) chains transfected into AtT-20 cells were secreted by the constitutive route (*24*). When HBP25's eluted from an insulin column were passed over an affinity resin containing purified rabbit Ig, the 25-kD proteins were recovered in the flow-through fractions (Fig. 3B). Similar results were also obtained with crude Golgi extracts that had not been passed over the prolactin column; the elution profile from insulin column was essentially identical to that of prolactin column (Fig. 1), and no protein was retained by immunoglobulin G (IgG), Hb, Myo, or BSA columns. Thus, the HBP25's bind selectively to proteins targeted to dense-core secretory granules, but not to constitutively secreted proteins.

Previous electron microscopic studies showed that at the trans-Golgi cisternae, where proteins are sorted into constitutive and regulated secretory vesicles, hormones become aggregated in the region of incipient granules (*14*). A possible explanation of such aggregation of hormones is that each cellular carrier can bind to more than one molecule of hormone and thereby cross-link them. To examine whether binding of HBP25's to hormones is multivalent, we passed Golgi extracts over an insulin column and then added prolactin. Some of the prolactin became retained on the column and was eluted upon dissociation by low *p*H (Fig. 4). The retention of prolactin by insulin column is not due to binding of hormones to each other, as has been suggested previously to explain hormone aggregation at the trans-Golgi. As shown in Fig. 4, prolactin did not bind to insulin columns that had not been previously loaded with HBP25's. In this case, all of the prolactin was recovered in the "wash-through" fractions, and none could be detected in the low *p*H eluates. These results suggest that binding of the HBP25's to hormones is at least bivalent.

Expression of HBP25's. To facilitate further analysis, we prepared polyclonal antiserum to the HBP25's purified by preparative SDS-PAGE (polyacrylamide gel electrophoresis) (*25*). Immunoblot analysis of crude Golgi extracts showed that the antibodies specifically recognize antigen or antigens in the 25-kD region (Fig. 5). Additional immunoblot analyses confirmed that the 25-kD antigens were the hormone-binding proteins: the antigens were found in wash-through fractions from IgG-, Hb-, Myo-, and BSA-Sepharose columns, but were retained and eluted at low *p*H from prolactin- and insulin-Sepharose columns (Fig. 3B).

Using the antibodies, we examined the expression pattern of HBP25's. Cross-reacting antigens were found in the pituitary cell

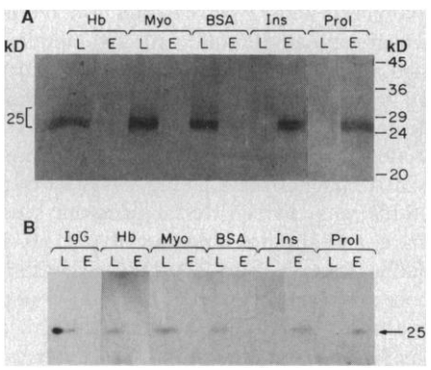

Fig. 3. Comparison of binding of the HBP25's to various columns. (**A**) The 25-kD proteins, isolated from a prolactin column as described in Fig. 1, were neutralized and again chromatographed on columns containing insulin (Ins), hemoglobin (Hb), or myoglobin (Myo). Prolactin (Prol) and BSA columns are included for comparison. For each column, the flow-through fractions collected during loading at neutral *p*H (indicated as lanes L) were compared with those collected during elution with *p*H 5.0 elution buffer I (indicated as lanes E). Proteins were analyzed by 10 to 18 percent SDS-PAGE and stained with Coomassie brilliant blue. (**B**) Immunoblotting of the column eluates with antiserum to purified HBP25's. Fractions from the various columns as described in (A) were separated by 10 to 18 percent SDS-PAGE and transferred to nitrocellular filter paper. The paper was incubated with a 1:20 dilution of rabbit antiserum to the 25-kD proteins (anti-25 kD), reacted with ^{125}I-labeled protein A, and analyzed by autoradiography. For lanes indicated as IgG, low *p*H eluates from an insulin column were neutralized and passed over an affinity column containing purified rabbit IgG, and the fractions were immunoblotted with anti-25 kD. The antigen binds to insulin and prolactin columns, but not to immunoglobulin, hemoglobin, myoglobin or BSA columns. Rabbit IgG was purified from nonimmune rabbit serum by protein A Sepharose (Repligen). IgG, human hemoglobin, horse myoglobin, BSA, porcine insulin, and sheep prolactin (the last five reagents from Sigma) were individually coupled to CNBr-activated Sepharose at 10 mg/ml (Pharmacia). Affinity chromatography was carried out as described for Figs. 1 and 2. Generation of polyclonal rabbit antiserum is described in the legend to Fig. 5. Immunoblotting was done essentially as described (*38*) with minor modifications. The paper was first incubated with 3 percent milk in phosphate-buffered saline (PBS), and the subsequent incubations with immunoreagents were in a mixture of 3 percent milk, 0.05 percent Tween-20, and PBS.

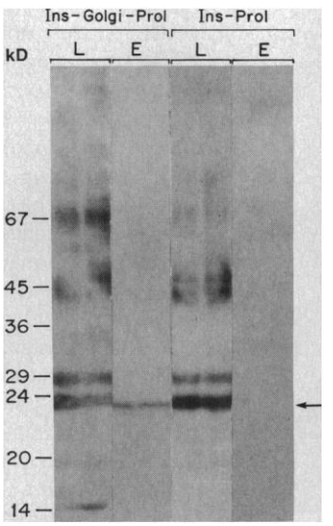

Fig. 4. Multivalent binding of HBP25's to hormones. Detergent-solubilized Golgi lysates were first loaded onto an insulin-Sepharose column to allow binding of HBP25's. To test whether the bound HBP25's could bind additional ligands, sheep prolactin (62.5 μg/ml in loading buffer) was passed over the column at neutral *p*H. The column was washed with neutral loading buffer, and then eluted with *p*H 5.0 elution buffer II to dissociate any bound prolactin. Fractions collected during loading of prolactin (indicated as lanes L) and those collected during *p*H 5.0 elution (indicated as lanes E) were analyzed on 10 to 18 percent SDS-PAGE and immunoblotted with antiserum to prolactin. For Ins-Golgi-Prol, the column was loaded with Golgi lysates before passage of prolactin. For Ins-Prol, the control column in which Golgi lysates were eliminated. Arrow on the right indicates the position of monomeric sheep prolactin. In the column that had been loaded with Golgi lysates, approximately 30 percent of the prolactin bound to the column and was recovered in the low *p*H eluates. In contrast, prolactin passed over a column which had not been loaded with Golgi lysates was recovered only in the flow-through fractions. In addition to monomeric prolactin, the antiserum also detected several species of larger molecular size that might represent aggregates of prolactin. Chromatography was performed as for Fig. 1. Immunoblotting was done with a 1:500 dilution of rabbit antiserum to sheep prolactin (U.S. Biochemicals).

line AtT-20, which is capable of regulated secretion (Fig. 6, lane 1). The antigens detected in AtT-20 cells have apparent molecular sizes similar to that of the dog pancreatic antigen. When compared next to each other on a 10 to 18 percent gel, the AtT-20 antigens migrate slightly more slowly than the pancreatic protein. In contrast, cross-reactive antigens were barely detectable in the pituitary cell line, GH_3 (Fig. 6, lane 2). This somatotroph-derived cell line has been shown to retain little of its ability to form storage granules in culture (*26*). However, treatment of these cells for 5 days with a mixture of hormones (estradiol, insulin, and epidermal growth factor) induces these cells to form regulated granules (*26*). To ascertain whether the amount of HBP25's was altered in cells that had been induced to accumulate regulated secretory granules, we treated GH_3 cells with the mixure of hormones and found that the cellular level of a 25-kD antigen was greatly enhanced. Quantitation of the immunoblot showed that the intracellular level of the 25-kD antigen was 44 times higher in induced cultures compared to uninduced cultures. Under these conditions, the number of storage granules in the cells was 50 times greater than that in control cells (*26*). In contrast, the levels of total cellular proteins and the lysosomal hydrolase, β-*N*-acetylglucosaminidase, were not appreciably affected in induced and uninduced cells; a 10-cm dish of control preparation contained 1.50 ± 0.20 (mean ± SE; $n = 2$) mg of total protein compared to 1.69 ± 0.08 ($n = 2$) mg for induced preparation, and 2.30 ± 0.04 ($n = 2$) glucosaminidase activity (micromoles of *p*-nitrophenol hydrolyzed per hour) compared to 2.49 ± 0.06 for induced preparation. These results showed that the level of hormone-binding proteins increased specifically in cells that were manufacturing more dense-core granules. The coordinate induction of HBP25's with the formation of regulated secretory granules is consistent with their possible role in granule assembly.

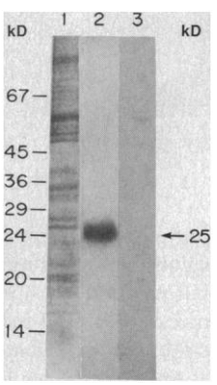

Fig. 5. Immunoblot analysis showing the specificity of antibodies to the HBP25's. Crude Golgi lysates were separated by 10 to 18 percent SDS-PAGE. (Lane 1) Total proteins (200-μl lysates) stained with Coomassie brilliant blue. (Lane 2) An identical sample immunoblotted with anti-25 kD. (Lane 3) An identical sample immunoblotted with pre-immune serum. Rabbit anti-25 kD was generated as follows. Dog pancreatic Golgi lysates were chromatographed on a prolactin column, and low *p*H eluates were loaded onto a 10 to 18 percent preparative SDS-PAGE. The 25-kD proteins (containing approximately three bands) were excised from the gel and electroeluted (*25*). Antigen (10 to 50 μg) was injected subcutaneously into five or more sites on the back of a New Zealand White rabbit. Injection was repreated six times at 2-week intervals. Serum was tested for antibody production by immunoblot analysis.

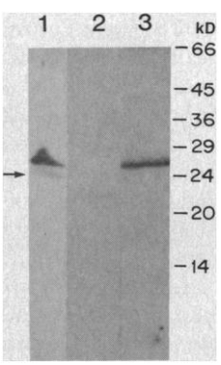

Fig. 6. Cross-reactivity of anti-25 kD in cultured cells. Extracts from AtT-20 cells and GH_3 cells were analyzed by SDS-PAGE and immunoblotted with anti-25 kD. (Lane 1) AtT-20. (Lane 2) GH_3 cells. (Lane 3) GH_3 cells treated with a mixture of hormones to induce formation of storage granules. Arrow on the left indicates the position of the canine HBP25's. One 10-cm dish of AtT-20 cells was harvested and a portion containing 1 mg of protein was boiled in SDS-gel sample buffer 10 minutes and analyzed on a 10 to 18 percent SDS-polyacrylamide gel. Immunoblotting with rabbit anti-25 kD was performed as described for Fig. 3. To study the effects of hormone-treatment on the level of HBP25's, two identical 10-cm dishes of GH_3 cells (subclone M2.1) were plated at subconfluent density. One of the dishes was treated with 1 n*M* estradiol, 300 n*M* insulin, and 10 n*M* epidermal growth factor for 5 days to induce storage granule formation (*26*). Cells were harvested (approximately 1 mg of protein per dish), and pellets were boiled in SDS-gel sample buffer for 10 minutes, separated on a 15 percent SDS polyacrylamide gel, and blotted with anti-25 kD.

The intracellular distribution of the HBP25's in AtT-20 cells (Fig. 7, A to C) was studied by immunocytochemistry. The polyclonal antiserum to the 25-kD antigen (anti-25 kD) gave a weak signal by indirect immunofluorescence. Thus, the more sensitive PAP (peroxidase–anti-peroxidase)-staining method was used to visualize the location of HBP25's. Specific staining was found concentrated in the perinuclear region (Fig. 7A). The staining was distributed over the same general area as the Golgi, although it appeared more diffuse than the staining pattern of wheat germ agglutinin (Fig. 7B). The antigens were not concentrated in mature storage granules since staining did not overlap with that for mature ACTH. The latter were localized at the tips of processes in AtT-20 cells (Fig. 7C). Staining of hormone-induced GH_3 cells with anti-25 kD also showed an intense perinuclear staining (Fig. 7D) similar to the Golgi staining by wheat germ agglutinin (Fig. 7E). No specific staining with anti-25 kD was detected in uninduced cells (Fig. 7F).

Possible role of the HBP25's in sorting. The hormone-binding proteins that we isolated from dog pancreatic tissues are likely candidates for the carriers that sort proteins into regulated secretory granules. They bind to the regulated hormones, prolactin and insulin, but do not show detectable affinities for immunoglobulin, hemoglobin, myoglobin, and BSA. Both immunoglobulin and albumin are natural constitutive secretory proteins, whereas globin, though normally a cytoplasmic protein, can be secreted constitutively if fused to a signal sequence. Thus, the proteins exhibit the expected ligand specificity. Furthermore, the levels of expression of the HBP25's correlate with the amount of storage granules manufactured. In cells induced to store regulated secretory granules, the HBP25's are greatly elevated.

While our purification scheme, in principle, could lead to the isolation of cell surface receptors that mediate the physiological responses to insulin and prolactin, it is unlikely that the HPB25's are physiological insulin or prolactin receptors. First, the molecular sizes of the previously identified prolactin receptor (40 to 45 kD) (*27*) and insulin receptor (130 kD) (*28–30*) are not in the same range as HBP25's. Second, localization of HBP25's in AtT-20 cells and induced GH_3 cells showed that most of the staining is intracellular, and not at the cell surface. Finally, the HBP25's bind to both insulin and prolactin, whereas physiological receptors are hormone-specific. At the trans-Golgi cisternae, where sorting takes place, proinsulin has not yet been processed to mature insulin (*31*). However, we used commercially available mature insulin, rather than proinsulin, because it was recently shown that deletion of the C peptide from proinsulin does not affect its ability to be sorted into regulated granules (*32*).

Two possible mechanisms have been proposed for sorting regulated secretory proteins away from constitutive proteins at the trans-Golgi cisternae. One is that the cellular carrier may be a membrane-bound receptor, which binds monomeric hormones and segregates them into clathrin-coated regions for incorporation into newly formed granules. However, electron-microscopic studies have shown that hormones aggregate at the site of sorting (*14*). This raises the second possibility, that the carrier may be a soluble cross-linking protein which causes aggregation of hormones. The aggregates could then be segregated by a membrane-bound receptor. The HBP25's could function in either or both of these processes. Upon freeze-thawing, the HBP25's were distributed approximately equally between membrane and soluble fractions. The membrane associated HBP25's were not released by sonication, but could be dissociated by washing at high *p*H. It is possible that HBP25's

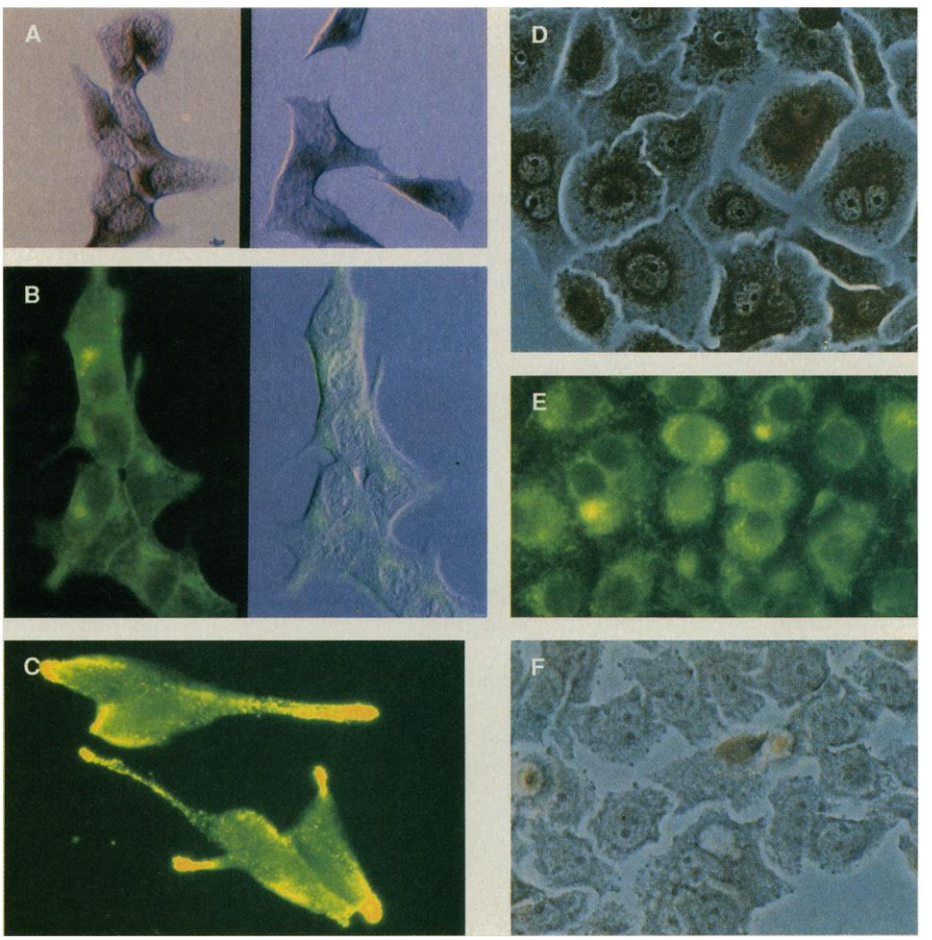

Fig. 7. (A to C) Immunolocalization of HBP25–cross-reactive antigens in AtT-20 cells. (**A**) PAP-staining of the HBP25 antigens in permeabilized AtT-20 cells. (**B**) Golgi apparatus stained with fluorescein isothiocyanate (FITC)–wheat germ agglutinin. At the left is a fluorescence image and at the right is a Nomarski image superimposed on the fluorescence image. (**C**) Mature regulated secretory granules detected by rabbit antiserum to ACTH and FITC-conjugated goat antiserum to rabbit IgG. (D to F) Immunolocalization of HBP25–cross-reactive antigens in GH_3 cells. (**D**) PAP-staining of the HBP25 antigens in permeabilized GH_3 cells treated with hormones to induce formation of storage granules. (**E**) Golgi apparatus stained with FITC-conjugated wheat germ agglutinin in hormone-treated GH_3 cells. (**F**) PAP-staining of HBP25 antigens in uninduced GH_3 cells. Cells were plated on glass cover slips coated with poly-D-lysine and laminin. Immunocytochemistry of HBP25's was carried out essentially as described (*39*). Cells were fixed in Bouin's fluid for 30 minutes, permeabilized for 5 minutes with 0.1 percent Triton X-100 in PBS, and reacted with rabbit anti-25 kD (1:100 dilution in PBS) for 1 hour, goat antiserum to rabbit Ig (Cappel, 1:10 in PBS) for 30 minutes at room temperature, and rabbit peroxidase–anti-peroxidase (Cappel, 1:100) for 30 minutes at 4°C. Rinsed cover slips were incubated with diaminobenzidine (Eastman Kodak) for 3 minutes, reacted with 2 percent OsO_4 for 10 minutes, and rinsed sequentially with distilled water, 70, 80, 95, and 100 percent ethanol, and xylene. To stain the Golgi apparatus, fixed cells were permeabilized for 15 minutes in PBT buffer (PBS, 1 percent BSA, 0.1 percent Triton X-100, 1 m*M* $MnCl_2$, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$), and incubated with FITC-conjugated wheat germ agglutinin (EY Laboratories, 50 μg/ml in PBT) for 30 minutes. Staining of mature secretory granules with anti-ACTH was done as described (*32*).

function both to aggregate hormones in the lumen, and to promote their association with the proper membrane for budding. Consistent with this hypothesis is the finding that HBP25's can cross-link two different hormones (Fig. 4). Whether each hormone also contains multiple binding sites for HBP25's is not yet known.

The fact that HBP25's do not appear to colocalize with mature ACTH (Fig. 7) suggests that they are probably not packaged in mature granules, but are likely to be recycled. Alternatively, they may be selectively degraded during maturation of storage granules. The reversible, *p*H-dependent binding of HBP25's to hormones offers a possible mechanism for their recycling. At the trans-Golgi cisternae where regulated proteins are segregated from constitutive proteins, the lumenal *p*H is near neutral (*33*). Loading of AtT-20 cells with the electron microscopic *p*H probe DAMP [3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine] (*34*) shows that this region does not accumulate DAMP significantly above the background (*35*). Thus, cellular carriers are expected to bind to hormones at neutral *p*H. This prediction is consistent with the observation that HBP25's bind to hormones at neutral *p*H. As granules pinch off from the Golgi, the lumen becomes acidified. Immature granules in islet β-cells have a lumenal *p*H of 5.5 to 6.5, and mature granules have a lumenal *p*H of 4.5 to 5.5 (*33*). Since HBP25's do not bind to hormones at low *p*H, the acidity of the lumen of the granules may cause the dissociation of hormones from their carriers. The empty carriers can then be recycled. Such a scheme predicts that neutralization of lumenal *p*H of the granules should prevent the dissociation of hormones from their carriers, thereby reducing the number of available carriers for further transport. It is interesting that when AtT-20 cells were treated with chloroquine, sorting of ACTH into regulated secretory granules was indeed inhibited (*36*). Thus, dissociation of HBP25's from hormones at low *p*H may be correlated with unloading of cargos at the target site and subsequent recycling.

An interesting question that remains to be answered is whether multiple binding proteins exist, each mediating a specific set of sorting events. Available evidence suggests that in some cells, different hormones are packaged into distinct storage granules (*37*). This raises the possibility that different carriers may exist to segregate different hormones. The dog pancreatic HBP25's consist of at least three molecular species. We have data showing that whereas all three bands bind to prolactin and insulin, only a subset of the 25-kD proteins binds to hGH. Thus, it is possible that the HBP25's belong to a family of proteins that mediate intracellular transport of regulated secretory proteins. We also have some evidence that the individual bands of the 25-kD triplet may be immunologically distinguishable, but the exact relationship among the three bands remains to be determined.

In conclusion, we have isolated a set of proteins from dog pancreatic tissues that exhibit properties expected of sorting carriers for regulated secretory proteins. These proteins bind to regulated hormones but not constitutively secreted proteins. They are present in the perinuclear regions of cells capable of regulated secretion. Furthermore, the level of these proteins is increased in cells that have been induced to form secretory granules. Further work is necessary before we can assert the biological function of these proteins; however, the evidence we have amassed so far is consistent with

their role in the molecular sorting of proteins to the regulated secretory pathway. Perhaps they are the long-sought sortases.

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