Constitutive and Basal Secretion from the Endocrine Cell Line, AtT-20

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Abstract. A variant of the ACTH-secreting pituitary cell line, AtT-20, has been isolated that does not make ACTH, sulfated proteins characteristic of the regulated secretory pathway, or dense-core secretory granules but retains constitutive secretion. Unlike wild type AtT-20 cells, the variant cannot store or release on stimulation, free glycosaminoglycan (GAG) chains. In addition, the variant cells cannot store trypsinogen or proinsulin, proteins that are targeted to dense core secretory granules in wild type cells. The regulated pathway could not be restored by transfecting with DNA encoding trypsinogen, a soluble regulated secretory protein targeted to secretory granules. A comparison of secretion from variant and wild type cells allows a distinction to be made between constitutive secretion and basal secretion, the spontaneous release of regulated proteins that occurs in the absence of stimulation.

In cells that have specialized regulated secretory vesicles, called secretory granules, sorting of the regulated secretory products away from constitutively secreted products and from lysosomal enzymes occurs in the *trans*-Golgi network (Griffiths and Simons, 1986; Tooze et al., 1987; Orci et al., 1987). The concentrated contents of the secretory granules are released from cells in response to external signals, whereas no signals are required for constitutive secretion. The mechanism of protein sorting between these two secretory pathways is not known; both a hormone-specific receptor mechanism (Chung et al., 1989) and selective condensation of secretory granule contents (Kelly, 1985; Gerdes et al., 1989) have been proposed.

Analysis of the secretory process is made easier when secretion-defective mutants are available. The steps involved in constitutive secretion have been elucidated in yeast using temperature-sensitive secretion mutants (Novick et al., 1980; Schekman, 1985). In mammalian cells, mutants have been described that influence secretion, but most of these mutations involve changes in the structure of the secretory protein itself, or changes in its posttranslational modification (for review see Tartakoff, 1983). Many examples of defective secretion involve proteins that are constitutively secreted, such as immunoglobulin chains (for review see Scharff et al., 1975) or viral glycoproteins (Tufaro et al., 1987). Mutations in the machinery of constitutive secretion are often lethal since the constitutive pathway is responsible for the normal membrane traffic vital for cell growth. Nonlethal mutations that affect the regulated secretory process have been reported for ciliated protozoa, for example in the exocytosis of trichocysts in *Paramecium* (Aufderheide, 1978; Lefort-Tran et al., 1981) and mucocysts in *Tetrahymena* (Orias et al., 1983; Maihle and Satir, 1985). In this paper we describe a mammalian cell variant that has lost its capacity for regulated secretion.

The mouse pituitary cell line, AtT-20, expresses the hormone, ACTH, and packages it in electron-dense secretory granules (Mains and Eipper, 1976). In addition to regulated secretion of ACTH, AtT-20 cells secrete proteins such as laminin from a constitutive secretory pathway (Burgess et al., 1985). In this paper we have characterized a variant of AtT-20 cells that does not express several soluble markers of the dense core secretory vesicle, including ACTH, and does not have morphologically identifiable dense core secretory granules. We are now able to experimentally distinguish in endocrine cells, two types of unstimulated secretion, basal release and constitutive secretion. In earlier estimates of the efficiency of sorting in AtT-20 cells we ignored basal secretion when measuring sorting into the regulated pathway (Moore and Kelly, 1985). The secretion properties of the variant cell line indicated that a considerable fraction of the protein sorted into the regulated pathway was secreted in the absence of stimulation by what we term basal secretion.

Materials and Methods

Materials

Synthetic DNA linkers, restriction endonucleases, and polynucleotide kinase were from New England Biolabs (Beverly, MA). DNA polymerase I (Klenow) and T4 DNA Ligase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). [^{35}S]methionine, [^{35}S]cysteine, and [^{35}S]sulfate were from Amersham Corp. (Arlington Heights, IL). Trans-[^{35}S]label was from ICN Radiochemicals (Irvine, CA). ^{125}I-ACTH, used as a tracer in the ACTH RIA was obtained from the Metabolic Research Lab at The Univer-

sity of California (San Francisco, CA). Affinity purified rabbit anti-ACTH antibodies used for immunofluorescence and RIA were described previously (Moore et al., 1983*b*). Rabbit anti-mouse κ chain antiserum and rabbit anti-guinea pig IgG were from ICN Biomedicals, Inc. (Irvine, CA). Guinea pig anti-porcine or anti-human insulin and rabbit anti-human insulin C peptide were obtained from Linco Inc. (St. Louis, MO). Rabbit anti-trypsinogen was obtained from Dr. Charles Craik (University of California, San Francisco, CA). Rabbit anti-sheep IgG was from Organon Teknika-Cappel (Malvern, PA). Rabbit anti-rat synaptic vesicle antiserum that recognized synaptophysin (p38) was prepared by Dr. Dan Cutler and Dr. Reg Kelly (Cutler and Cramer, 1990). Mouse monoclonal anti-synaptophysin was from Boehringer Mannheim Biochemicals. Protein A-bearing *Staphylococcus aureus*, Cowan strain bacterial cells (SAC)[1] (Pansorbin or Zysorbin) were from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA) or Zymed Labs (San Francisco, CA). 8-bromo-(8-Br) cAMP, ouabain, and 4-methyl umbelliferyl β-D-xyloside were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

The MPC-11 myeloma derivative, 4TOO1 (Margulies et al., 1976), resistant to 2.5 mM ouabain and 5 μg/ml 6-thioguanine but sensitive to HAT medium (100 μM hypoxanthine, 30 μM thymidine, and 10 μM aminopterin) was originally derived by Dr. Matthew D. Scharff (Albert Einstein College of Medicine, Bronx, NY) and obtained from Dr. Sherie L. Morrison (University of California, Los Angeles, CA). Cells were grown at 37°C in DME H21, (4.5 g glucose/liter) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), penicillin/streptomycin, and kept in an atmosphere of 5% CO_2. AtT-20 F2 cells were as described (Mains and Eipper, 1976; Gumbiner and Kelly, 1982). AtT20/D16V-WT#6 cells (Sabol, 1980) were from Laurel Thomas (Oregon Health Sciences University, Portland, OR). AtT-20 cells were grown at 37°C in DME H21 containing 10% FCS, penicillin/streptomycin, and kept in an atmosphere of 15% (for F2 cells) or 10% (for WT#6 cells) CO_2.

Construction of Expression Vectors

The pRSV trypsinogen expression vector was as described previously (Burgess et al., 1987). Other expression vectors containing genomic clones carrying their endogenous polyadenylation signals were constructed in a derivative of an RSV-LTR plasmid containing the chloramphenicol acetyltransferase (CAT) gene (Gorman et al., 1982). In these vectors, the CAT gene was removed and replaced with either a Bam HI or a Hind III site, using DNA linkers. pBR322-MPC-11 κ (Kelley et al., 1982), containing an 8.5-kb Bam HI fragment with the entire rearranged mouse Ig κ chain genomic clone from the MPC-11 myeloma (Laskov and Scharff, 1970), was obtained from Dr. Brain Van Ness (University of Iowa, Iowa City, IO). The 8.5-kb κ chain genomic clone was cleaved with Pvu II, which removed unwanted 5′ sequences and brought the start site of the kappa chain gene within 30 bp of the 5′ end of the DNA fragment. This truncated Pvu II-Bam HI fragment was adapted with Bam HI linkers and cloned into an RSV-LTR-containing expression vector at the Bam HI site. pBR322-human proinsulin, pInsC2 (Laub and Rutter, 1983), was obtained from Dr. Michael Walker and Dr. William J. Rutter (University of California, San Francisco, CA). The proinsulin gene contained in this plasmid is a chimera between a cDNA at the 5′ end and 3′ genomic sequences carrying endogenous polyadenylation signals. The proinsulin gene on a 1.2-kb Eco RI fragment was removed from pInsC2 and cloned into an RSV-containing expression vector using Hind III linkers. All large scale plasmid preps were isolated from HB101 bacterial cells by the cleared lysate technique (Clewell and Helinski, 1972) and plasmids were purified through two equilibrium gradients of cesium chloride using standard procedures (Maniatis et al., 1982).

DNA Transfections

DNA transfections of AtT-20 cells was by a calcium phosphate precipitation protocol (Moore et al., 1983*b*). For stable transformation, 100 μg (for AtT-20 F2) or 50 μg (for AtT-20 WT#6) of various plasmid DNAs and 20 μg of a selectable DNA pSV2-neo (Southern and Berg, 1982), or pTK-hygromycin resistance plasmid (pHS-53; from Dr. Dan Littman, University of California, San Francisco, CA), were mixed and applied as a calcium phosphate precipitate to 10-cm dishes (~3 × 10^5 cells/dish) of AtT-20 cells. Stable clones were selected in either 0.25 mg/ml (for F2) or 0.5 mg/ml (for WT#6) of the antibiotic G418 (Geneticin; Gibco Laboratories, Grand Island, NY) or with 0.175 mg/ml hygromycin (Boehringer Mannheim Biochemicals). Drug-resistant clones were screened for production of the desired protein by metabolic labeling followed by immunoprecipitation of cell extracts and culture media with the appropriate antibodies and fixed protein A–containing bacterial cells. Immunoprecipitates were analyzed with SDS-PAGE followed by autoradiography. Transfected wild type AtT-20 F2 cells expressing trypsinogen are as described (Burgess et al., 1987). AtT-20 WT#6 cells expressing proinsulin were generated by Dr. Sam Green (University of California, San Francisco, CA) using the human proinsulin-containing expression vector described above.

Isolation of Variants

Variants of AtT-20 cells, HYA.15.6 and HYA.15.10, were isolated as slow growing clones that survived several rounds of drug selection during a somatic cell hybridization procedure (Kohler and Milstein, 1976; Margulies et al., 1976) between AtT-20 F2 cells (HAT resistant, ouabain sensitive) and the mouse myeloma, 4TOO1 (HAT sensitive, ouabain resistant). Equal numbers of both cell types were mixed in suspension centrifuged at low speed (2,000 *g*) to form a pellet. The cells were resuspended in serum-free DME containing 35% polyethylene glycol 4,000 (PEG), pH 8.2, incubated for 90 s and then immediately diluted with 40 ml of fresh medium. Cells were removed from the PEG by low-speed centrifugation and then resuspended in DME containing 10% FCS, HAT, and 2.5 mM oubain. The selection medium was changed every 48 h until >99% of the cells were killed. Slower growing clones exhibiting the qualities described in the results were recovered and subcloned additional times to ensure their homogeneity.

Metabolic Labeling and Immunoprecipitation

Sulfated proteins were labeled by incubating cells for 18 h at normal growth conditions of 37°C and 10–15% CO_2, in sulfate-depleted DME H21 containing 5 mCi [^{35}S]sulfate, penicillin, streptomycin, glutamine, and 10% dialyzed FCS as described (Moore et al., 1983*a*). Cells were then incubated in serum-free DME H21 for two 1.5-h intervals, the latter interval in the presence or absence of 5 mM 8-Br-cAMP. Samples were precipitated with cold 10% TCA containing 1 mg/ml deoxycholate, washed twice with cold acetone, and redissolved in SDS-PAGE sample buffer. Analysis was performed on 12–20% polyacrylamide gradient gels, which were soaked in 0.5 M sodium salicylate containing 30% methanol, dried, and exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY).

Sulfated, free GAG chains were generated as described (Burgess and Kelly, 1984) by preincubating cells for 2.5–3 h in DME H21 growth medium containing 0.5 mM 4-methylumbelliferyl β-D-xyloside and then incubating the cells in sulfate-depleted DME H21 containing xyloside and 5 mCi [^{35}S]sulfate. Cells were chased by incubating for 2.5 h in serum-free medium containing xyloside. The medium was collected, and then analyzed directly on 12–20% polyacrylamide gels that were processed for autoradiography as described above. Pulse-chase experiments to follow free GAG chains were performed by preparing confluent six-well dishes (12 wells), preincubating for 1 h in DME containing 0.5 mM xyloside, shortening the labeling time in 5 mCi [^{35}S]sulfate to 1 h, washing the cells with PBS, and incubating for 15, 30, 45, 60, 90, 120, 150, or 210 min in complete growth medium. At 60 min of chase, 5 mM 8-Br-cAMP was added to one well of cells and the media collected at 150 min. At 150 min of chase, cAMP was added to a second and the media collected at 210 min. At the end of the chase intervals, media were removed from the cells, dialyzed for 24 h at 4°C against three changes of 50 mM NH_4HCO_3, dried in a Speed Vac (Savant Instruments, Hicksville, NY) for 6 h, dissolved in SDS-PAGE sample buffer, and then analyzed on 12–20% polyacrylamide gels as described above. Cell extracts were prepared by direct lysis in NDET buffer (1% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.4; Burgess et al., 1985), removing the nuclei, adding SDS-PAGE sample buffer, and loading portions directly on polyacrylamide gels. GAG chains were quantified by cutting out the parts of the gels containing the GAG chains, dissolving in NCS tissue-homogenizing solution (Amersham Corp.) or TS-1 tissue solubilizer (Research Products International Corp., Mt. Prospect, IL), and counting in a liquid scintillation counter.

To screen transfected clones of cells for the production of trypsinogen or Ig κ light chain 10^4–10^5 cells were incubated for 5 h at 37°C in 15% CO_2 in DME H21 lacking methionine and cysteine and supplemented with 2%

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; 8-Br, 8-bromide; GAG, glycosaminoglycan; PEG, polyethylene glycol; SAC, Cowan strain bacterial cells.

FCS and 100–200 μCi of either [^{35}S]methionine, [^{35}S]cysteine, or Trans [^{35}S]label. The culture media and cell extracts were recovered and adjusted to a final concentration of 0.3% SDS and NDET. Samples were precleared by incubation for 30 min at room temperature with 100 μl of prewashed SAC (Pansorbin or Zysorbin). The material that nonspecifically bound to the SAC was removed by centrifugation and the samples were incubated for 16 h at 4°C with specific antiserum, either rabbit anti-mouse κ or rabbit anti-rat trypsinogen. The amounts and types of antiserum to be used for immunoprecipitations were optimized using derivatives of the mouse myeloma MPC-11 expressing κ light chain and transfected wild type AtT-20 cells expressing trypsinogen (Baumal et al., 1973; Burgess et al., 1987). Soluble immunoprecipitates were recovered by incubating with fixed SAC for 30 min at room temperature. The preparation of samples for analysis by SDS-PAGE and autoradiography were as described (Burgess et al., 1985).

To examine the secreted proteins in the medium after stimulation with the secretagogue, 8-Br-cAMP, 10^6–10^7 cells were treated as described (Burgess et al., 1985, 1987). Trypsinogen-expressing wild type AtT-20 cells or variant HYA.15.6.T.3 cells were incubated first for 16 h in depleted DME containing 200 μCi [^{35}S]cysteine, 10% FCS, glutamine, penicillin, streptomycin, and 1/5th volume complete medium, and then for a 1-h interval in fresh labeling medium containing [^{35}S]cysteine at the same specific activity. After the labeling period, cells were washed twice with PBS and chased in normal media for three 2.5-h intervals. During the third interval one portion of cells was incubated in the presence of 5 mM 8-Br-cAMP to stimulate release from the regulated secretory pathway. The media and cell extracts were collected at each time interval and immunoprecipitated with rabbit anti-trypsinogen, anti-proinsulin or anti-kappa antiserum as described. Immunoprecipitates were analyzed on SDS-PAGE gels and the amount of material that was secreted was quantified by cutting out the bands from the gels, eluting the labeled proteins in tissue-solubilizing solution at 60°C for 16 h and counting in a liquid scintillation counter and by scanning the autoradiograph of the gel with a soft laser densitometer (LKB Instruments, Inc., Bromma, Sweden). Proinsulin secretion were examined by essentially the same procedure except that immunoprecipitates were washed in distilled water to remove detergent and analyzed on 12–20% gradient SDS-PAGE gels. The identity of the proinsulin and mature processed insulin bands were distinguished on gels by molecular weight and by using two antiserum, a rabbit anti-human C peptide (recognizes proinsulin only) and a guinea pig anti-mature insulin (recognizes both forms). κ chain secretion by transfected AtT20 cells was determined as described for trypsinogen except that 100 μCi [^{35}S]methionine was used for the labeling and cells were chased for two 3-h intervals, the latter interval in the presence or absence of 8-Br-cAMP. The amounts of the endogenous hormone, ACTH, that were released into the media during the stimulation interval was determined by a solid-phase ACTH radioimmunoassay that has been previously described (Moore et al., 1983*b*). Protein determinations were assayed by an Amido Schwartz dye-binding assay (Moore et al., 1983*b*).

Synaptophysin was detected by immunoprecipitation of cell extracts labeled for 16 h with Trans [^{35}S]label with either a rabbit anti-rat synaptic vesicle antiserum (Cutler and Cramer, 1990) that recognized synaptophysin or with preimmune serum, followed by SAC.

Immunofluorescence and EM

Anti-ACTH immunofluorescence was performed as described (Matsuuchi et al., 1988). Cells were grown on poly-D-lysine-coated glass coverslips, incubated with rabbit anti-ACTH antibodies and sheep anti-rabbit IgG coupled to fluorescein (Cappel Laboratories, Cochranville, PA). Antisynaptophysin (p38) immunofluorescence was done on fixed and 0.01% Triton X-100 permeabilized cells with the monoclonal antibody, SY38 (Boehringer Mannheim Biochemicals) diluted 1:2, followed by fluorescein-coupled goat anti-mouse γ1 (diluted 1:10) as described (Matsuuchi et al., 1988). EM was performed as described (Matsuuchi et al., 1988).

Results

AtT-20 Variants Lacking ACTH

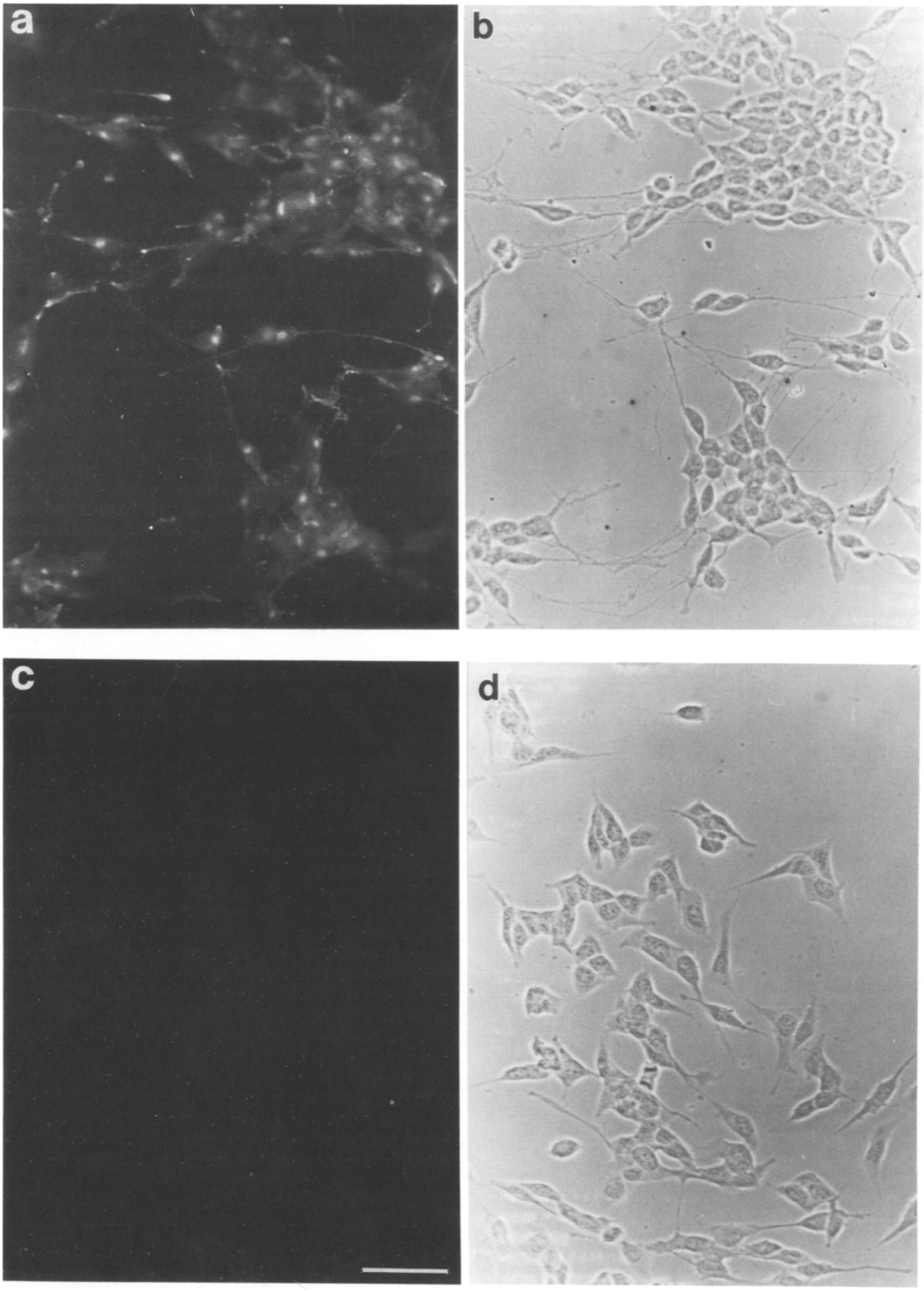
In an attempt to generate antibody-secreting endocrine cells, the mouse pituitary cell line AtT-20, which is ouabain sensitive and HAT resistant, was fused to the mouse plasmacytoma, 4TOO1, which is ouabain resistant and HAT sensitive. Clones were selected that survived several rounds of selection in medium containing ouabain (2.5 mM) and HAT. The surviving clones differed from wild type cells in that they grew slowly and extended thin branching processes several cell bodies in length. Individual subclones were tested by immunofluorescence, after fixation and detergent permeabilization of cells, for the expression of the endogenous hormone expressed by AtT-20 cells, ACTH. Metabolically labeled clones were also tested by immunoprecipitation for expression of the antibody produced by the plasmacytoma, IgG2b,κ. Of 33 subclones that were recovered, none expressed antibodies, 30 expressed ACTH, two were mixed, containing both ACTH expressing and nonexpressing cells, and one appeared to be an ACTH nonexpressor. None of the subclones retained the ouabain resistance of the myeloma parent.

The ACTH nonexpressor was subcloned two additional times to ensure that no cells in the culture were expressing ACTH (HYA.15.6), then further characterized. Sibling clones of cells that expressed ACTH were also isolated during the subcloning of HY.15.6 for use as ATCH-expressing control cells (HYA.15.10). AtT-20 cells normally extend processes when plated on glass or plastic substrates. This process can be enhanced by incubating cells in growth medium containing 5 mM 8-Br-cAMP (Burgess and Kelly, 1985). When HYA.15.6 or HYA.15.10 cells were plated on glass coverslips, however, they both extended long, thin processes without stimulation with the secretagogue, 8-Br-cAMP. ACTH could be detected by immunofluorescence in HYA.15.10 cells but not in HYA.15.6 cells (Fig. 1). A sensitive radioimmunoassay was used to detect ACTH in cell extracts. Wild type AtT-20 cells contained 29.9 ng ACTH/μg cell protein, HYA.15.10 cells contained 1.4 ng ACTH/μg cell protein, and HYA.15.6 cells contained less than 0.10 ng ACTH/μg cell protein (the limit of detection of this assay), at least 300-fold less than wild type cells.

Absence of the Regulated Pathway in the AtT-20 Variant

In normal AtT-20 cells, ACTH is condensed and packaged into secretory granules, which have electron-dense cores when observed in electron micrographs. To see if dense core secretory granules were present or absent in the HYA.15.6 variant cells, sections of epon-embedded cells were examined in the electron microscope. Electron micrographs of 25 random fields of ACTH-negative HYA.15.6 or ACTH-positive HYA.15.10 cells were taken and the numbers of dense core granules counted. Photos of ACTH-positive cells (HYA.15.10) contained 681 morphologically identifiable dense core vesicles. In contrast, photos of the ACTH-negative cells (HYA.15.6), contained only one dense structure that could have been a dense core vesicle.

If the variant line had lost the regulated pathway, it should not be able to store, and secrete on stimulation the other soluble components of dense core secretory granules. These include chromogranin A (Iacangelo et al., 1988), secretogranins (Rosa et al., 1985), sulfated proteins such as B15 (Moore et al., 1983*a*), and the "sulfated staircase" which represent proteoglycans that are sulfated on glycosaminoglycan (GAG) side chains (Moore et al., 1983*a*; Burgess and Kelly, 1984). Many of these components were not expressed by the HYA.15.6 variant. The sulfated proteoglycans and proteins,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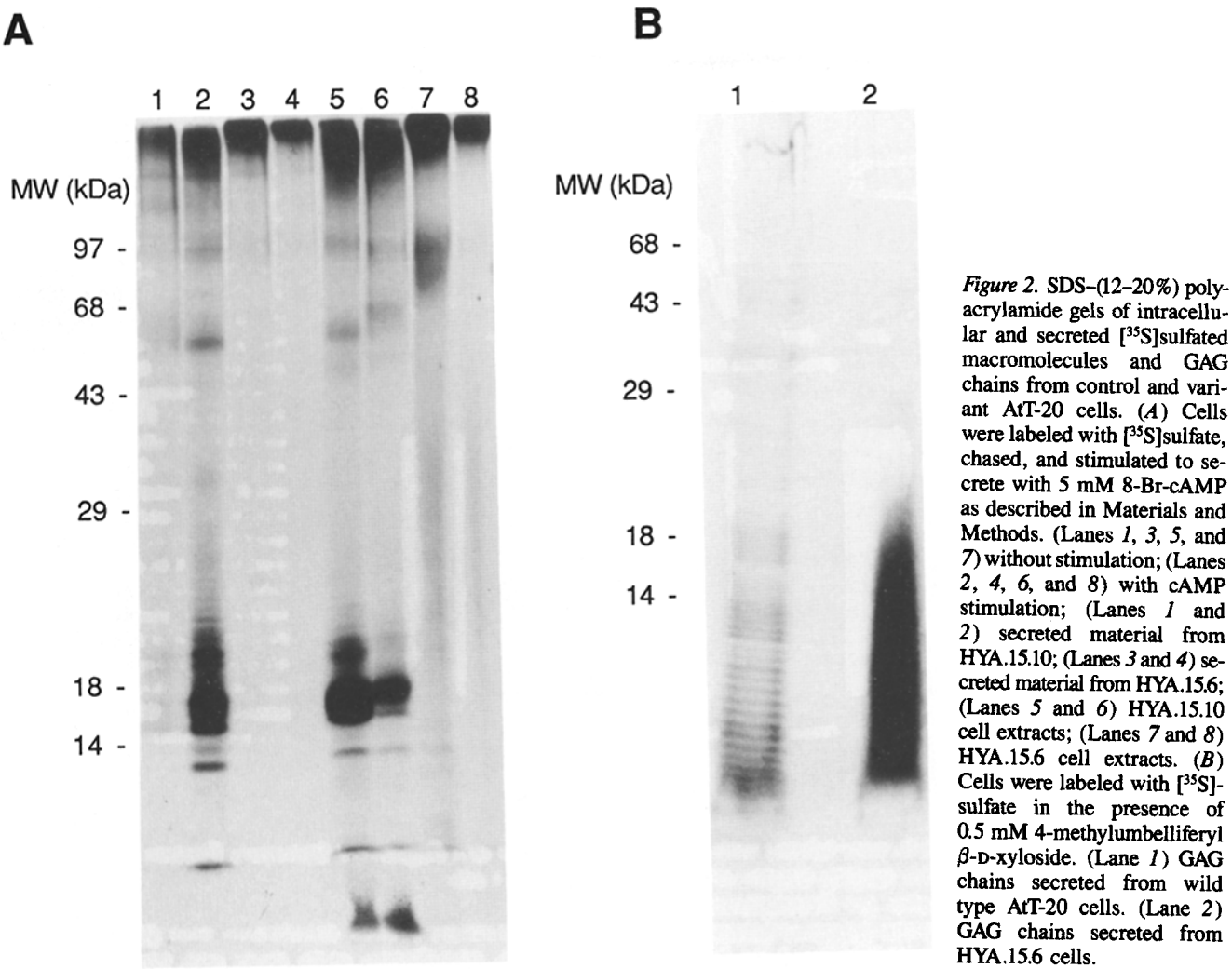

Figure 2. SDS–(12–20%) polyacrylamide gels of intracellular and secreted [^{35}S]sulfated macromolecules and GAG chains from control and variant AtT-20 cells. (*A*) Cells were labeled with [^{35}S]sulfate, chased, and stimulated to secrete with 5 mM 8-Br-cAMP as described in Materials and Methods. (Lanes *1*, *3*, *5*, and *7*) without stimulation; (Lanes *2*, *4*, *6*, and *8*) with cAMP stimulation; (Lanes *1* and *2*) secreted material from HYA.15.10; (Lanes *3* and *4*) secreted material from HYA.15.6; (Lanes *5* and *6*) HYA.15.10 cell extracts; (Lanes *7* and *8*) HYA.15.6 cell extracts. (*B*) Cells were labeled with [^{35}S]-sulfate in the presence of 0.5 mM 4-methylumbelliferyl β-D-xyloside. (Lane *1*) GAG chains secreted from wild type AtT-20 cells. (Lane *2*) GAG chains secreted from HYA.15.6 cells.

sorted into the regulated secretory pathway and released on stimulation of cells containing the regulated secretory pathway, HYA.15.10 (Fig. 2 *A*, lanes *1* and *2*), were not made or released into the medium by the HYA.15.6 variant (Fig. 2 *A*, lanes *3* and *4*). The only sulfated material in extracts of HYA.15.6 cells was higher molecular weight material that did not enter the gel and some material that migrated as a diffuse band (Fig. 2 *A*, lanes *7* and *8*). Although some sulfated material migrating at 97 kD could be seen decreasing in the cell extracts after stimulation, a corresponding appearance in the stimulated medium could never be reproducibly detected. In addition, neither chromogranin A nor its mRNA could be easily detected in HYA.15.6 cells because expression was at such low levels (Grimes, M., and R. B. Kelly, unpublished results).

Although the dense core vesicle-specific proteoglycans and other sulfated molecules were absent in the HYA.15.6 variant cells, the machinery to synthesize the glycosaminoglycan side chains of proteoglycans was still intact. This was shown by preincubating cells in medium containing 0.5 mM 4-methyl-umbelliferyl xyloside and labeling cells, in the continued presence of xyloside, with [^{35}S]sulfate. Xylosides compete with the protein core of proteoglycans for attachment of free GAG chains. The ^{35}S-labeled free GAG chain xyloside conjugates in cell extracts and media were recovered and analyzed on SDS–polyacrylamide gels. Wild type AtT-20 cells, grown in the xyloside analogue, secreted into the medium a ladder of GAG chains, migrating between the protein molecular weight markers of 2 and 18 kD (Fig. 2 *B*, lane *1*). The HYA.15.6 variant cells, grown under the same conditions, secreted a ladder of sulfated GAG chains of roughly the same molecular weight although the ladder was less discrete (Fig. 2 *B*, lane *2*).

To test the possibility that secretory granules were present in the variant, but lacked identifiable markers, the storage and release of free GAG chains were used as a nonspecific granule content marker. In earlier work, ^{35}S-sulfated GAG chains appeared to segregate as a bulk phase marker into both regulated and constitutive pathways (Burgess and Kelly, 1984). To measure GAG storage and release from the regu-

Figure 1. Indirect immunofluorescence and phase images of control and variant AtT-20 cells. Cells were permeabilized and stained for ACTH (*a* and *c*) or observed by phase microscopy (*b* and *d*). HYA.15.10 (*a* and *b*); HYA.15.6 (*c* and *d*). Bar, 200 μm.

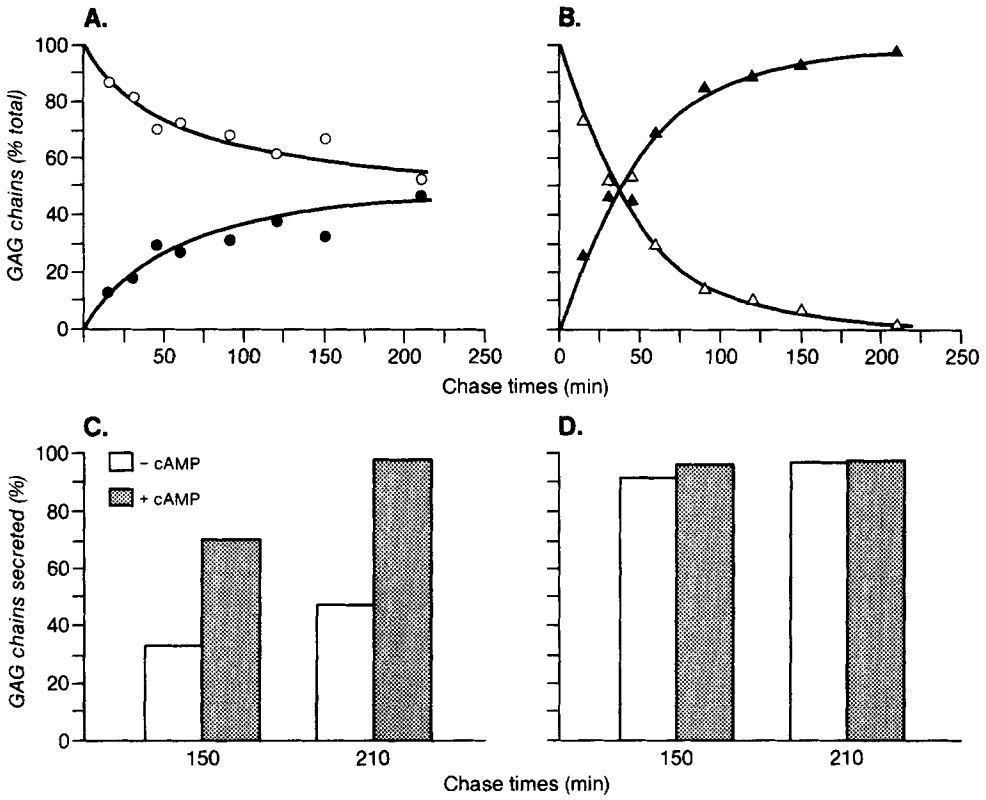

Figure 3. Kinetics of secretion of free GAG chains from wild-type and variant AtT-20 cells. (*A* and *B*) Wild type AtT-20 (*A*) and variant HYA.15.6 (*B*) cells were labeled for 1 h with [^{35}S]-sulfate and chased for 15, 30, 45, 60, 90, 120, 150, or 210 min. Secreted (●,▲) and intracellular (○,△) GAG chains were recovered from SDS-polyacrylamide by dissolving the gel in tissue solubilizer and quantified by liquid scintillation counting. (*C* and *D*) Wild type AtT-20 (*C*) and variant HYA.15.6 (*D*) cells, pulsed and chased as described in *A* and *B*, were taken at 60 or 150 min of chase and either stimulated to secrete GAG chains with 5 mM 8-Br-cAMP or not. GAG chains in the secreted media were recovered at either 150 min of chase (for the 60-min addition) or at 210 min of chase (for the 150-min addition). (□) Without cAMP; (▨) with cAMP.

lated pathway, wild type AtT-20 cells were labeled with a 1-h-pulse of [^{35}S]sulfate and chased for varying lengths of time. The [^{35}S]GAGs were identified after gel electrophoresis as the ladder of radioactive material that migrated between the protein molecular weights of 2.3–14 kD (Fig. 2 *B*). In wild type cells, 40% of the [^{35}S]GAGs were secreted into the medium with a half time of ~30 min, while the remainder of the GAGs was stored intracellularly (Fig. 3 *A*). After a chase period, treatment with 8-Br-cAMP increased the amount of GAG chains released into the media twofold, suggesting that GAGs were indeed stored in the regulated secretory pathway (Fig. 3 *C*). In contrast, variant cells released 98% of their [^{35}S]GAG chains, with a halftime also of ~30 min (Fig. 3 *B*). No stimulation of secretion of the remaining 2% of the GAG chains could be seen after the addition of 8-Br-cAMP (Fig. 3 *D*). Longer incubation times of cells in xyloside and [^{35}S]sulfate more extensively labeled the stored pools of [^{35}S]GAG chains in wild type cells, since they have a half-time longer than 1 hour. No evidence of storage of GAG chains or their regulated release could be detected in the variant cells even under these conditions. Wild type AtT-20 cells were over twenty times more efficient at storing [^{35}S]GAGs than the variant and the GAGs were stored as efficiently as trypsinogen, ACTH, and insulin. Either the variant did not have secretory granules into which GAGs may flow, or the GAGs could not enter the granules because of missing components of the sorting machinery. The apparent efficiency of sorting of the GAG chains into the regulated pathway is not consistent with the earlier assumption (Burgess and Kelly, 1984) that they can be used as a bulk phase marker.

Expression of a Regulated Secretory Protein in Variant Cells

If empty secretory granule membranes were present in the variant cells, and absence of the dense core was because of the lack of specific contents (as opposed to free GAG chains) to be packaged in the membranes, then secretory granules might be induced in the variant by introducing a protein normally packaged into vesicles of the regulated pathway. To test this possibility, trypsinogen, a protein that is sorted into regulated granules was expressed in HYA.15.6 variant cells by DNA-mediated transfection. Trypsinogen is targeted in transfected wild type cells to the same regulated secretory compartment that houses the endocrine hormones, ACTH, and insulin (Burgess et al., 1985, 1987; Orci et al., 1987). To test for the formation of a regulated pathway in trypsinogen transfected AtT-20 variants, cells were labeled for 16–20 h in medium containing [^{35}S]methionine or cysteine, chased for several intervals, and then stimulated to release the labeled material in the regulated pathway (Moore and Kelly, 1985; Burgess et al., 1985). In agreement with previous reports, trypsinogen secretion from wild type cells was characteristic of the regulated secretory pathway (Burgess et al., 1985; Moore et al., 1983*b*). There was a slow release of labeled trypsinogen in the absence of stimulation. Only 13% of the total labeled trypsinogen was released in the first chase period and the secretion rate fell off with a half time of 5 h. A 5.3-fold increase in release of trypsinogen was obtained when the secretagogue 8-Br-cAMP was present during the last chase period (Fig. 4 *A*). In contrast, trypsinogen secretion from the HYA.15.6.T.3 variant was much more rapid.

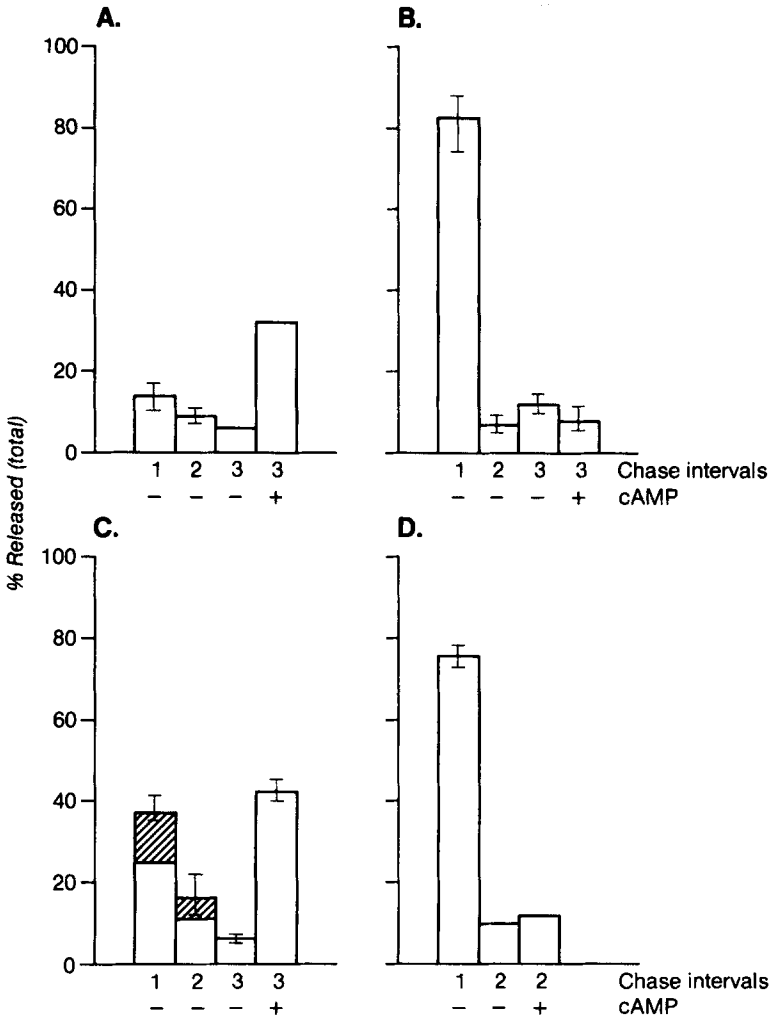

Figure 4. Kinetics of trypsinogen, proinsulin/insulin, and mouse immunoglobulin κ secretion from either transfected wild-type AtT20 cells or from transfected variant HYA.15.6.T.3 cells. Cells were labeled with [^{35}S]Met or [^{35}S]Cys for 18 h to approach steady-state labeling conditions, Two (*D*) or three (*A*, *B*, and *C*) consecutive 2.5-h chases were done after the labeling period (refers to numbers *1*, *2*, and *3* chase intervals), with the last chase interval done in the presence or absence of 5 mM 8-Br-cAMP. Secreted and intracellular proteins were recovered by immunoprecipitation. (*A*) Trypsinogen secreted from transfected AtT-20 cells. (*B*) Trypsinogen secreted from transfected variant HYA.15.6 cells. (*C*) Proinsulin (▨) and mature insulin (□) secreted from AtT-20 cells. (*D*) Mouse immunoglobulin κ chain (from the MPC-11 mouse myeloma tumor) secreted from AtT-20 cells.

90% of the trypsinogen was released during the first chase interval, consistent with a half time of secretion of ~40 min. The amount of trypsinogen that was secreted was not increased by exposure to 8-Br-cAMP (Fig. 4 *B*). Expression of the regulated secretory protein, trypsinogen, by the variant cells does not, therefore, induce formation of the regulated secretory pathway or reveal the presence of empty secretory granules, lacking content proteins.

The unstimulated release of trypsinogen by the variant, corresponding to a half time of secretion of ~40 min, is considerably faster than unstimulated release by wild type AtT-20 ($t_{1/2}$ = 5 h). The rate of trypsinogen release from unstimulated variant cells was compared to the rate of constitutive secretion of immunoglobulin chains by wild type cells. In plasma cells, secretion of antibodies has been shown to be constitutive, with no evidence for their storage or their stimulated release (Tartakoff and Vassalli, 1978; Tartakoff et al., 1977). Antibodies are convenient constitutive secretory proteins to study because the genes for the immunoglobulin heavy and light chains expressed by many plasma cell tumor lines have been cloned. A cell line of particular interest, the MPC-11 plasmacytoma, expresses both IgG2b heavy chain and κ light chain but over-expresses the latter. The κ light chains not assembled with heavy chain are secreted freely into the medium (Laskov and Scharff, 1970), making κ chain a good candidate for a simple, soluble product to use as a constitutive secretory marker in nonlymphoid cells such as AtT-20 cells. AtT-20 cells transfected with the MPC11 κ light chain gene made high levels of κ chain, and secreted it with kinetics that were identical to that of immunoglobulin secretion in normal plasma cell lines. In transfected cells, 75% of the labeled κ light chain was secreted during the first chase interval and the half time of secretion was ~1 h. Stimulation with 8-Br-cAMP did not enhance the amount of light chain secreted (Fig. 4 *D*). Since transfected trypsinogen was secreted from the AtT-20 variant at a rate indistinguishable from that of the constitutively secreted κ-light chain in transfected wild type cells (Fig. 4, *B* and *D*), it is very likely that unstimulated secretion in the variant is from the constitutive secretory pathway.

If constitutive secretion of trypsinogen and κ chain has a half time of ~1 h, as the data from the variant show, the slow unstimulated release of trypsinogen from wild type cells (Fig. 4 *A*) must have another explanation. One possibility is that unstimulated release in wild type cells is not due entirely to constitutive secretion but also because of basal release. Basal release is secretion from the regulated pathway in the absence of prior stimulation with a secretagogue. If a

secreted prohormone is processed to its mature form after sorting into the regulated pathway (Orci et al., 1987*b*), then basal release will appear as the unstimulated release of mature hormone. When the secretion of insulin forms was examined in wild type cells (Fig. 4 *C*), mature insulin was indeed released in the absence of stimulation. During the chase, the rate of [^{35}S]labeled insulin secretion dropped with a half life of $\sim$3 h, while the rate of proinsulin secretion fell with a half time of only 40 min. The kinetics suggest that mature insulin is released basally from secretory granules and proinsulin from the constitutive pathway. Consistent with this explanation is the observation that whereas exposure to 8-Br-cAMP increased the rate of insulin secretion sevenfold, it had no effect on the rate of proinsulin secretion. Proinsulin-transfected HYA.15.6 variant cells secreted proinsulin and neither stored it nor processed the prohormone to the mature form (data not shown). Comparison of the variant and wild type cells clearly demonstrates therefore, two forms of unstimulated release, constitutive and basal.

Discussion

In the initial experiments on pathways of secretory protein sorting in AtT-20 cells, the regulated pathway was functionally defined by secretion of the ACTH hormone in response to a secretagogue while the constitutive pathway was defined by the externalization of a viral membrane protein, gp70, which did not require exposure to a secretagogue (Gumbiner and Kelly, 1982). Unresolved in this work and in subsequent studies was whether constitutive secretion accounted for all unstimulated release, especially the slow release observed for "regulated" proteins, endogenous as well as transfected (Burgess et al., 1985; Moore and Kelly, 1985). The AtT-20 variant was clearly lacking the regulated secretory pathway and all secretion from these cells was rapid and constitutive. The characteristics of secretion by the variant allow us to propose that there must be two pathways of unstimulated release in wild type cells: constitutive secretion, as described in our earlier work, and basal release from compartments that come after sorting into the regulated pathway. Since the regulated pathway is missing from the variant, basal release must also be missing. Basal release may be because of the fusion of mature regulated secretory granules with the plasma membrane in the absence of stimulation or the budding of vesicles from immature granules as proposed by von Zastrow et al. (1987). Whatever the mechanism of basal release, it accounts for a large fraction of the total secreted protein and has important consequences for the analysis of sorting in neuroendocrine cell lines. Thus measuring the ratio of stimulated to unstimulated release need not be a measure of sorting but a measure of what fraction of the material sorted into the regulated pathway that can be released by stimulation with secretagogues. Furthermore, the sorting index used by Moore and Kelly (1985) to measure protein sorting efficiencies, by ignoring basal release, underestimates the ability of AtT-20 cells to correctly sort proteins into the regulated pathway.

Basal release from endocrine tissue may be physiologically relevant. If endocrine cells in an organism have a basal level of secretion then they can respond to both positive and negative stimuli, to raise or lower hormone levels in the blood. The presence of basal secretion in tumor cell lines is perhaps because of a property of normal cells rather than to an aberration in the control of the regulated secretory process.

Although the variant was recovered from a somatic cell hybridization between AtT-20 cells and mouse myeloma cells, it is unlikely to represent a true hybrid between AtT-20 cells and the myeloma cells since it lacks properties of the myeloma parent, immunoglobulin expression, and resistance to oubain (Margulies et al., 1976). In contrast, the variant exhibited many properties of the AtT-20 parent. The cells survived exposure to HAT medium, were adherent with extended processes, and expressed synaptophysin (p38; data not shown) a membrane protein shared between neurons and endocrine cells (Navone et al., 1986; John et al., 1985; Franke et al., 1986; Weideman and Franke, 1985). The variant also targeted the synaptophysin it made to synaptic vesicle-like structures (data not shown), a characteristic property of neuroendocrine cell lines (Clift-O'Grady et al., 1990). HYA.15.10, a subclone that retained the ability to express ACTH, also contained dense core secretory granules, and retained the ability to release material from the regulated pathway. The AtT-20 cells that survived drug selection may be spontaneous variants that were resistant to short exposure to ouabain, perhaps because of their slow growth rate.

The HYA.15.6 variant exhibited multiple defects, including the loss of secretory granules with an electron-dense core, and the loss of several granule content proteins, ACTH, sulfated proteins (B15), and granule-specific sulfated proteoglycans. The variant could reflect an early developmental state before the expression of the secretory granule content proteins is switched on, assuming that expression of granule contents is required for dense core secretory granule formation. We cannot determine whether, in the absence of dense core formation, empty secretory granule membranes still form, but if they do they are not capable of storing and releasing on stimulation the exocrine protein trypsinogen.

Since the regulated pathway-deficient variant could not store free GAG chains, this storage property (and release of free GAG chains after stimulation) in wild type cells must be because of the regulated pathway. In wild type AtT-20 cells, almost half of the [^{35}S]GAG chains made in a 1-h pulse, remained stored in a releasable compartment at the end of a long chase (Fig. 3). The storage of GAGs in the regulated secretory pathway was of surprisingly high efficiency, comparable to that found for ACTH, human growth hormone (Moore and Kelly, 1985), trypsinogen (Burgess et al., 1985), and insulin (Kelly et al., 1989). Apparently, the GAG chains are not true bulk-phase markers as proposed by Burgess and Kelly (1984). A tripeptide (*N*-octonoyl-ASN-TYR-THR-NH_2) that behaves as a bulk-phase marker (Wieland et al., 1987) is excluded from the regulated secretory pathway in insulin-secreting cells (Gold et al., 1988). GAGs may be sorted from the *trans*-Golgi network into the secretory granules either by a GAG-specific receptor, similar to the hormone-specific receptor (Chung et al., 1989), or by association with a protein that is sorted. The latter mechanism has been invoked to explain sorting of an anti-secretogranin I antibody into secretory granules (Rosa et al., 1989). An alternative possibility is that in AtT-20 cells the machinery for synthesizing and sulfating GAG chains remain active in the immature granule after protein sorting events have occurred. Consistent with this alternative in our finding in AtT-20 cells

that sulfated-labeled proteins and oligosaccharides appear to be sorted more efficiently than methionine or cysteine-labeled proteins (Matsuuchi, L., and R. B. Kelly, unpublished results). Preferential secretion from parathyroid tissue of sulfate-labeled chromogranin A is also seen and was explained by proposing that sulfation occurs after the sorting of chromogranin into secretory granules (Gorr and Cohn, 1990). In pulse-labeled PC12 cells, in contrast, no sulfation of immature or mature secretory granules was seen (Tooze and Huttner, 1990). To resolve this point, similar experiments need to be done in wild type and variant AtT-20 cells.

We thank Debbie Crumrine for her assistance with the EM. We also thank Michael R. Gold, Sam Green, Mark Grimes, Adam Linstedt, and Aaron Turkewitz for their critical reading of the manuscript and Leslie Spector for her help in preparing the manuscript.

This work was supported by National Institutes of Health grants DK33937 (R. B. Kelly and L. Matsuuchi), AM35895 (L. Matsuuchi), and NS-09878 (R. B. Kelly).

Received for publication 9 July 1990 and in revised form 26 November 1990.

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