An Antibody Specific for an Endoproteolytic Cleavage Site Provides Evidence That Pro-opiomelanocortin Is Packaged into Secretory Granules in AtT20 Cells before Its Cleavage

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Abstract. We have raised a rabbit antiserum against a synthetic peptide corresponding to the cleavage site between β-lipotropic hormone and the ACTH moieties of murine pro-opiomelanocortin (POMC). After affinity purification, the anti-cleavage site antibody immunoprecipitates POMC from extracts of AtT20 cells but it does not immunoprecipitate the ACTH in such extracts or any of the other products of cleavage of POMC. By contrast, an antiserum raised against pure swine ACTH immunoprecipitates both POMC and ACTH from AtT20 cell extracts. Using the anti-cleavage site antibody we have shown that all the POMC synthesized during a 15-min pulse-labeling with [^{35}S]methionine is cleaved at this site within 1 h. By immunoelectron microscopy we show that ~25–30% of peripheral secretory granules in AtT20 cells can be labeled with the anti-cleavage site antibody while anti-ACTH antiserum labels all these granules. This establishes that at least some POMC is packaged into secretory granules before its proteolytic cleavage.

PRO-OPIOMELANOCORTIN (POMC)[1] is a polyprotein which contains the sequences of several biologically active peptides including ACTH, β-endorphin, and α-melanocyte-stimulating hormone. The amino acid sequence of murine POMC has been predicted from the nucleotide sequence of POMC cDNA (22, 30). In AtT20 cells, a line of murine anterior pituitary tumor cells (10), cleavage of POMC liberates ACTH, β-lipotropic hormone (βLPH), and an amino-terminal peptide (5, 17, 25). Some of the βLPH is cleaved further to yield β-endorphin while the amino-terminal peptide is cleaved to yield from its carboxy end the joining peptide whose carboxy terminus is α-amidated (8). The ACTH does not, however, undergo further cleavage. In the intermediate lobe of the pituitary, however, ACTH, as well as βLPH and the amino-terminal fragment are all cleaved again (25). All of these cleavages of POMC, and its products, occur at the sites of pairs of basic amino acids, various combinations of lysine and arginine residues. Each cleavage eliminates the two basic amino acids at that site but the remainder of the polypeptide chain is conserved. Antibodies against the products of cleavage are likely, therefore, also to recognize the precursor molecule. The cleavage sites themselves, however, must be specific for POMC since they are eliminated by cleavage, which involves excision of the two basic amino acids. Pursuing this reasoning we raised a rabbit antiserum to a synthetic peptide with a sequence corresponding to eight amino acids at the ACTH–βLPH cleavage site of murine POMC, and affinity purified the antiserum using a synthetic peptide corresponding to six amino acids of this site. The resultant antibody proved to be specific for POMC and here we describe its characterization and results obtained using it to study the sorting of POMC into the secretory granules of AtT20 cells.

1. *Abbreviations used in this paper*: KLH, Keyhole Limpet hemocyanin; βLPH, β-lipotropic hormone; POMC, pro-opiomelanocortin.

Materials and Methods

Peptide Synthesis

Three peptides were synthesized as indicated in Fig. 1. The longest of them, a nonapeptide, had the sequence Lys-Leu-Glu-Phe-Lys-Arg-Glu-Leu-Glu and corresponds to the eight amino acids of the cleavage site between the ACTH and βLPH moieties of murine POMC (22, 30) with an additional amino-terminal lysine residue. This peptide was used for immunization of rabbits (see below). The second peptide had the sequence Lys-Glu-Phe-Lys-Arg-Glu-Leu and was used to affinity purify the antisera raised against the longest peptide (see below). Finally a peptide, Glu-Leu-Glu-Gly-Glu, corresponding to the amino terminus of murine βLPH (22, 30), was made and used as described in the text.

The peptides were synthesized in a continuous flow instrument constructed in our laboratory (9). Synthesis was performed on a 1% cross-linked polystyrene support using fluorenylmethoxycarbonyl amino acids (3) and in situ activation with benzotriazolyl-tris-(dimethylamino)-phosphoniumhexafluorophosphate (4). The synthetic peptides were purified by high-pressure liquid chromatography.

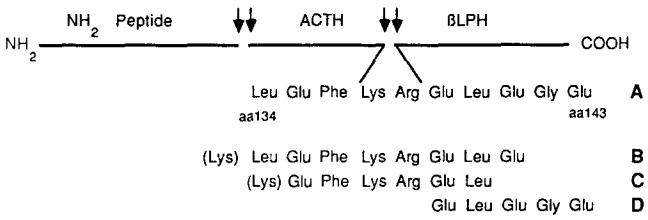


Figure 1. A diagram of murine POMC showing the cleavage sites between the amino-terminal peptide and the ACTH and βLPH moieties (the amino-terminal signal peptide responsible for leading the polypeptide chain into the lumen of the rough endoplasmic reticulum is not shown since it is cleaved off during translocation); (*A*) the amino acid sequence around the ACTH-βLPH cleavage site; (*B, C, D*) the three peptides that were chemically synthesized.

Antibody Production

We followed exactly the procedure described by Kreis (18) in which the antigen, in our case the nonapeptide and also swine ACTH, is coupled using electron microscopic grade glutaraldehyde to Keyhole Limpet hemocyanin (KLH) obtained from Calbiochem-Behring Corp. (La Jolla, CA). The antigen-KLH was then emulsified with complete Freund's adjuvant (Gibco, Grand Island, NY) before use for immunization. Two antisera were raised in rabbits, one against the peptide corresponding to the βLPH-ACTH cleavage site (see above), the other against swine ACTH (Serva, Heidelberg). An antiserum against swine ACTH was also raised in a guinea pig. The anti-ACTH antisera were not affinity purified since immunofluorescence and immunoblotting tests showed them to be specific for ACTH and of a high titer. The antiserum against the cleavage site peptide was affinity purified on a column of CNBr-activated Sepharose 4B (Pharmacia, Uppsala) to which was coupled the shorter synthetic peptide (Lys-Glu-Phe-Lys-Arg-Glu-Leu). The affinity-purified antibody is referred to here as anti-POMC-CS.

Cell Culture

AtT20D16V cells were grown in Falcon plastic ware in DME supplemented with 3.5 g/liter of glucose and 10% FCS or horse serum (Gibco). The cells were replated after dispersion of clumps with 0.52% trypsin and 0.05% EDTA every 5-7 d. Under these culture conditions the cell population doubles in ~40 h (28).

Indirect Immunofluorescence Microscopy

AtT20 cells grown on glass coverslips were fixed, permeabilized, and labeled with antibodies using the procedures described by Ash et al. (1). As first antibodies we used the affinity-purified POMC-CS and either rabbit or guinea pig anti-ACTH antisera as described in the figure legends. The second antibodies were affinity purified rhodamine-conjugated sheep anti-rabbit and fluorescein-conjugated goat anti-guinea pig antibodies. In some experiments, as indicated in the legends, the first antibodies were preincubated at room temperature for 30 min with either 1 mg/ml swine ACTH or 1 mg/ml of the pentapeptide, Glu-Leu-Glu-Gly-Glu, or 1 mg/ml of the heptapeptide used to affinity purify the anti-POMC-CS. The ACTH and the peptides were also present at 1 mg/ml during the 20-min labeling with the first antibodies.

Mouse anterior pituitaries were dissected out and fixed for 10 min in 4% paraformaldehyde in 200 mM Pipes, pH 7.2, at 4°C and then in 8% paraformaldehyde in the same buffer for 50 min at room temperature. The tissue blocks were then infused with 2.1 M sucrose and frozen in liquid nitrogen. 1-μm thick sections were cut of the fixed frozen tissue, mounted on glass slides, and labeled with the antibodies using the procedure of Ash et al. (1).

Immunoelectron Microscopy

AtT20 cells were fixed for 10 min in 4% paraformaldehyde in 200 mM Pipes buffer, pH 7.2, and then for 50 min at room temperature in 8% paraformaldehyde in the same buffer. The cells were scraped from the dishes, washed, infiltrated with 2.1 M sucrose, and frozen in liquid nitrogen. Thin sections were cut and labeled according to established procedures (14, 26, 27). The antibodies used were anti-POMC-CS and guinea pig anti-ACTH antisera as specified in the legends. After incubation with these antibodies, the sections were labeled with protein A conjugated to either 5, 8, or 10 nm gold. The sections were contrasted with uranyl acetate before being examined in Philips electron microscopes.

Immunoprecipitation

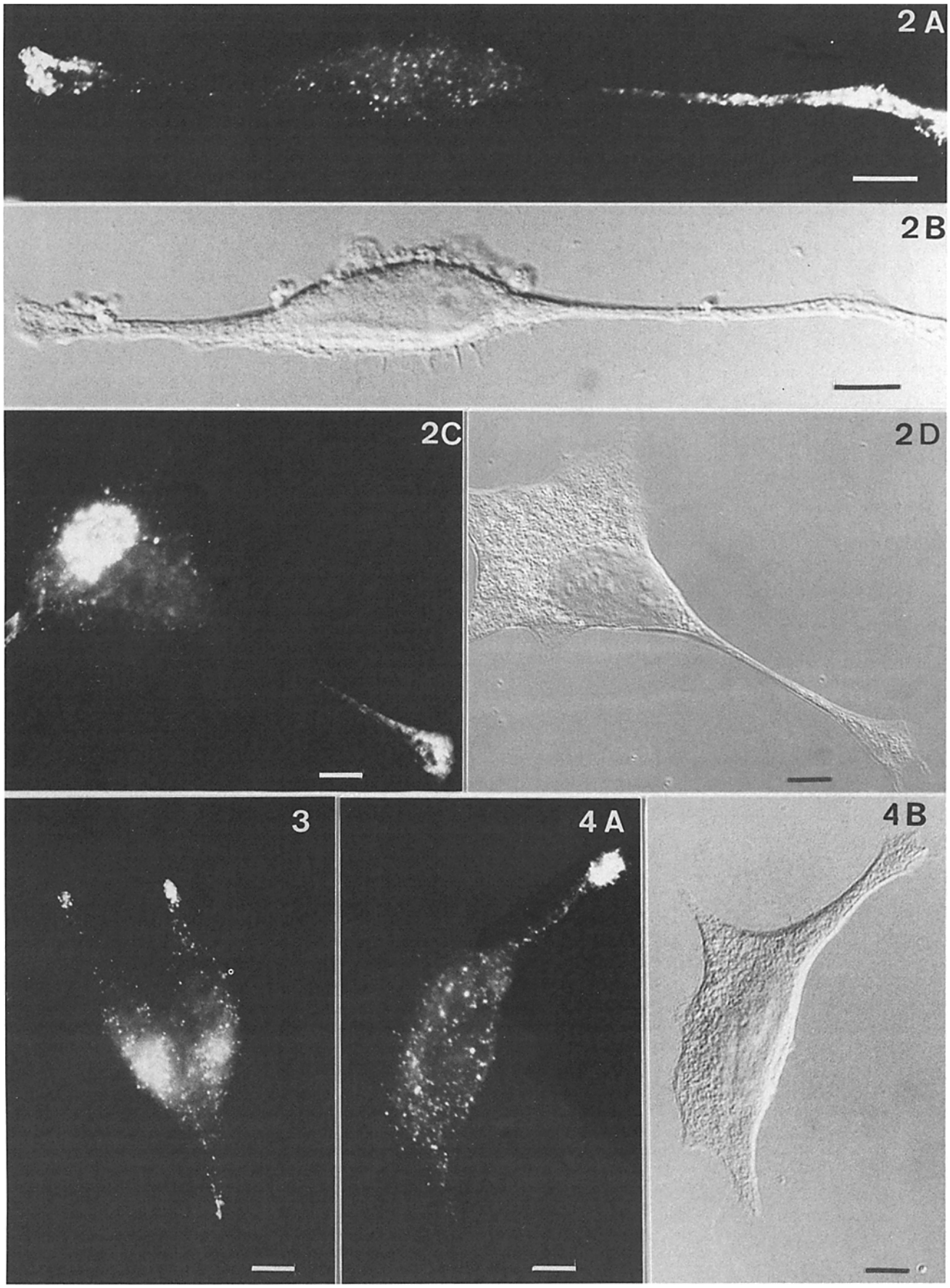
AtT20 cells grown in 6-cm tissue culture petri dishes were labeled with 250 μCi of [^{35}S]methionine in 1 ml of methionine-free growth medium (MEM-Met containing dialyzed FCS) for the times indicated in Fig. 6. After labeling, some cultures were chased for 1 h by the addition of 4 ml of normal growth medium supplemented with 0.15 mg/ml of unlabeled methionine. For immunoprecipitation analysis, cells in each dish were solubilized in 1 ml of a solution containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% SDS, 40 μg/ml PMSF. DNA was sheared by repeated passage through a 21-gauge needle fitted to a 1-ml syringe. After a 5-min centrifugation in an Eppendorf centrifuge, Triton X-100 was added to each supernatant, from a 20% stock solution in H_2O, to yield a final concentration of 2%. The centrifugation step was then repeated and the supernatants were retained. 10 μg of affinity-purified anti-POMC-CS or 10 μl of anti-ACTH antiserum was added to each sample followed by 20 μl of a 50% suspension of protein A-Sepharose (Pharmacia). These samples were then incubated for 16 h at 4°C on an end over end rotator. The Sepharose was subsequently washed five times in a solution containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 40 μg/ml PMSF, followed by two washes in 50 mM Tris-HCl, pH 7.4. The immunoprecipitates were then solubilized in 100 μl of SDS gel sample buffer and the samples fractionated on a 10-18% polyacrylamide gradient gel. Gels were impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed at −80°C.

Results

Production of Antibody against Cleavage Site Peptide

In murine POMC, the amino acid sequence at the cleavage site between the ACTH and βLPH moieties is Leu-Glu-Phe-Lys-Arg-Glu-Leu-Glu (see Fig. 2). This sequence is also present in rat POMC and closely related sequences occur in bovine and human POMC. A synthetic peptide comprising an amino-terminal lysine residue followed by these eight amino acids was synthesized and linked, using glutaraldehyde, to KLH. The additional amino-terminal lysine residue was added to facilitate this linkage. One of two rabbits immunized with KLH-peptide emulsified in complete Freund's adjuvant gave, after two boosts, an antiserum that labeled cytoplasmic granules in AtT20 cells above a high nonspecific background. The second rabbit's serum did not label AtT20

Figures 2-4. (*Fig. 2 A*) An AtT20 cell labeled with anti-POMC-CS and rhodamine-conjugated second antibody. Note the labeling of the tips of the cell projections and the light perinuclear labeling; (*Fig. 2 B*) the same cell viewed with Nomarski optics; (*Fig. 2 C*) for comparison, an AtT20 cell labeled with rabbit anti-ACTH antiserum; (*D*) the same cell under Nomarski optics. (*Fig. 3*) Two AtT20 cells incubated first for 20 min at room temperature with guinea pig antiserum against ACTH at fivefold the concentration used to label cells for ACTH and then incubated with anti-POMC-CS followed by rhodamine-conjugated anti-rabbit second antibody. Preincubation with guinea pig anti-ACTH antiserum does not block labeling with anti-POMC-CS. Perinuclear labeling of the Golgi region by anti-POMC-CS is particularly clear in these two cells. (*Fig. 4 A*) An AtT20 cell labeled with anti-POMC-CS which was incubated at room temperature for 30 min with 1 mg/ml swine ACTH before the labeling and during the 20 min of labeling. The incubation of anti-POMC-CS with ACTH does not block its ability to label the cells. (*Fig. 4 B*) The same cell viewed with Nomarski optics. Bars, 10 μm.


2 A
2 B
2C
2 D
3
4 A
4 B

cell granules and was discarded. The positive antiserum was found, by dot blot analysis (not shown), to cross-react slightly with ACTH. It was therefore affinity purified on a column consisting of the shorter peptide, Lys-Glu-Phe-Lys-Arg-Glu-Leu, coupled to cyanogen bromide Sepharose. The last six amino acids of this peptide correspond to the last two amino acids of ACTH, the dibasic cleavage site and the first two amino acids of βLPH. The affinity-purified antibody eluted from this column, designated anti-POMC-CS, no longer recognized ACTH. It was subsequently characterized to establish its specificity for the cleavage site in POMC.

Indirect Immunofluorescence Labeling with Anti-POMC-CS

In AtT20 cells processed for indirect immunofluorescence microscopy with the affinity-purified anti-POMC-CS, granules in both the tips of projections from the cells and the Golgi region were labeled (Figs. 2 *A* and 3). This pattern of labeling resembles but, particularly in the Golgi region, is less intense than that obtained with guinea pig or rabbit anti-ACTH antisera (Fig. 2 *C*). To show that the POMC-CS was not labeling either ACTH or βLPH in the secretory granules we did the following experiments. (*a*) In a double labeling procedure AtT20 cells were incubated with guinea pig anti-ACTH antiserum but no second antibody and then with anti-POMC-CS and rhodamine anti-rabbit second antibody. The preincubation with antiserum against ACTH did not block subsequent labeling with POMC-CS (Fig. 3). (*b*) Anti-POMC-CS was incubated at room temperature for 30 min with swine ACTH at 1 mg/ml. AtT20 cells were then labeled with this anti-POMC-CS in the presence of 1 mg/ml ACTH. The ACTH did not block the labeling of secretory granules in AtT20 cells by anti-POMC-CS (Fig. 4). In contrast incubation of anti-ACTH antiserum with 1 mg/ml ACTH completely blocked labeling of secretory granules (not shown). (*c*) This experiment was repeated using at 1 mg/ml a synthetic peptide (Glu-Leu-Glu-Gly-Glu) corresponding to the first five amino acids of murine βLPH instead of ACTH. Again, incubation of anti-POMC-CS with this peptide, or with the peptide and ACTH together, did not block labeling of secretory granules in AtT20 cells (Fig. 5). (*d*) We incubated the anti-POMC-CS with 1 mg/ml of the peptide used for its affinity purification, namely Lys-Glu-Phe-Lys-Arg-Glu-Leu. This completely abolished the labeling of AtT20 cells (not shown). On the other hand, incubation of anti-ACTH antisera with each of the three synthetic peptides used in this study at 1 mg/ml did not block labeling of the ACTH in the secretory granules (not shown).

These results established that anti-POMC-CS labeled an epitope in secretory granules of AtT20 cells which is not present in ACTH or the first five amino acids of βLHP. The antibody thus appeared to be specific for POMC. This was proven by immunoblotting and immunoprecipitation experiments.

Immunoprecipitation of POMC by Anti-POMC-CS

After the characterization of anti-POMC-CS by immunofluorescence microscopy we established by dot blots on nitrocellulose strips that the antibody failed to bind to swine ACTH (not shown). We then used the anti-POMC-CS and a rabbit antiserum against ACTH in immunoprecipitation analyses of AtT20 cells labeled for various times with [^{35}S]methionine (Fig. 6). After a 15-min pulse-label, both of these antibodies specifically precipitated only POMC, the ACTH and βLPH precursor molecule. However, after a 60-min incubation ("chase") in the presence of excess unlabeled methionine the anti-ACTH antiserum immunoprecipitated mature ACTH whereas the anti-POMC-CS failed to precipitate specifically any labeled protein. If, however, we labeled cells continuously for 75 min, the duration of the previous pulse and chase, we found that while anti-POMC-CS still only precipitated POMC, the anti-ACTH antiserum precipi-

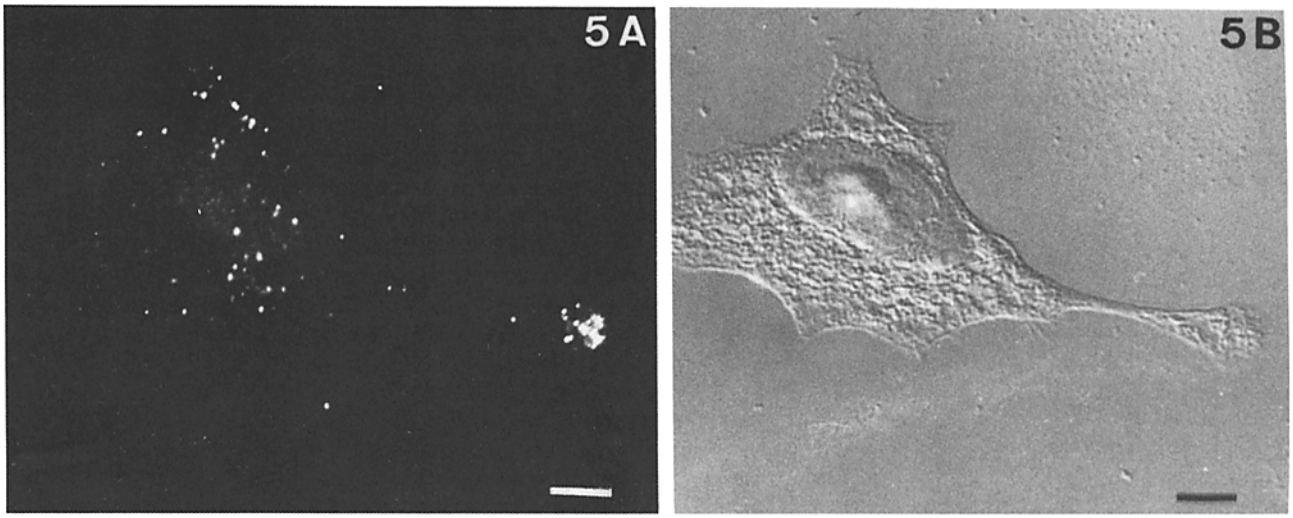


Figure 5. (*A*) An AtT20 cell labeled with anti-POMC-CS which had been incubated for 30 min at room temperature with 1 mg/ml of the pentapeptide Glu-Leu-Glu-Gly-Glu together with 1 mg/ml of swine ACTH. Incubation of anti-POMC-CS with this peptide, the amino-terminal sequence of murine βLPH, does not block its ability to label the cells. We consistently observed some reduction in labeling of AtT20 cells when either anti-POMC-CS or anti-ACTH antiserum was preincubated with 1 mg/ml of the pentapeptide. This we attribute to nonspecific effects of the presence of such a high concentration of such an acidic peptide before and during labeling. (*B*) The same cell viewed by Nomarski optics. Bars, 10 μm.

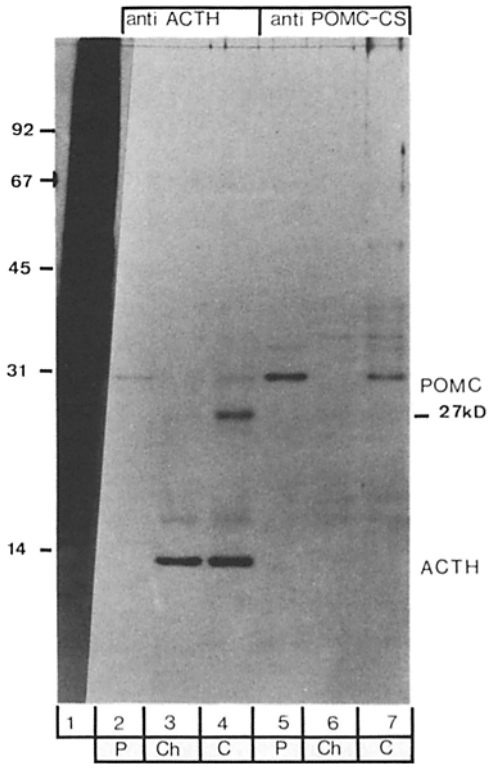


Figure 6. Immunoprecipitations with anti-POMC-CS and anti-ACTH antiserum using either anti-ACTH (lanes *2–4*) or anti-POMC-CS (lanes *5–7*). Cells were pulse-labeled with [^{35}S]methionine for 15 min (*P*), pulse-labeled for 15 min and chased for 1 h (*Ch*), or labeled continuously for 75 min (*C*). After a 15-min pulse only POMC is precipitated by both anti-ACTH (lane *2*) and anti-POMC-CS (lane *5*). After a 1-h chase all of the POMC is processed to yield mature ACTH (lane *3*) and βLPH, neither of which is precipitated by the anti-POMC-CS (lane *6*). After 75 min continuous label anti-ACTH precipitated both POMC and ACTH but also an intermediate ~27,000-mol-wt peptide (lane *4*). Anti-POMC-CS on the other hand precipitates only POMC (lane *7*). This suggests that the 27,000-mol-wt peptide seen in lane *4* results from the cleavage of βLPH from POMC. Lane *1* shows the total ACTH extract after the 15 min pulse label. 100 times this amount was used in each immunoprecipitation. It is worth noting that the anti-ACTH apparently has a lower affinity for POMC than for mature ACTH (compare lanes *2*, *3*, and *5*). The bands with molecular weights greater than POMC appeared variably in our immunoprecipitates.

tated not only POMC and ACTH but also a third polypeptide of intermediate molecular weight; from its mobility on the gels we estimate its molecular weight to be about 27,000.

We conclude from these results that anti-POMC-CS is specific for POMC in AtT20 cells and secondly that POMC is completely processed within 1 h of its synthesis. A third possible conclusion is that the cleavage of the site in POMC between βLPH and ACTH, against which anti-POMC-CS is directed, occurs on average before the cleavage of the site between ACTH and the amino-terminal peptide, thereby generating an intermediate, with an apparent molecular weight of 27,000, containing the ACTH sequence but lacking the ACTH-βLPH cleavage site. This, however, is contingent upon the demonstration that the 27-kD peptide contains the POMC amino terminus and that it can be "chased" to yield ACTH. These experiments have not so far been performed.

Secretory Granules Contain POMC

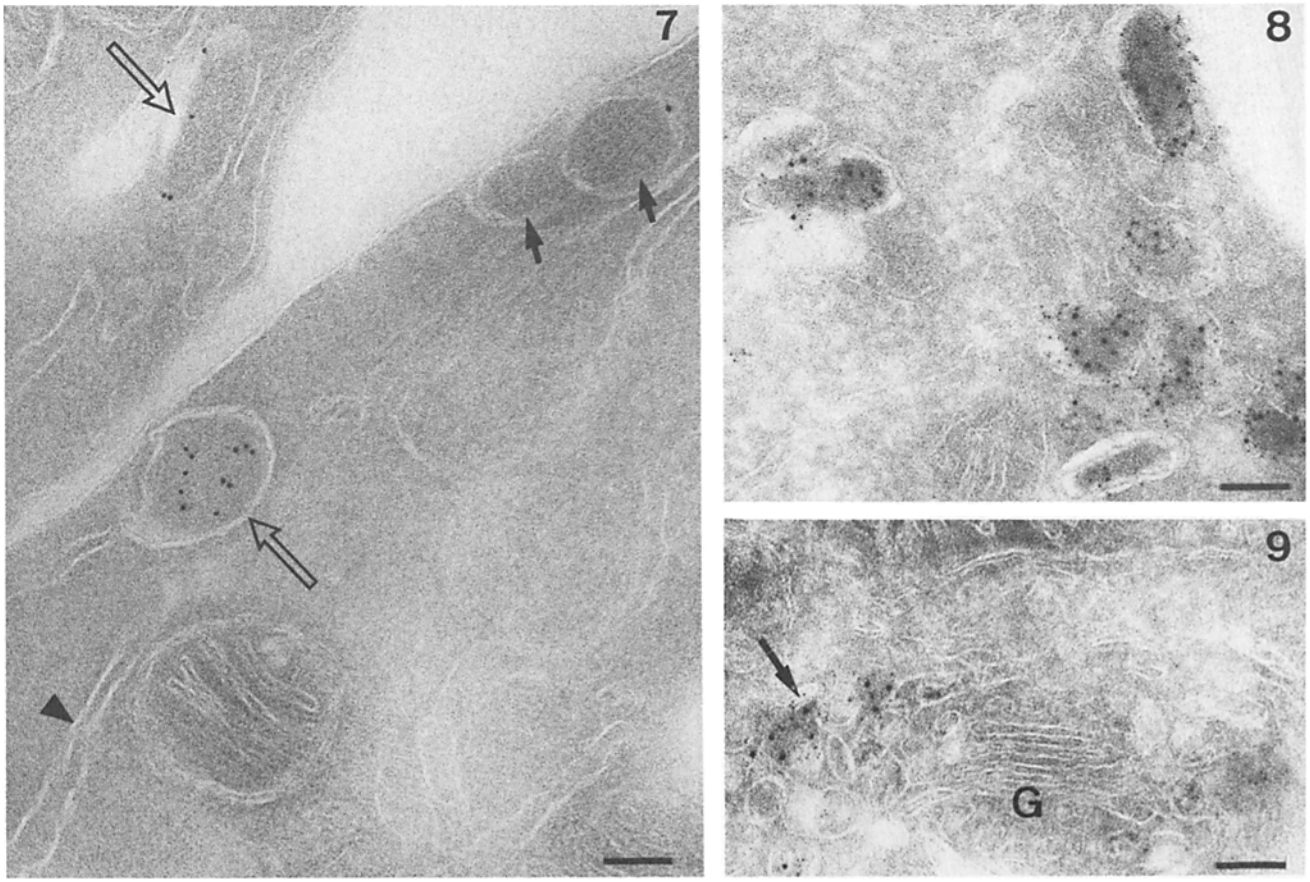
The pattern of indirect immunofluorescence labeling of AtT20 cells with anti-POMC-CS strongly suggested that the antibody was recognizing a component of secretory granules. To prove this at the level of resolution of the electron microscope we cut cryosections of AtT20 cells and labeled them by the immunogold procedure with either anti-POMC-CS, guinea pig anti-ACTH antiserum, or both antibodies in a double labeling procedure. While the anti-ACTH antiserum when used alone labeled all the peripheral secretory granules (not shown), the anti-POMC-CS antibodies labeled only some of them (Fig. 7). Counts of the proportion of granules labeled with anti-POMC-CS, in which only granules with three or more gold particles over their cores were scored as positive, indicated that between 25 and 30% of the granules at the cell periphery contain POMC. This establishes that at least a quarter to a third of peripheral secretory granules contain POMC that has not been cleaved at the site between ACTH and βLPH, which is apparently the first site to undergo cleavage during maturation of POMC in these cells. The double labeling of the granules with the anti-POMC-CS and guinea pig anti-ACTH antibodies (Fig. 8) confirmed the results of indirect immunofluorescence, which showed that the epitope recognized by anti-POMC-CS is not competed for by anti-ACTH antiserum. We were also able to confirm at the level of resolution of the electron microscope the labeling by anti-POMC-CS and anti-ACTH antiserum of immature peri Golgi secretory granules (Fig. 9).

Crossreactivity of Anti-POMC-CS Antibody

Anti-POMC-CS is specific for POMC in AtT20 cells as evidenced by the results of immunoprecipitation experiments and electron microscopic immunocytochemistry described above. We have used the antibody for indirect immunofluorescence labeling of 1-μm thick sections of mouse anterior pituitary tissue. The corticotrophs, which can easily be recognized by their irregular shape and by labeling with the guinea pig anti-ACTH antiserum (Fig. 10 *A*), are also labeled by anti-POMC-CS in a double labeling experiment as expected (Fig. 10 *B*). However, in the anterior pituitary sections a second type of cell, clearly distinct from the corticotrophs, is strongly labeled by anti-POMC-CS but not by anti-ACTH antiserum (Fig. 10 *B*). We have not yet identified this other positive cell type; such identification will require additional light and electron microscopic immunocytochemical studies.

Discussion

The experiments presented here show that using quite short synthetic peptides it is possible to obtain an antibody specific for a single dibasic amino acid cleavage site in a prohormone, POMC, which has eight potential cleavage sites of this type. The antibody specifically immunoprecipitates POMC from extracts of AtT20 cells and labels specifically peripheral secretory granules in these cells. In sections of mouse anterior pituitary processed for indirect immunofluores-



Figures 7–9. (*Fig. 7*) Secretory granules in AtT20 cells labeled with anti-POMC-CS. This field shows four secretory granules below the plasma membrane of two AtT20 cells. Two of the granules (*open arrows*) are labeled to different extents (four gold particles over one and 11 over the other) while two others (*solid arrows*) are unlabeled. The single gold particle over one of these two granules is not considered significant. Mitochondria are not labeled. The arrowhead indicates a cisterna of the rough endoplasmic reticulum. As is usual in cryothin sections the ribosomes are not visible. (*Fig. 8*) Secretory granules in AtT20 cells double labeled first with anti-POMC-CS followed by protein A conjugated to ~10 nm gold and then with anti-ACTH antiserum followed by 5 nm gold. In this selected field all of the secretory granules are labeled with both large and small gold indicating the presence of POMC as well as ACTH in all of them. Counts of a larger representative sample, however, showed that only 25–30% of all peripheral granules contain POMC. (*Fig. 9*) A Golgi region in a frozen thin section of an AtT20 cell. The section was labeled with anti-ACTH antiserum followed by 5 nm gold and then anti-POMC-CS followed by 10 nm gold. The Golgi stack (*G*) is unlabeled by the immunogold technique, as in a previous study where the reasons for this are discussed (29), but peri Golgi immature secretory granules (*arrow*) are labeled by both antibodies. Bars, 0.1 μm.

cence, the anti-POMC-CS labels in addition to the corticotrophs synthesizing POMC a second class of cells.

One obvious possibility is that the anti-POMC-CS is recognizing another cleavage site in a different polypeptide hormone precursor synthesized in these clearly different cells. The other less interesting possibility is that we are seeing a chance crossreactivity with some other protein specific to these cells. Indeed, we have noticed that our affinity-purified anti-POMC-CS antibody shows a very specific, but presumably trivial crossreactivity with the intermediate filament protein vimentin. This was demonstrated by immunoprecipitation, immunoblotting, and immunofluorescence microscopy (not shown). While this may restrict the use of the antibody, the conclusions arrived at in this paper remain valid since AtT20 cells and epithelial neurosecretory cells of the anterior pituitary do not contain vimentin. In this context it is interesting to note that a number of groups have reported monoclonal and antipeptide antibodies against diverse proteins, e.g., thy1 and pp60^{src} (6, 21), which apparently cross react with vimentin. Presumably vimentin exhibits structural features which are common to a variety of proteins.

Our immunoprecipitation experiments establish that within 1 h of its synthesis POMC in AtT20 cells is cleaved at the site between ACTH and βLPH. Furthermore, comparison of the results of immunoprecipitations of SDS extracts of [^{35}S]methionine-labeled AtT20 cells using anti-ACTH antiserum and anti-POMC-CS is consistent with previous findings (7, 19, 24) that this site is cleaved before the cleavage between the amino-terminal peptide and the ACTH moiety. We have also shown that 25–30% of the secretory granules at the cell periphery in cryosections of AtT20 cells can be labeled above background using anti-POMC-CS and the immunogold labeling procedure. Combining these two sets of observations we can reach two conclusions concerning the sorting and packaging of POMC into secretory granules of the regulated pathway of AtT20 cells. First our results clearly indicate that at least some POMC is sorted and packaged into the cores of the secretory granules before its cleavage at the ACTH-βLPH site. And, since this site is cleaved first, this POMC must be sorted and packaged before it has undergone any proteolytic maturation. This observation is consistent with biochemical evidence that in cells of the rat intermedi-

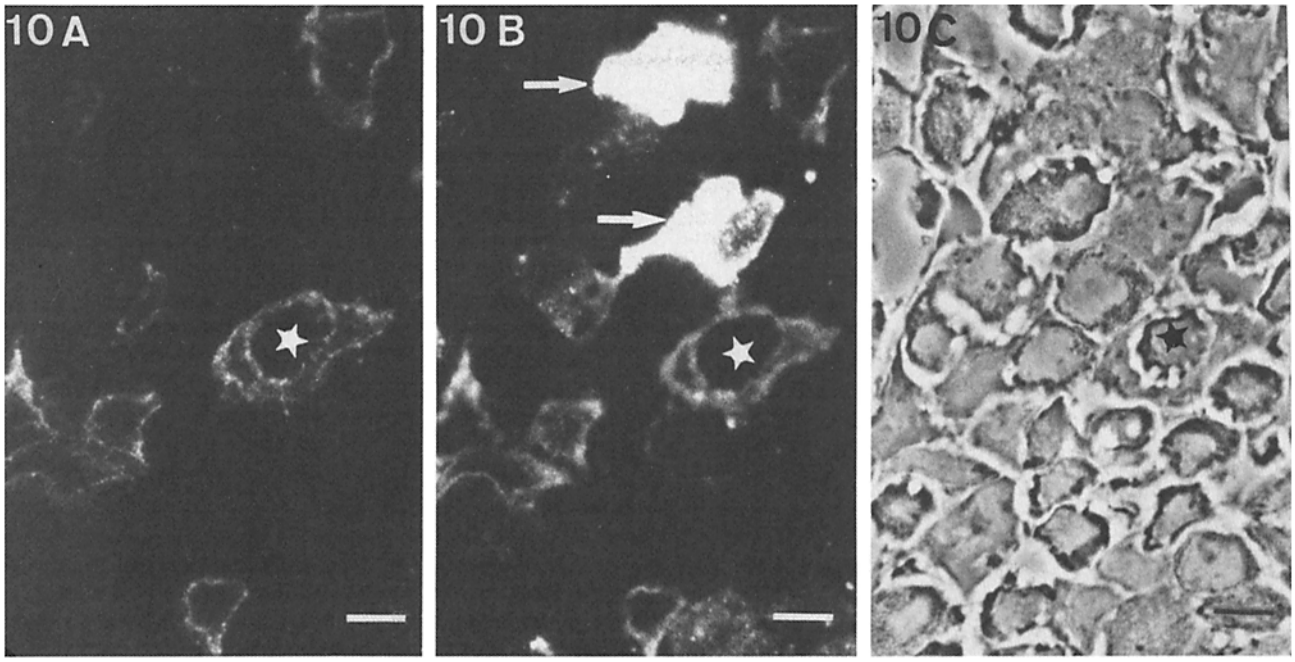


Figure 10. A 1-μm-thick frozen section of mouse anterior pituitary double labeled with guinea pig anti-ACTH antiserum and fluorescein-conjugated second antibody (*A*) followed by anti-POMC-CS and rhodamine-conjugated second antibody (*B*). Corticotrophs are labeled by both antibodies. The similarity in the pattern of labeling of these cells by the two antibodies indicates that peripheral secretory granules, which line up in a layer below the plasma membrane and therefore after labeling reveal the outline of the cell, contain POMC. In addition to the corticotrophs a second cell type is labeled with anti-POMC-CS (arrows in *B*) but not by anti-ACTH antiserum. We have not yet identified this cell type but its shape and distribution indicate that it is one of the other neurosecretory cells in this tissue. (*C*) Phase-contrast micrograph of the same field as shown in *A* and *B*. A star marks the same corticotroph in all three micrographs. Bars, 10 μm.

ate pituitary some POMC enters secretory granules before cleavage (12). In this respect, AtT20 cells also resemble neurosecretory neurons in the pituitary producing either vasopressin or oxytocin, where at least some cleavage of prohormone occurs in the secretory granules while they are moving down the long axons of these cells (2, 11). Moreover, in pancreatic cells producing insulin, packaging also precedes cleavage of the prohormone (23). We cannot, of course, conclude that sorting and packaging of POMC in AtT20 cells obligatorily precedes its cleavage. On the other hand sorting before cleavage is the simplest interpretation of our findings and also the fact that all three cleavage products of POMC are present in the secretory granules of AtT20 cells (15). The identical pattern of immunofluorescence labeling by anti-POMC-CS and anti-ACTH antiserum of the corticotrophs in mouse anterior pituitary tissue indicates that the peripheral secretory granules that line the inner face of the plasma membrane of these cells also contain some POMC.

The packaging of POMC into the cores of secretory granules occurs in the *trans*-most compartment of the Golgi apparatus (29) and is followed by migration of the secretory granules to the cell periphery. Since POMC is cleaved within an hour of its synthesis, peripheral secretory granules containing POMC must be <1 h old, if the POMC in the peripheral granules continues to undergo cleavage. Presumably that is the case because mature secretory granules, the densest population of granules isolated from AtT20 cells, contain the three POMC cleavage products but they do not contain POMC itself (15).

The immunogold labeling technique is quantitative (13) and is particularly valuable for determining the relative amounts of an antigen in one compartment; for example secretory granules. The three- to four-fold variation in the extent of labeling of peripheral secretory granules in the same thin sections of the same or adjacent cells (Fig. 7) indicates, therefore, a three- to fourfold variation in the amount of POMC in individual granules. We interpret this to mean that we are looking at a heterogeneous population of secretory granules in which cleavage of POMC is progressing to completion. Further immunocytochemical studies of the processing of POMC and the formation of secretory granules in AtT20 cells using anti-POMC-CS are under way. At the outset of this work we hoped that an antibody specific for POMC would provide a probe for the identification by immunoelectron microscopy of post-Golgi vesicles of the constitutive exocytic pathway that externalize uncleaved POMC (16). However, to date we have failed to identify such vesicles with anti-POMC-CS in frozen thin sections. It may be that the amount of POMC in individual vesicles is below the threshold of detection by the immunogold technique. Adding chloroquine to cells and diverting all the POMC into the constitutive pathway (20) may be one way to improve the chances of detecting the constitutive exocytic vesicles.

AtT20 cells and POMC provided a convenient model system in which to establish the feasibility of producing antibodies against specific dibasic endoproteolytic cleavage. The results suggest that our approach may be generally useful and that it should be possible to obtain antibodies specific for such cleavage sites in other prohormones using synthetic peptides.

We are grateful to our colleagues Dr. T. Kreis, for advice concerning the conjugation of the peptide to Keyhole Limpet hemocyanin, and Drs. P. Rosa and W. Huttner, for dissecting out the mouse anterior pituitaries. We thank Ines Benner for typing the manuscript.

Received for publication 2 December 1986, and in revised form 12 March 1987.

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