Aminopeptidase B: a processing enzyme secreted and associated with the plasma membrane of rat pheochromocytoma (PC12) cells

Agnès Balogh¹, Sandrine Cadel¹, Thierry Foulon¹, Renée Picart², Arsène Der Garabedian¹, Annie Rousselet³, Claude Tougard² and Paul Cohen¹,*

¹Laboratoire de Biochimie des Signaux Régulateurs Cellulaires et Moléculaires, Unité de Recherche Associée au Centre National de la Recherche Scientifique 1682, Université Pierre et Marie Curie, 96 Boulevard Raspail, F-75006 Paris, France
²Unité 36 de l’Institut National de la Santé et de la Recherche Médicale, Groupe de Biologie de la Cellule Neuroendocrine, Collège de France, 11 Place Marcelin Berthelot, F-75231 Paris cedex 05, France
³Laboratoire de Biologie du Cycle Cellulaire et de la Motilité, UMR 144 du CNRS, Institut Curie, 26, rue d’Ulm, F-75248 Paris, cedex 05, France
*Author for correspondence (e-mail: pacohen@ccr.jussieu.fr)

Accepted 3 November 1997: published on WWW 23 December 1997

SUMMARY

Aminopeptidase B (Ap-B) is a Zn²⁺-dependent exopeptidase which selectively removes Arg and/or Lys residues from the N terminus of several peptide substrates. Isolated and characterized from rat testes, this ubiquitous enzyme may participate in the final stages of precursor processing mechanisms. To test this hypothesis, we have investigated the secretion and subcellular localization of this enzyme in a rat cell line of pheochromocytoma (PC12 cells). By using a combination of biochemical and immunocytochemical methods, the following observations were made: (i) the level of aminopeptidase B detectable in the cell culture medium increased with time; (ii) 8-bromo-adenosine 3’-5′-cyclic monophosphate and the Ca²⁺ ionophore A23187 both stimulated enzyme liberation in the culture medium; (iii) brefeldin A, an inhibitor of vesicular transport from the endoplasmic reticulum to the Golgi apparatus, decreased enzyme secretion in a time-dependent manner; (iv) whereas nocodazole, a microtubule depolymerizing agent, inhibited enzyme secretion, cytochalasin D, a microfilament disruption agent, had no effect on released aminopeptidase B level; (v) immunofluorescence demonstrated the presence of aminopeptidase B in the Golgi apparatus; (vi) immunofluorescence, electron microscopy and tests of enzyme activity on intact cells showed an association of the peptidase with the external face of the plasma membrane. Together these data strongly argued in favour of the enzyme secretion by PC12 cells. It is concluded that aminopeptidase B may participate in processing events occurring either during its intracellular transport along the secretory pathway or at the plasma membrane level, or both.

Key words: LTA₄ hydrolase, 8-Bromo-adenosine 3’-5′-cyclic monophosphate, Brefeldin A, Ca²⁺ ionophore, β NGF, Golgi apparatus, Nocodazole

INTRODUCTION

Several processing endoproteases, possibly involved in the maturation of hormone precursors, have been shown to produce cleavage of basic amino acid doublets on their N-terminal side (Devi and Goldstein, 1985; Parish et al., 1986; Azaryan and Hook, 1994; Chesneau et al., 1994). N-terminally extended forms of various peptide hormones bearing an extra basic residue, Arg or Lys, and resulting from proteolytic processing of their corresponding precursors, have been previously described (Regoli and Barabé, 1980; Pedersen and Brownie, 1983; Misono et al., 1984; Eberlein et al., 1992; Hui et al., 1994). These observations gave rise to the idea that an aminopeptidase B activity might be involved in the subsequent processing steps leading to the excision of the additional basic residues at the N terminus of the precursor fragments generated by endoproteolytic action (Cohen, 1987; Cadel et al., 1995). This concept received only little attention since the efforts focused primarily on endopeptidases cleaving at the C terminus of basic amino acid doublets (for review see Benjannet et al., 1991; Seidah and Chrétien, 1992) and on the subsequent participation of carboxypeptidase E or H (Cp-H: EC 3.4.17.10; Fricker, 1988; Fricker et al., 1990).

Aminopeptidase B (Ap-B: EC 3.4.11.6) was originally defined as an exopeptidase able to trim basic amino acid residues from the NH₂ terminal of various peptide substrates (Hopsu et al., 1964; Cadel et al., 1995). Purification of a 72 kDa Ap-B from rat testis (Cadel et al., 1995) allowed us to demonstrate that this metallopeptidase clearly exhibited a strict selectivity for Arg and/or Lys residues at the N terminus of peptides. Ap-B was inhibited by cation chelating agents, as well as by bestatin and arphamenines A and B. Moreover, antibodies raised against the isolated peptidase showed that this component of the seminiferous tubules, appearing at late stages of spermatogenesis (Cadel et al., 1995), is widely distributed in a number of tissues, including endocrine and non-endocrine...
Recent elucidation of the primary structure demonstrated that this Zn²⁺-metallopeptidase exhibits strong homology with an important enzyme of the arachidonic acid pathway, leukotriene A₄ hydrolase (Cadel et al., 1997). The exact physiological role of the enzyme (Kawata et al., 1980; Söderling and Mäkinen, 1983; Gainer et al., 1984; Mantle et al., 1985; Gluschankof et al., 1987; Gomez et al., 1988; Mc Dermott et al., 1988; Flores et al., 1993) has not so far been determined. However, reports by various authors including from our laboratory (Gainer et al., 1984; Gomez et al., 1988; Castro et al., 1989; Cadel et al., 1995) have given substance to the idea that Ap-B might be a processing exopeptidase and could participate in precursor maturation in the secretory pathway or/and at the extracellular level of specialized cells.

We have now used the rat pheochromocytoma cell line, PC12, as a model of neuroendocrine cells, to study the possible association of Ap-B with their secretory machinery. PC12 is a noradrenergic clonal cell line derived from a rat adrenal medullary tumour (Greene and Tischler, 1976). In response to treatment with nerve growth factor (NGF) and via membrane receptors (Halegoua and Patrick 1980; Radeke et al., 1987), PC12 cells develop neuronal properties characterized by the formation of neurite processes and synaptic cell contacts (Tischler and Greene, 1975; Greene and Tischler, 1976). Moreover, the PC12 cell line is a convenient system for studying the mechanisms by which various factors may regulate secretory function, since these cells present at least two secretory pathways, of which one is regulated (Tooze and Hutten, 1990).

Using a combination of biochemical and immunocytochemical techniques we demonstrate that Ap-B is secreted and that it is localized in the Golgi apparatus and associated with the plasma membrane of PC12 cells. These findings strongly support the idea that Ap-B is a secretory enzyme which could participate in processing mechanisms occurring during its intracellular transport along the secretory pathway and/or at the plasma membrane of these neuroendocrine cells.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Nerve growth factor (NGF), Dulbecco's modified Eagle's medium (DMEM), horse and foetal calf sera (HS, FCS), goat anti-rabbit IgG conjugated to alkaline phosphatase and NBT/BCIP reagent were purchased from Gibco BRL (Grand Island, NY). Leupeptin, brefeldin A, nocodazole and cytochalasin D, cells were treated after 30 minutes starvation (0.15% sera) in 500 μl DMEM without serum. For the experiments run at 20°C, 1.5x10⁵ cells/well were preincubated for 30 minutes then incubated for 2 hours at 20°C in 1 ml DMEM. For the analysis of NGF action, cells were treated with or without 50 ng/ml NGF in 500 μl DMEM supplemented with 10% HS and 5% FCS. For the A23187 experiments, cells were serum starved 30 minutes then preincubated 45 minutes with DMEM without serum at 37°C.

**Immunoblot analysis**

PC12 cells were scraped into phosphate-buffered saline (PBS) and homogenized, at 4°C, with an Elvejem mini Potter in 50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM magnesium acetate, supplemented with 50 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM PMSF, 2.5 mM DTT and 1 mM EDTA. Then, crude extracts were centrifuged at 19,000 g for 1 hour at 4°C. Cell culture medium was removed and concentrated 10-fold (about 50 μl). Protein concentrations were determined by the Bradford procedure (Bradford, 1976). Aliquots of intra- (10 μg) and extracellular (10 μl) proteins were run under denaturing conditions on 8% polyacrylamide gels.

Electrotransfer of proteins to nitrocellulose membranes (0.45 μm; Schleicher and Schuell, Dussel, Germany) was performed with a semi-dry blotting apparatus (Hoefer Sci. Instruments, San Francisco, CA). Ap-B was detected with a rabbit polyclonal anti-Ap-B serum at 1:2,000 dilution as previously described (Cadel et al., 1995). The visualization of the antigen-antibody complex was carried out by two different methods: (i) using alkaline phosphatase coupled antibody and NBT/BCIP mixture; (ii) using horseradish-peroxidase coupled antibody and enhanced chemiluminescense detection (ECL). Bands obtained by both techniques were scanned with an Arcus plus Scanner (Agfa Gevaert, UK) coupled to a Macintosh computer using PhotoLook 2.07.2 (Agfa Gevaert UK) software. The signal intensities of the resultant profiles were evaluated using Adobe Photoshop 3.01, then integrated by Scan Analysis (Biosoft UK and USA). Histograms represent the mean values of a representative experiment which were performed at least three times. In the experiments, the band intensities obtained did not differ by more than 15-20% when either alkaline phosphatase or ECL reactions were used. The amount of Ap-B protein was expressed as the percentage of the value obtained with non treated cells, taken as 100%, which was defined as the arbitrary unit.

All western blots concerning secretion experiments were also treated with anti-alpha-tubulin antibody to show that no release of this intracellular protein occurred.

**Immunofluorescence microscopy**

PC12 cells were labeled by two different methods: (i) in order to observe total Ap-B labeling, cells were fixed in 3% paraformaldehyde in PBS for 2 hours at room temperature then washed 3 times in PBS and in 50 mM NH₄Cl. The cells were permeabilized in PBS solution containing 0.2% BSA, 0.005% Triton X-100 for 20 minutes then incubated, for 60 minutes, with a rabbit anti-Ap-B serum affinity purified according to the immunoblotting technique of Harlow and Lane (1988). Cells were rinsed 3 times with PBS then incubated with goat anti-rabbit IgG conjugated with FITC for 60 minutes. The anti-Golgi antibody was used in combination (v:v) with the purified anti-Ap-B antibody in double-labeling experiments; (ii) to selectively label Ap-B at the cell surface, living PC12 cells were rinsed twice in PBS at 4°C. Cells were washed 3 times with PBS at 4°C, they were fixed in 3% paraformaldehyde, 0.25% glutaraldehyde in PBS for 20 minutes at 4°C. Cells were washed with 0.1% NaBH₄ in PBS then incubated 60 minutes with goat anti-rabbit IgG conjugated with tetramethylrhodamine. Cells were examined either with an epifluorescence microscope (Leica
DMRB) coupled with a chilled CCD camera (Hamamatsu Photonics, Hamamatsu city, Japan) interfaced with a Power Macintosh 8500/120.

**Immunoelectron microscopy**

The immunoperoxidase procedure was performed on PC12 cells in Petri dishes as previously described (Tougard et al., 1980; Tougard and Picart, 1986). Briefly, cells were fixed with PLP for 2 hours at room temperature, washed, preincubated in 50 mM NH₄Cl. After incubation for 90 minutes with specific rabbit anti-Ap-B polyclonal antibodies, cells were incubated 90 minutes with goat Fab anti-rabbit IgG conjugated with peroxidase. After washing, post-fixation in 1% glutaraldehyde, detection of peroxidase activity using 3,3'-diaminobenzidine tetrahydrochloride, and post-fixation in 1% osmium tetroxide, cells were then embedded in situ in Epon (Brinkley et al., 1967). Observation at the light microscope level allowed us to select areas of immunoreactive cells which were then sectioned (Picart and Tixier-Vidal, 1974). Ultrathin sections were examined under the electron microscope without further staining.

**Enzyme activity**

The activity of Ap-B was determined routinely, using L-Arg-β-naphthylamide (L-Arg-β-NA) substrate, as previously described (Cadel et al., 1995). To measure the activity possibly associated with the external side of the plasma membrane, PC12 cells (6×10⁵) were grown on Petri dishes (3.5 cm diameter) for two days. The culture medium was removed and cells were washed three times with 1 ml PBS at room temperature. Then a further 1 ml of PBS containing 200 μM of L-Arg-β-NA was added and, after 30 minutes at 37°C, the amount of digested substrate, representing the total peptidase activities, was evaluated as above. To determine the specificity of Ap-B cleavage, tests were performed in the presence of 10 μM arphenamine B, a specific inhibitor of Ap-B activity. In order to evaluate the percentage of activity corresponding to the level of secreted Ap-B, the samples were centrifuged at 850 g for 10 minutes to eliminate suspended cells and then the supernatant was removed by suction. Ap-B activity released in the buffer was measured as above after addition of L-Arg-β-NA substrate. The fraction of Ap-B activity corresponding to the membrane-associated protein was evaluated by subtracting from the total Ap-B activity the level of Ap-B activity secreted by cells.

**RESULTS**

**Expression of Ap-B in PC12 cells**

Previous studies have shown that rat testis is the main organ for Ap-B protein expression (Cadel et al., 1995). In PC12 cells the Ap-B mRNA was also 2.2 kb long and, as expected its expression level was lower in pheochromocytoma cells than in male gonad (data not shown).

To investigate the hypothetical regulation of Ap-B expression during PC12 differentiation, cells were cultured for 12-96 hours in the absence, or in the presence, of nerve growth factor (NGF; 50 ng/ml). Then mRNA and cellular protein extracts were subjected to northern and western blot analyses, respectively. No significant difference could be observed in the Ap-B intracellular expression levels during NGF treatment (not shown). It can be concluded that NGF does not play an important role in Ap-B expression at the transcriptional and translational levels during PC12 differentiation, therefore most of the following experiments were performed with undifferentiated PC12 cells.

**Secretion of Ap-B by PC12 cells**

As mentioned above, the cleavage specificity of Ap-B strongly suggested that this enzyme might be involved in processing mechanisms. Therefore this exopeptidase would be localised in the secretory pathway and/or at the cell surface and in the culture medium. Analysis of the PC12 cell culture medium showed that the Ap-B protein was indeed present indicating that the enzyme is secreted (Fig. 1). This secretion increased with time without any treatment. Secreted Ap-B was detectable after 1 hour and its level increased 4-fold after 2 hours, and 6-fold after 6 hours. As the secretion level of the enzyme reached a detectable amount after 2 hours, this time was used for further studies on Ap-B secretion. All western blots concerning secretion experiments were treated with anti-alpha-tubulin antibody to show that no release of this intracellular protein occurred. This indicated that Ap-B release in the cell medium was not due to cell disruption, but indeed, to enzyme secretion (not shown). To characterize the nature of Ap-B secretion the effect of 8-bromo-adenosine 3'-5'-cyclic monophosphate (8-Br-cAMP) on the enzyme level in the extracellular medium was examined. This membrane permeant analogue of cAMP is known to exert its effect on the secretion by regulated exocytosis. Exposure of PC12 cells to different concentrations of 8-Br-cAMP allowed us to determine that 0.5 mM was the optimal concentration of secretagogue to enhance Ap-B secretion (more than 5-fold; Fig. 2). This enhanced secretion decreased when the 8-Br-cAMP concentration further increased. To determine an optimal time for 8-Br-cAMP action, cells were treated for 1 or 2 hours with this compound. The data in Fig. 3B show that the effect of 8-Br-cAMP reached a maximum (more than 5-fold increase) after 2 hours. There was no effect of 8-Br-cAMP (Fig. 3A) on the intracellular Ap-B content.

The antiviral antibiotic breelfadin A (BFA) was used to investigate the effect of an inhibitor of intracellular transport on Ap-B secretion. This fungal metabolite blocks protein
transport from the endoplasmic reticulum to the Golgi complex. At a concentration of 10 μg/ml BFA, it was observed that the secreted Ap-B level decreased within 30 minutes (Fig. 4). The maximum effect (6-fold decrease) was reached after 60 minutes.

The Ca\(^{2+}\) ionophore, A23187, was used to determine whether Ap-B secretion could be induced in a calcium-dependent manner. When 1 μM of the ionophore was added for 15 minutes to the incubation medium containing 1.8 mM Ca\(^{2+}\), the drug produced a 4-fold increase in the released enzyme level (Fig. 5). As mentioned above, control indicated that this brief ionophore treatment did not produce cell disruption.

Finally either cytochalasin D or nocodazole were used as actin or tubulin depolymerizing agents to show whether Ap-B secretion was dependent or not on the cytoskeleton network. Data in Fig. 6 indicated that whereas 10 μg/ml nocodazole had a significant effect in inhibiting Ap-B secretion, 1 μg/ml cytochalasin D did not decrease the extracellular Ap-B level after 2 hours treatment. Under these experimental conditions, PC12 cells adopted a round-shaped morphology, indicating that the drug concentrations used were adequate.

**Ap-B localization by immunofluorescence microscopy**

To further support the biochemical evidence for Ap-B secretion and to investigate the enzyme distribution in PC12 cells, several immunocytochemical techniques were used. Immunofluorescence was performed at first to localize Ap-B in PC12 cells. Preliminary experiments were conducted using different fixation methods.

Only the 3% paraformaldehyde fixation and cell permeabilization by 0.005% Triton X-100 gave a significant labeling. This fixation and previous affinity purification of the
anti-Ap-B serum allowed us both to localize Ap-B and to partially colocalize it with the CTR 433 antigen in the Golgi apparatus (Fig. 7). CTR 433 antibody was obtained from a library of monoclonal antibodies against centrosomes isolated from human lymphoblasts and was previously characterized as a marker of the medial compartment of the Golgi apparatus (Jasmin et al., 1989). Labeling obtained from cells fixed at 37°C showed Ap-B localization in the Golgi apparatus (Fig. 7A-A'). Note the yellow color of the Golgi stacks indicative of a colocalization of Ap-B and CTR 433 antigens. In order to demonstrate, by immunofluorescence technique, that Ap-B follows the secretory pathway PC12 cells were incubated either at 20°C or treated with nocodazole. This reduced temperature is known to block the transport of secretory products and membrane proteins at the level of the trans-Golgi network (Saraste et al., 1986). When cells were preincubated for 2 hours at 20°C prior to fixation, Ap-B labeling was restricted to the Golgi stacks (Fig. 7B and B'). Nocodazole is known to block the early secretory pathway and induces a fragmentation in the
Golgi apparatus. Fig. 7C-C’ shows the effect of nocodazole on the morphology of the Golgi apparatus and/or on the Ap-B labeling. As in the case of the 20°C treatment, Ap-B was found colocalized with CTR 433 antigen.

To discriminate Ap-B present at the cell surface from cellular Ap-B total content, 3% paraformaldehyde/0.025% glutaraldehyde fixation was employed on the PC12 living cells incubated at 4°C with anti-Ap-B serum, in order to minimize antibody internalization (see Materials and Methods). When external Ap-B was selectively labeled (Fig. 8B), a strong heterogeneity of the cell population labeling was observed. Only 5 to 7% of the cells exhibited a bright labeling whereas the majority of the cells indicated a weaker surface localization of the protein. However, this fluorescence was significantly brighter than the signal given by the preimmune serum (Fig. 8A).

**Ap-B subcellular distribution as revealed by immunoperoxidase at the electron microscope**

A conspicuous specific immunolabeling was observed on some domains of the plasma membrane of numerous PC12 cells (Fig. 9). It was not restricted to cell contacts. This staining was observed in cells treated or not with NGF. However, all cells did not exhibit this marked labeling. Quantification of the number of immunolabeled cells was difficult since this number varies from one cluster of cells to another (from
Aminopeptidase B secretion and localization

Ap-B activity associated with the membrane of PC12 cells

Further evidence for membrane attachment of Ap-B was obtained by testing enzyme activity towards L-Arg-β-NA on the supernatant solution of PC12 cells (see Materials and Methods). Total secreted and membrane-associated enzyme activities containing in addition to Ap-B other related, or different, enzyme activities, were found to produce about 66 pmole/minute of β-NA. Consequently, tests were performed in the presence of 10 μM arphamenine B, a specific inhibitor of Ap-B cleavage, to establish that 74% of this activity could be attributed to both secreted and membrane-associated Ap-B. These results also confirmed that other aminopeptidases, different from Ap-B (like Ap-N for example) were associated with the cell surface and hydrolysed the substrate.

The fraction of membrane-associated Ap-B activity was evaluated by measuring the level of secreted enzyme (see Materials and Methods) and subtracting it from total Ap-B activity. The fraction corresponding to the secreted enzyme generated about 32 pmole/minute of product and was totally inhibited by arphamenine B. Since this enzyme activity represented 66% of the total Ap-B activity, the remaining (16 pmole/minute) was attributed to membrane-associated Ap-B in PC12 cells.

DISCUSSION

Previous reports have established Ap-B as a Zn\(^{2+}\)-dependent exopeptidase strictly specific for Arg and Lys residues at the N terminus of various peptides (Cadel et al., 1995, 1997). Since a 72 kDa immunologically related form of Ap-B was detected in brain cortex as well as in other rat tissues (Foulon et al., 1996, 1997), these data argued in favour of a ubiquitous distribution of the enzyme together with the above mentioned enzyme properties of a role in propeptide and proprotein processing mechanisms. The present results indicate unequivocally that, in PC12 cells, Ap-B is in part associated with the Golgi apparatus and secreted. Moreover, the enzyme is both localized and active at the level of the plasma membrane.

It was previously suggested that Ap-B might be secreted by various cells including Jurkat T cells (Belhacène et al., 1993) and bovine pars intermedia pituitary cells (Gainer et al., 1984; Castro et al., 1989). Indeed, the primary structure of the enzyme indicates the presence, at the N terminus, of a putative signal peptide which could be responsible for this property (Cadel et al., 1997). The present observations clearly demonstrate that Ap-B is secreted by PC12 cells both in a constitutive and a regulated manner as shown for the distinct molecular forms of acetylcholinesterase (Schweitzer, 1993). Whereas Ap-B secretion increased with time without stimulation, this effect could be significantly stimulated by various secretagogues including 8-Br-cAMP and A23187. These observations and the significant inhibition of Ap-B secretion by brefeldin A, a drug known to affect the secretory process, argued in favour of an Ap-B transit via the endoplasmic reticulum to the Golgi apparatus. The detection of Ap-B in the Golgi apparatus and in some secretory granules clearly indicates that these structures participate in the transport of the enzyme to the cell surface. This result reinforced some previously reported observations such as the detection of Ap-B activity in secretory granules of rat cortical cells (Gluschankof et al., 1987) and of bovine pars intermedia pituitary cells (Gainer et al., 1984), and the presence of Ap-B protein in the Golgi apparatus of young spermatozoides in rat testis (Cadel et al., 1997).

Finally it should be noticed that, in the presence of Ca\(^{2+}\) ions in the culture medium, the calcium ionophore A23187 had an intense effect on enzyme liberation. Consequently, it can be hypothesized that Ap-B might exert a processing function at the cell surface, possibly on co-secreted substrate(s) under a concomitant Ca\(^{2+}\)-dependent cellular signal.

Rat PC12 cells, which are derived from a tumour of adrenal medulla, share morphological and cytochemical properties with chromaffin cells and neurones (Greene and Tischler, 1976). One common feature is the presence of hormone or neurotransmitter in secretory granules within these cells. Interestingly, PC12 cells have no detectable processing activity in agreement with the fact that they do not express significant levels of PC1 or PC2 (Galanopoulou et al., 1993). Indeed, a number of hormones and neuropeptides in this cell line are stored mainly in their precursor forms (Carraway et al., 1993; Dittie and Tooze, 1995).

Although prohormone convertases, with the exception of furin, have been reported to be absent from these cells (Galanopoulou et al., 1993), carboxypeptidase E (CPE), which is known to complement the action of these endopeptidases, is largely present in PC12 (Das et al., 1992). These observations led us to hypothesise that CPE and, possibly, other propeptide processing enzymes, could function after their secretion (Darby and Smyth, 1990). In this manner, peptide precursors might be processed by enzymes located at the plasma membrane level of the producing cells. Similarly, Ap-B, which is secreted as a full length (72 kDa), active form, indistinguishable from the intracellular enzyme, could be involved in such a mechanism since it exhibits an activity in the extracellular medium. Moreover, the fact that Ap-B is active over a broad pH range and shows an optimal activity at neutral pH, argues in favour of such a role. Strikingly, fluorescence and electron microscopy examination of Ap-B subcellular localization indicated an association with the membrane of PC12 cells. Although previous reports provided preliminary evidence for such an association in Jurkat T cells (Belhacène et al., 1993), there was no clear demonstration for such a concentration as seen in PC12 cells in the present report. Furthermore, western blot analyses confirmed this Ap-B association with the membrane after cell disruption and fractionation of their various compartments (data not shown). This plasma membrane localization of Ap-B was also clearly detected in rat prolactin cells in culture (C. Tougard et al., unpublished observations). Together these observations are suggestive of plasma membrane targeting of Ap-B via the secretory pathway. The question then arises of how Ap-B could be attached to the membrane. Since there was no evidence for a well defined putative trans-membrane domain in the Ap-B...
primary structure (Cadel et al., 1997), the mode of attachment of the enzyme to the membrane is at present unknown. Further studies should indicate if this peptidase is associated in some fashion to cell surface receptors. Consequently, it can be hypothesised that Ap-B may act at the extracellular level as a processing exopeptidase.

We thank Drs Michel Bornens, Evelyne Coudrier (Institut Curie) and Bruno Goud (Institut Curie) for helpful discussions and Dr Michel Bornens for the CTR 433 anti-Golgi antibody. Photographs were kindly supplied by Eric Etienne (Collège de France). This work was supported in part by funds from the Centre National de la Recherche Scientifique (CNRS, URA 1682), Université Pierre et Marie Curie, Rhône-Poulenc-Rorer (Programme Bioavenir) and the Institut National de la Santé et de la Recherche Médicale. A.B. and S.C. were supported in part by funds from the Centre National de la Recherche Scientifique (CNRS, URA 1682), Université Pierre et Marie Curie, and supported in part by funds from the Centre National de la Recherche Scientifique (CNRS, URA 1682), Université Pierre et Marie Curie.

REFERENCES


