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Characterization of Pro-opiomelanocortin Processing in Heterologous Neuronal Cells that Express PC2 mRNA

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Abstract—We have investigated processing of monkey pro-opiomelanocortin (POMC) following transfection into heterologous neuronal Neuro 2A (N2A) cells. In several separately transfected stable cell lines (termed N2A/POMC2-like; n = 4), POMC was processed to βE only, by direct cleavage from the precursor. Thus, these cell lines did not produce βE in the orderly manner observed in the pituitary, that is, via the intermediate peptide βLPH. Analysis of one representative N2A/POMC2 cell line revealed that the extent of processing to βE appeared to be negatively correlated with precursor expression level, suggesting that the processing enzyme(s) in these cells was present in limiting amounts. Northern analysis of PC1 and PC2, two recently cloned processing enzymes, showed that N2A/POMC2 cells expressed low levels of PC2 mRNA, but no detectable PC1 mRNA. These data suggest that (1) the order of processing observed in the pituitary is not exclusively determined by tertiary folding of the precursor, but rather by the complement of processing enzymes in a particular cell, and (2) if PC2 is responsible for POMC processing in N2A/POMC2 cells, this enzyme, expressed in limiting amounts, appeared to show selectivity for the βE amino terminal processing site.

Introduction

Pro-opiomelanocortin (POMC) is the precursor of several different peptides with distinct biological activities, including the stress hormone, adrenocorticotropic hormone (ACTH), and the opioid peptide, βEndorphin$_{1-31}$ (βE).[1] These component peptides are excised from POMC by enzymatic cleavage at specific dibasic amino acid residues (e.g. lys-arg), a process which is common to many precursor proteins. POMC and its component peptides are found in a number of peripheral tissues such as the pituitary, pancreas and gastrointestinal tract and in neurons in the brain emanating from the arcuate nucleus and nucleus tractus solitarius (NTS). Although there is only one POMC gene, the extent to which this molecule is processed to yield different peptides varies depending on the tissue in which it is expressed.[1,2] In addition, this precursor is processed in an orderly manner. For the purpose of this study, processing of the carboxy terminal half of the precursor (comprising ACTH and βLPH), in the rat species will be described (for review see ref. 2). The initial phases of proteolytic cleavage are consistent
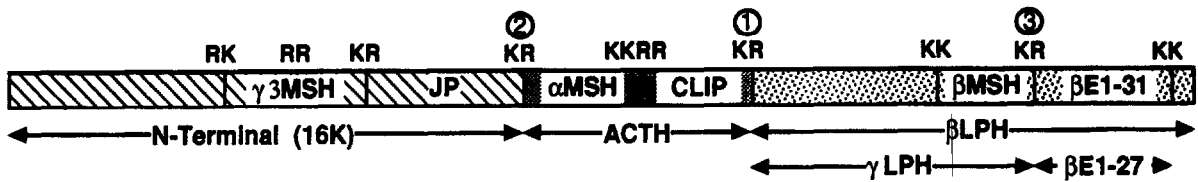
Fig. 1 Structure of POMC. This figure shows the POMC precursor and its component peptides, as well as the dibasic amino acid cleavage sites flanking each component peptide (e.g. KR = lys-arg). Certain cleavage sites labelled by circled numbers are referred to in the text.

across tissues. In all tissues, the first sites to be cleaved within POMC (sites 1 and 2, Fig. 1) result first in the production of βLPH, followed by the production of ACTH. The extent to which subsequent cleavages occur then differs between different tissues. In the anterior pituitary, there is little further processing, except for cleavage of ~30% of the βLPH molecules (at site 3, Fig. 1) to give γLPH and βE. Thus, the major carboxy terminal end products in this tissue are ACTH, βLPH and some βE. In the intermediate pituitary, the POMC precursor is processed more extensively. ACTH is processed to yield ACTH$_{1-13}$ and CLIP, and βLPH is fully processed to γLPH and βE. In addition, the βE moiety is further processed at its carboxy terminus to truncated forms of this peptide. Other modifications in the intermediate pituitary include N-acetylation and C-terminal amidation of ACTH$_{1-13}$ (resulting in α-melanocyte stimulating hormone; αMSH) and acetylation of βE peptides. In the brain, POMC processing appears to resemble that seen in the intermediate pituitary, in that the smaller peptides predominate. However, whereas in the arcuate nucleus non-acetylated βE and αMSH are the major end products,[3,4] in the NTS acetylated peptide forms predominate.[5]

What is responsible for the orderly and tissue-specific processing of POMC? While the orderly pattern of POMC maturation could either be a function of the conformational folding of the precursor, or a function of the cleavage site specificity of the POMC processing enzyme(s), the tissue-specific pattern of cleavage is presumably due to differential expression or regulation of these enzymes. Two candidate neuropeptide processing enzymes, termed PC1 (or PC3) and PC2, have recently been cloned from pituitary and insulinoma libraries.[6–10] RNA transcripts of these clones have been shown to be present in a num-

ber of tissues and cell lines, suggesting that they play a widespread role in precursor processing. With respect to POMC-producing tissues, it has been shown that anterior pituitary corticotrophs contain abundant PC1 mRNA, but low levels of PC2 mRNA, while in the intermediate pituitary, PC2 message is more abundant than PC1 message.[9,11,12] These findings, in conjunction with POMC processing data, suggest that PC1 may be predominantly important in the initial cleavages of POMC resulting in the production of βLPH and ACTH, whilst PC2 may be predominantly important in the later stages of processing resulting in the production of βE and αMSH. More direct data on the cleavage site specificity of PC1 and PC2 were obtained by co-expressing the precursor with each enzyme in host cell lines.[13,14] It was found that co-expression of POMC with PC1 resulted in the production of ACTH and βLPH, suggesting that PC1 favored cleavage at the amino terminal of ACTH and βLPH. In comparison, co-expression of the precursor with PC2 resulted in production of βE, αMSH and an ACTH peptide extended with either joining peptide (JP)[13] or γLPH.[14] The latter finding implies that PC2 has a broader range of cleavage site specificity than PC1, since it cleaved more sites in the POMC precursor. However, it should be noted that the enzymes were highly expressed in these studies, due to virally-mediated DNA transfer, and that this condition may not be representative of the true cleavage site specificity of PC1 and PC2 when expressed in their natural tissues.

An alternative approach to studying prohormone processing enzymes is to examine peptide end products following transfection of precursor cDNA into a variety of heterologous cell lines. The results of these studies aid in determining those elements of processing controlled by the intrinsic precursor

structure, or by the complement of endogenous processing enzymes within a particular cell. This type of approach has been used to study processing of several propeptides.[15-19] In this paper, we have examined processing of POMC by the propeptide convertase(s) in a heterologous neuronal cell line, Neuro 2A (N2A). This cell line, derived from a mouse neuroblastoma, does not synthesize endogenous POMC, and synthesis of other neuropeptides awaits demonstration. However, it has been shown that N2A cells can express transfected POMC cDNA and process the precursor to authentic βLPH and βE.[16] Given their neuronal origin and their ability to carry out post-translational processing, N2A cells appeared to be a good model for studying POMC processing in the brain. In pursuing this line of study, we have uncovered an unusual pattern of POMC processing. Our data show that several separately transfected N2A cell lines (which we have termed N2A/POMC2-like) processed POMC to βE only, by direct cleavage from the precursor; no βLPH was produced. This is a novel pattern of processing, not seen in tissues which normally produce POMC, and is in contrast to previous data showing that transfected N2A cells processed POMC to βLPH and βE.[16] We have also observed the latter pattern of processing in transfected N2A cell lines. However, for the purpose of this study, we have focussed on those N2A cell lines which processed POMC to βE only.

Materials and methods

Construction of expression vectors

The POMC expression vector was constructed by subcloning an 870 base pair HindIII-SalI cDNA restriction fragment, containing the monkey POMC coding sequence,[20] into the cloning region of a plasmid employing the inducible mouse metallothionein I promoter (pMtneo; obtained from Dr J. Dixon, University of Michigan, USA). This vector contained the gene coding for neomycin phosphotransferase, confering resistance to the cytotoxic antibiotic G418 (geneticin). The resulting plasmid plus insert was termed pMtneo-POMC.

Maintenance, transfection, and selection of cell lines

N2A cells were obtained from ATCC (Rockville, MD, USA) and grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (GIBCO). The day prior to transfection, cells were plated at a density of 0.5×10^6 cells/plate in 10 cm tissue culture plates. Transfection was carried out by calcium phosphate co-precipitation, as described by Chen and Okayama,[21] using supercoiled plasmid DNA (20 μg) purified by cesium chloride gradient ultracentrifugation. 72 h after transfection, plasmid-containing cells were selected by addition of 0.5–0.75 mg/ml G418 to the media. Non-plasmid-containing cells died after 2–3 weeks exposure to G418. Following selection, individual cell clones were subcultured to separate flasks and maintained in 0.5 mg/ml G418. Cell cultures were passaged (subcultured) every 5–7 d (P1 representing the first passage following selection of stable cell line). 16 h prior to harvesting, the metallothionein promoter was induced, and hence POMC expression was stimulated, by addition of 85 μM ZnCl₂ to the media.

Extraction and molecular sieving of peptide products

N2A cells were scraped from tissue culture flasks, pelleted and homogenized in 0.2 M HCl/acetone (1:3) to extract peptide products. The cell extract was then passed over Sep-Pak C18 cartridges (Waters) and the eluant dried by rotary evaporation. Lyophilized cell extracts (from 20–30 million cells) were then fractionated on a Sephadex G50 column (1.5 × 90 cm; Pharmacia) equilibrated in 1% formic acid/0.01% BSA. The column was developed at a flow rate of 0.25 ml/min and 1.8 ml fractions were collected. The following molecular weight markers (10 mg/sample volume) were used: Blue Dextran to mark the void volume, cobalt chloride to mark the total volume and 13 K cytochrome C.

Measurement of POMC peptides by radioimmunoassay (RIA)

Following Sephadex G50 column chromatography, individual fractions were assayed for different POMC-derived peptides using specific, well characterized RIA's. The following antisera were used: Brenda (raised in our laboratory), directed against the midportion of the βE molecule (residues 17-27), with 100% cross-reactivity with POMC, βLPH, βE₁₋₃₁, βE₁₋₂₇ and βE₁₋₂₆; Nancy Beth (raised in our

laboratory), directed against the N-acetylated terminal of βE, with 100% cross-reactivity with all N-acetylated forms of βE; anti-βMSH (obtained from Dr N. G. Seidah, Clinical Research Institute of Montreal, Canada), directed against human βMSH, with 100% cross-reactivity with monkey POMC, βLPH, γLPH and βMSH; and anti-ACTH (raised in our laboratory), directed against residues 15–24 of this molecule, with no cross-reactivity with αMSH and 8% cross-reactivity with CLIP. The βE, N-Acetyl-βE and ACTH RIA's were performed as described previously.[5] The βMSH RIA was performed using the same conditions as the βE assay, with a final antibody dilution of 1:50 000.

HPLC analysis of peptides

N2A/POMC2 cells were harvested as described above and homogenized in 0.2 M HCl/acetone (1:3). The cell extract was then clarified by centrifugation, dried by rotary evaporation, and resuspended in 0.1% trifluoroacetic acid (TFA) with 0.001% BSA. This suspension was sieved through a Sephadex G50 column equilibrated in 0.1% TFA/0.001% BSA and the resulting fractions assayed for βE-immunoreactivity (βE-ir). Those fractions containing βE-sized immunoreactivity were pooled, dried, and resuspended in 0.1% TFA. The samples were then subjected to weak cation exchange HPLC, as described by Dores et al.[5] HPLC column fractions were dried and subjected to βE and N-Acetyl-βE RIAs. Standards (human) used to identify βE peptides were: N-Acetyl βE_{1-27}(AcβE_{1-27}; +3 charges); N-Acetyl βE_{1-26} (AcβE_{1-26}; +3 charges); βE_{1-27} (+4 charges); βE_{1-26} (+4 charges); N-Acetyl βE_{1-31} (AcβE_{1-31}; +5 charges); βE_{1-31} (+6 charges). It should be noted that while human βE_{1-31} differs from monkey βE_{1-31} in its extreme carboxyl terminal end, both sequences have the same number of positive charges and will elute similarly on cation exchange HPLC.

Northern analysis of PC1 and PC2 mRNA

The presence of PC1 and PC2 mRNA in N2A/POMC2 cells was assessed using specific, radio-labelled cRNA probes made from mouse PC1 and PC2 cDNA vectors (kindly donated by Drs R. Day and N. G. Seidah, Clinical Research Institute of Montreal, Canada). For comparison, these neuronal cells were probed alongside two other cell lines:

AtT20, derived from a mouse corticotrophic tumor, and Rin m5F, derived from a rat insulinoma, both of which express PC1 and PC2 mRNAs.[6,8,9]

Radiolabelled antisense cRNA probes were generated from cDNA's corresponding to the 3′ coding regions of PC1 and PC2 by in vitro transcription with T7 polymerase in the presence of 250 µCi (α-^{32}P)UTP. Total cytoplasmic mRNA was extracted from N2A, AtT20 and Rin m5F cells as described by Sambrook et al.[22] The RNA samples (50 µg) were size-fractionated on a 6% formaldehyde/1.5% agarose gel, then passively transfered to a Nytran filter and air dried. The filter was pre-hybridized (2 h, 60°C) in 50% formaldehyde, 400 mM Na_2PO_4, 1 mM EDTA, 1 mg/ml BSA and 5% SDS, pH 7.0. Radiolabelled PC1 or PC2 probe was added to the hybridization buffer and incubated at 60°C for 18 h. The filter was then washed (0.1 × SSC, 0.1% SDS and 1 mM EDTA, 70°C for 2 h) and exposed to X-ray film (Kodak x-Omat XAR5).

Results

POMC processing in transfected N2A cells

POMC peptide products, following extraction from transfected cells and separation by Sephadex G50 chromatography, were assayed for peptide-immunoreactivity by specific RIAs. Separate transfections of N2A with pMtneo resulted in cell lines with two POMC processing patterns. One set of transfected cell lines (n = 3) processed POMC to βLPH and βE (data not shown), while several separately transfected cell lines (termed N2A/POMC2-like; n = 4) processed POMC to βE only. The exact factors responsible for the two different processing patterns remain unclear. However, we have focussed our studies on the latter, novel pattern of processing, since the former processing pattern has been described before.[16]

The processing pattern of passage 4 (P4) from a representative N2A/POMC2 cell line is shown in Figure 2a–c. Following βE RIA, two predominate βE-immunoreactive species were observed which corresponded to 31 K POMC and 3.5 K βE; virtually no 11.5 K βLPH peak was observed. This pattern of processing may have resulted from rapid and complete processing of βLPH to βE, or from direct
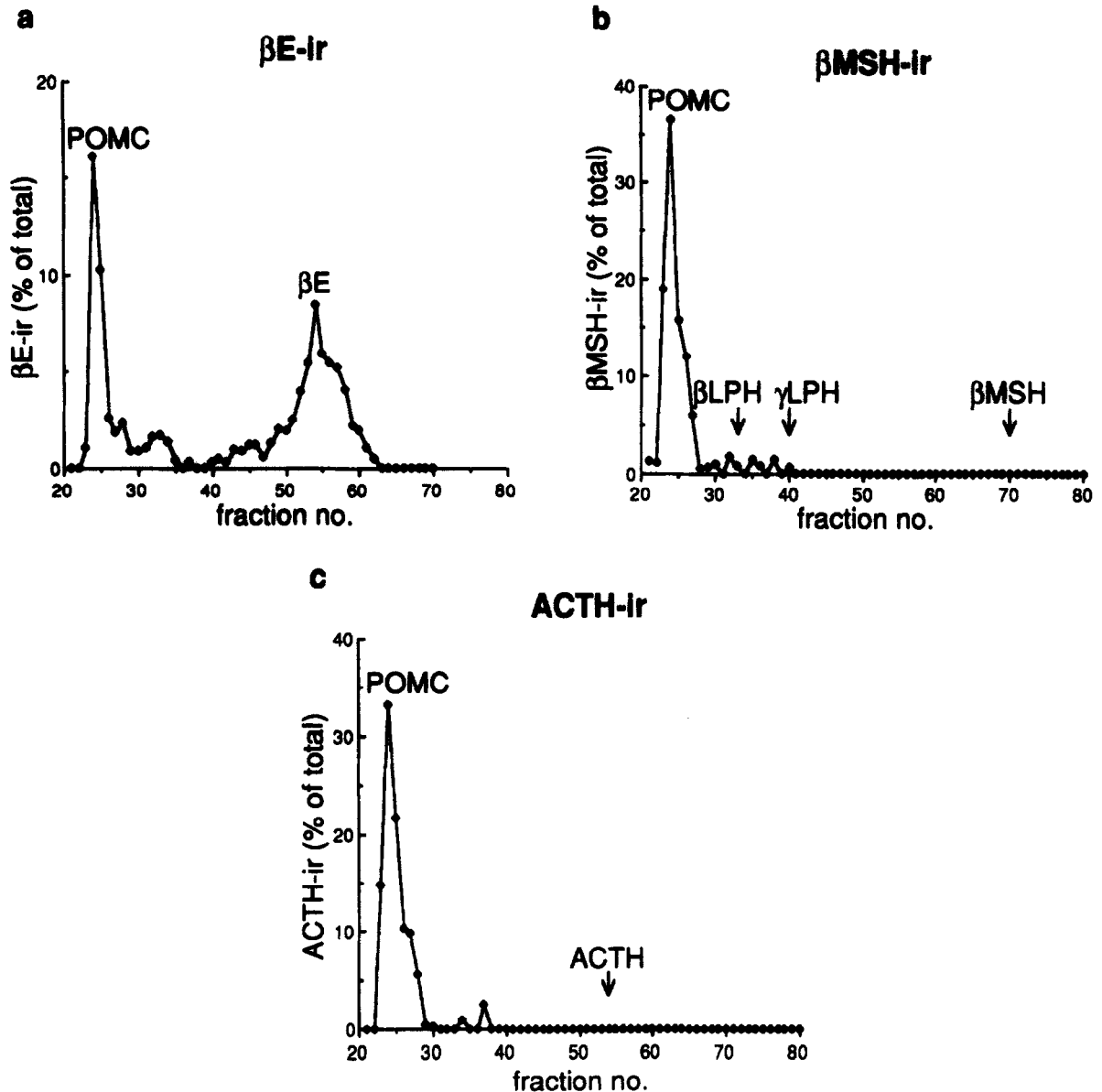
Fig. 2 POMC processing in N2A/POMC2 cells. This figure shows βE-ir (a), βMSH-ir (b) and ACTH-ir (c) chromatographic profiles from a representative N2A/POMC2 cell line. The βE-immunoreactive profile (a) shows that POMC was processed to βE only; virtually no βLPH was produced. When the same fractions were assayed for βMSH-ir (b), no γLPH or βMSH (βLPH cleavage products) were observed, suggesting that βLPH was not an intermediate to βE synthesis and therefore that βE was cleaved directly from the precursor. ACTH RIA on these fractions (c) showed that POMC was not processed to $ACTH_{1-39}$.

cleavage of βE from the POMC precursor. In order to investigate this issue, we subjected the same set of fractions to βMSH RIA, to look for the presence or absence of γLPH and βMSH, two cleavage products of βLPH. These peptides would have to exist in concentrations equimolar to βE if processing had proceeded via the intermediate peptide βLPH. The resulting βMSH sieving profile (Fig. 2b) exhibited only one immunoreactive species, corresponding to 31 K POMC. Thus, the absence of γLPH or βMSH in this cell line suggests that βE was cleaved directly from the precursor.

Table Proportions of βE-immunoreactive peptides in different passages from a representative N2A/POMC2 cell line

| Passage no.	βE-ir (% of total)			Total βE-ir
	POMC	βLPH	βE	(fmoles/10⁶ cells)
3	31.4	—	68.6	5.3
9	36.3	—	63.7	7.8
26	60.4	—	39.6	12.9
33	44.4	—	55.6	11.4

ACTH RIA on these N2A/POMC2 cells resulted in one immunoreactive peak, eluting in the position of the POMC precursor (Fig. 2c). This peak presumably consisted of intact POMC precursor and the peptide fragment remaining following cleavage of βE (~28 K). The absence of any $ACTH_{1-39}$-sized immunoreactivity in this cell line confirmed that the carboxy terminal processing site of ACTH was not cleaved, as predicted from the βE and βMSH profiles from the same cell line (Fig. 2a & b), and also implies that the amino terminal processing site of ACTH was not cleaved.

Cell lines exhibiting N2A/POMC2-like processing patterns remained stable with continuous passaging, consistently producing βE only. This finding is illustrated in Table for a second N2A/POMC2-like cell line.

HPLC analysis of βE-ir

βE-sized material was analyzed in a representative N2A/POMC2 cell line by weak cation exchange HPLC analysis. Using a mid-portion βE RIA (Brenda), four peaks of immunoreactivity were detected (Fig. 3) which corresponded to $AcβE_{1-27}$ (+3 charges), $βE_{1-27}$ (+4 charges), $AcβE_{1-31}$ (+5 charges) and $βE_{1-31}$ (+6 charges). Analysis of the same fractions with a RIA which recognized N-acetylated derivatives of βE (Nancy Beth) confirmed that the peaks of immunoreactivity eluting in fractions 28–35 and 57–61 represented $AcβE_{1-27}$ and $AcβE_{1-31}$ respectively (shaded peaks in Fig. 3).

In this particular cell line, 62% of the βE-ir corresponded to $βE_{1-27}$ peptides ($βE_{1-27}$ and $AcβE_{1-27}$), while 21% corresponded to full length peptides ($βE_{1-31}$ and $AcβE_{1-31}$). It should be noted, however, that HPLC analysis of βE-sized material from a different N2A/POMC2 cell line resulted in a profile where the majority of the immunoreactivity (69%)
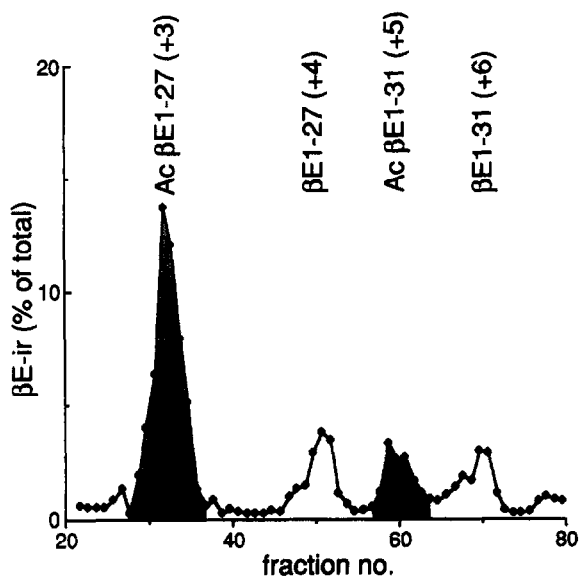

Fig. 3 Ion exchange HPLC analysis of βE-ir in N2A/POMC2 cells. βE-sized material from N2A/POMC2 cells was dried, resuspended in 0.1% TFA and chromatographed on a TSK CM-2SW ion exchange column as described in Methods. (a) Chromatographed βE-sized material was assayed with a mid-portion directed βE antiserum (Brenda; solid line) and the N-Acetyl βE antiserum (Nancy Beth; shaded areas). The elution position of the following standards is shown: $AcβE_{1-27}$ (+3 charges); $βE_{1-27}$ (+4 charges); $AcβE_{1-31}$ (+5 charges); $βE_{1-31}$ (+6 charges).

corresponded to full length βE peptides, while only 25% represented $βE_{1-27}$ peptides. In both cases, more of the truncated peptide, $βE_{1-27}$, was present in acetylated form (> 70%) than the full length peptide (~50%).

Correlation of POMC processing to βE with precursor expression level

The effect of precursor expression level on processing to βE is shown in Figure 4. In this analysis, processing to βE was defined as the percentage that βE peptide represented of total POMC expression (that is, total βE-ir; the sum of the amount of POMC, βLPH and βE peptides in fmoles/10⁶ cells). This graph represents data from several passages of a representative N2A/POMC2 cell line (see Table for data). The data show that the higher the level of POMC expression, the lower the degree of processing to βE (Fig. 4, r = –0.93). These data suggest that the processing enzyme in this N2A/POMC2 cell line was present in limiting amounts with respect to the amount of precursor expressed.
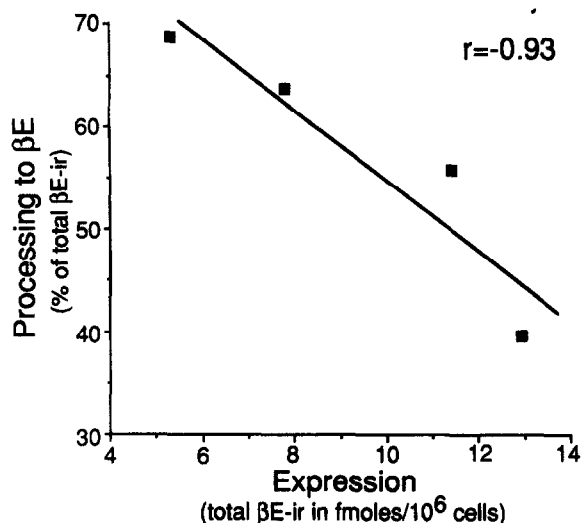
Fig. 4 Correlation of POMC processing to βE with precursor expression level. POMC processing to βE (as a percentage of total βE-ir) was plotted against POMC expression level (i.e. total βE-ir; the sum of the amount of POMC, βLPH and βE in fmoles/10⁶ cells) for several passages from a representative N2A/POMC2 cell line (data shown in Table). The correlation coefficient (r value) is indicated. The graphs show that those passages with higher levels of POMC expression exhibited less processing to βE peptide.
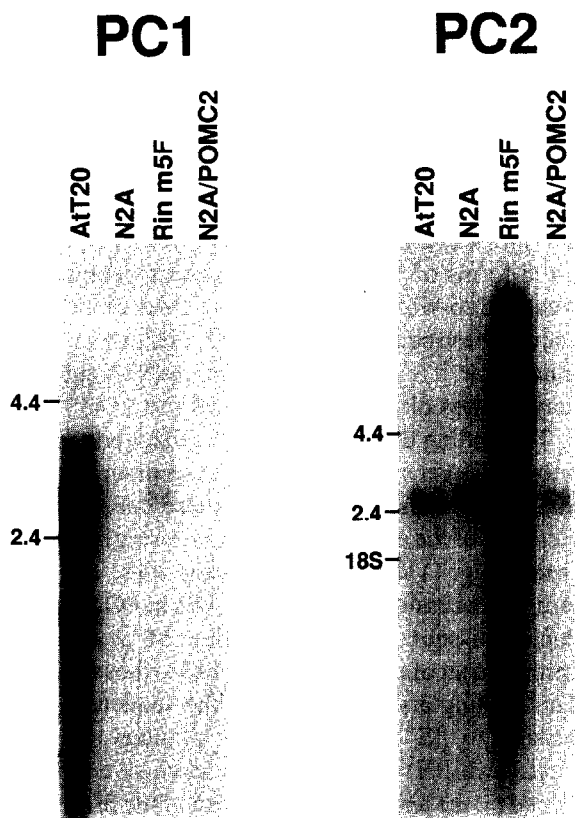
PC1 and PC2 mRNA expression in N2A/POMC2 cells

PC1 and PC2 mRNA expression were determined in untransfected and transfected N2A cells, AtT20 cells and Rin m5F cells by Northern gel analysis (Fig. 5). With respect to PC1 mRNA, one major band was observed in AtT20 and Rin m5F lanes which migrated just above the 2.4 kilobase (kb) marker. PC1 transcripts were most abundant in AtT20 cells and very low in Rin m5F cells. There was no detectable PC1 mRNA in untransfected N2A cells or in N2A/POMC2 cells. This transcript remained undetected following Northern analysis of poly(A⁺) mRNA from these cells (data not shown). Analysis of PC2 mRNA in the different cell types revealed one major band, migrating at a slightly lower molecular weight than PC1. PC2 mRNA was abundant in Rin m5F cells, but present in low amounts in AtT20 cells. Both untransfected N2A and N2A/POMC2 cells expressed low levels of PC2 mRNA.

Discussion

In this paper we have shown that several stably transfected N2A cell lines (termed N2A/POMC2-like)

processed transfected monkey POMC to βE-sized products only; this appeared to occur via direct cleavage of βE from the precursor since no biosynthetic intermediates or peptide products from any other region of the precursor could be detected. This is in contrast to several separately transfected stable cell lines where POMC was processed to βLPH and βE, as described previously.[16] The reason for the two different processing patterns is unclear and has not been addressed in this study. Instead, we have focussed on the novel processing pattern seen in N2A/POMC2-like cell lines. In this type of cell line we found that (a) the βE-sized material corre-

Fig. 5 Northern analysis of PC1 and PC2 mRNA in N2A/POMC2 cells: comparison with untransfected N2A, AtT20 and Rin m5F cells. Total cytoplasmic mRNA was extracted from the above cell lines, size-fractionated on an agarose gel and transfered to a Nytran filter. PC1 and PC2 mRNA were detected by hybridization to radiolabelled PC1 or PC2 cRNA probes and visualized by exposure to X-ray film. Both PC1 and PC2 mRNA migrated just above the 2.4 kb marker, although PC2 mRNA was slightly smaller in size than PC1 mRNA. N2A/POMC2 cells expressed low levels of PC2 mRNA, but no detectable PC1 mRNA.

sponded to elution of βE standard peptides by HPLC analysis, (b) processing to βE appeared to be dependent on the level of POMC expression, and (c) that these cells expressed low levels of PC2 mRNA, but no detectable PC1 mRNA.

Ion exchange HPLC analysis of βE-sized material in N2A/POMC2 cells demonstrated the presence of authentic βE_{1-31} and βE_{1-27} peptides. The ratio of truncated/full length peptides varied (from 62% to 25%) in two cell lines analysed. This variation was probably due to differences in the ratio of transfected precursor to processing enzyme, but this remains to be tested. With respect to full length βE-ir, ~50% appeared to be N-acetylated, whereas somewhat more of the truncated peptide (>70%) was present in acetylated form. A similar phenomenon was observed for acetylation of βE peptides in rat intermediate pituitary[23] and caudal medulla tissue.[5] This is probably due to acetylation being a more rapid process than carboxy-terminal truncation, such that βE_{1-31} is acetylated first and then cleaved to βE_{1-27} and βE_{1-26}.[24] That we observed acetylated and truncated forms of βE differs from results obtained by Noel and co-workers[16] who did not observe the presence of these peptides. While the explanation for this is not clear, it is possible that different N2A sublines may possess different biochemical features.

The finding that N2A/POMC2 cells produced βE only, by direct cleavage from the precursor, implies that the processing enzyme in these cells favored cleavage of the amino terminal processing site of βE (site 3, Fig. 1) above other cleavage sites. This pattern of processing is unlike that seen in POMC-producing tissues and is indicative of a different complement of processing enzymes to these tissues. This finding also suggests that the sequential processing to βE observed in the pituitary (that is, through βLPH) is unlikely to be exclusively determined by the tertiary conformation of the precursor, but rather is controlled predominantly by the processing enzymes themselves. Analysis of processing in several passages from a representative N2A/POMC2 cell line showed that the higher the level of precursor expression the lower the extent of processing to βE. The phenomenon of higher levels of precursor expression (and hence lower enzyme/precursor ratio) causing a decrease in processing has been observed before[25] and suggests that the amount of POMC expression in N2A/POMC2 cells may have saturated the N2A processing enzyme, that is, that the processing enzyme was present in limiting amounts.

With the cloning of PC1 and PC2, we were able to look for the expression of mRNAs coding for these enzymes in transfected N2A cells. As positive controls, we examined mRNA expression in AtT20 and Rin m5F cells, both of which express PC1 and PC2 mRNA and are known to process peptide precursors. AtT20 cells express endogenous POMC and process this precursor to similar end products as those found in the anterior pituitary,[2] that is, βLPH, ACTH and some βE. These cells, like anterior pituitary corticotrophs, contain abundant PC1 mRNA and low levels of PC2 mRNA.[6] Rin m5F cells, on the other hand, have been shown to process transfected POMC to a greater extent than anterior pituitary corticotrophs, the major end products (βE and αMSH) being similar to those found in the intermediate pituitary (ref. 26 and N. C. Day, unpublished data). With respect to enzyme mRNA expression in Rin m5F cells, both PC1 and PC2 were cloned from this cell line.[9] Although the relative abundance of these mRNAs was not reported, it has been shown that mouse and human insulinomas express abundant PC2 mRNA, but low levels of PC1 mRNA.[6,8]

Our analysis of PC1 and PC2 mRNA expression in AtT20 cells confirmed the above findings, showing that these cells contained an abundance of PC1 compared to PC2 mRNA and that these transcripts were similar in size to those reported previously.[6,8] In contrast, Rin m5F cells expressed abundant PC2 and very low levels of PC1 mRNA, indicating that rat insulinoma cells express these enzymes in similar proportions as mouse and human insulinoma cells.[6,8] Analysis of mRNA expression in N2A/POMC2 cells revealed that this cell line expressed low levels of PC2 mRNA, but contained no detectable PC1 mRNA (even following Northern analysis of poly(A$^+$) mRNA from transfected cells; data not shown). The PC2 transcript in N2A/POMC2 cells was similar in size and abundance as that seen in AtT20 cells.

The findings of the present study lead to some implications about the cleavage site specificity of PC2 and the enzyme complements required for processing to different POMC peptides. Previous stud-

ies of the cleavage site specificity of PC1 and PC2 have involved co-expression of the enzymes with POMC in host cell lines, using vaccinia virus-mediated DNA transfer.[13,14] The results of these studies suggested that PC1 favored cleavage at the amino terminal of ACTH and βLPH, while PC2 exhibited a broader range of specificity, cleaving additional sites in the POMC precursor. From these studies, the question arises as to the purpose of expressing PC1 in POMC-producing tissues, when PC2 appears to possess the ability of cleaving those sites which PC1 cleaves, as well as additional sites. We would like to suggest that the high level of expression afforded with the vaccinia virus method of transfection may not have been representative of the cleavage site specificity of PC2 when expressed in limiting amounts. In our studies, the POMC processing enzyme(s) in N2A/POMC2 cells did appear to be expressed in limiting amounts. Furthermore, expression of PC2 mRNA in these cell lines suggests that this propeptide convertase may have been responsible for the observed pattern of processing. It follows then that in the conditions prevailing in N2A/POMC2 cells, PC2 may have favored cleavage of the βE amino terminal processing site above other processing sites. Thus, this enzyme may indeed exhibit greater specificity than previously reported in POMC/PC2 co-expression studies.[13,14] Our findings also suggest that production of βE via the βLPH intermediate, as occurs in the pituitary,[24,27] probably requires the action of an additional enzyme, such as a PC1-like enzyme, which favors cleavage at the amino terminal processing site of βLPH. In support of this hypothesis is the observation that PC1 is expressed alongside PC2 in POMC-producing cells of the anterior and intermediate pituitary (albeit in different ratios), suggesting that PC1 is required in addition to PC2 in order to achieve the orderly pattern of POMC maturation observed in these tissues.

Acknowledgements

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