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Partial processing of the neuropeptide Y precursor in transfected CHO cells

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The activation of regulatory peptides by post-translational modification of their biosynthetic precursors is generally thought to occur only in neuroendocrine cells. We have selected clones of Chinese hamster ovary cells, a non-neuroendocrine cell line, which were transfected with a eukaryotic expression vector coding for the precursor for neuropeptide Y. Although the majority of the immunoreactive NPY was found in the form of pro-NPY, some degree of intracellular proteolytic processing of the precursor occurred in all clones. Part of the intracellular NPY immunoreactivity was even correctly amidated. Extracellular degradation of pro-NPY in the tissue culture medium generated immunoreactivity which corresponded in size to NPY. It is concluded that precursor processing can occur in non-neuroendocrine cells both as a biological process within the cells and as apparent processing, degradation in the tissue culture medium.

Neuropeptide Y; Peptide amidation; Peptide processing; Transfection

1. INTRODUCTION

In recent years, investigations of post-translational modifications of regulatory peptides have changed from rather passive descriptions of the natural biosynthesis towards more active studies using transfection of cell lines. New questions concerning e.g. the structure-function relationship of precursor molecules can now be addressed by expressing the cDNA coding for a peptide precursor and mutants of this in neuroendocrine cell lines. The first transfection experiments with peptide precursors were performed in COS cells, a non-neuroendocrine cell line, using vectors in which the cDNA for pre-proinsulin was placed under the transcriptional control of the SV-40 promoter [1,2]. The transfected cells produced proinsulin by transient expression of the plasmids. The production of unprocessed prohormone in non-neuroendocrine cell lines was confirmed in transient expression experiments and in stably transfected clones of CHO cells for pancreatic polypeptide [3], 3T3 cells for somatostatin [4], BHK cells for glucagon [5], BSC-40 cells for enkephalin [6], and in BHK cells for vasopressin [7]. However, in an early study, Warren and Shields did observe apparent processing of pro-somatostatin in COS cells [8].

As the basis of protein engineering studies on the precursor for neuropeptide Y (NPY), we initially used the fast growing, non-endocrine Chinese hamster ovary (CHO) cells to optimize vector constructions, etc. [3]. In the present paper, we present evidence for intracellular cleavage and partial amidation of the NPY precursor in stably transfected CHO cells, as well as evidence for degradation of the precursor by the tissue culture medium. In both cases, the cleavage of the NPY precursor mimics the precursor processing of neuroendocrine cells.

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2. MATERIALS AND METHODS

2.1. *DNA constructions*

The dicistronic mammalian expression vector p753 has 4 functional elements, as shown in fig.1. These elements were cloned in *Eco*RI/*Bam*HI digested pML [9]. A human ubiquitin gene (Ub C) promoter [10] was cloned at the 5'-end of the expression unit. (Lambda)HUb13 [10] was digested with *Xho*I and the resulting 1.5 kb promoter containing fragment was cloned in *Sal*I digested pBR322 to generate pBR322ubi. The 1.5 kb *Xho*I fragment of the human ubiquitin gene has *Bgl*II sites just 3' to the initiating methionine codon and 0.1 kb 3' to the upstream *Xho*I site. pBR322ubi was digested with *Bgl*II, then digested with *Bal*31 to eliminate 6 nucleotides at the 3'-end of the fragment. Hereby the methionine codon was removed. *Hind*III linkers were added, and the resulting 1.4 kb promoter fragment was subcloned in *Hind*III digested pIC19R [11]. From such a plasmid the ubiquitin promoter was isolated as a 1.4 kb *Eco*RI/*Bam*HI fragment and used in the construction of p753.

Human NPY cDNA was isolated from pNPY3-75 [12] as a 0.38 kb *Bss*HII-/*Hin*fI fragment, treated with the klenow fragment of DNA polymerase to repair the staggered ends, and then ligated to *Bam*HI linkers. The resulting 0.38 kb *Bam*HI fragment was used in the final construction of p753. The neomycin phosphotransferase gene from pKm2 [13] was isolated as a 1 kb *Bgl*II/*Sma*I fragment. The staggered ends at the *Bgl*II site were repaired with klenow DNA polymerase, and the neogene was subcloned in a *Hind*III digested pIC19R vector with repaired ends. From a subclone, the neogene was isolated as a 1

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kb *Bam*HI/*Bgl*II fragment and used in the construction of p753. At the 3'-end of the construct, the early polyadenylation signal of SV40 was cloned as a 0.24 kb *Bam*HI/*Bcl*I fragment.

2.2. *Transfection procedure*

Chinese hamster ovary (CHO) cells were maintained in RPMI 1640 containing 10% fetal calf serum (Gibco) with 5% CO_2. 1.8×10^6 cells were seeded in 100 mm Petri discs and the transfection was carried out after 24 h by the calcium phosphate precipitation technique [14] using 2 μg pSV$_2$-Neo and 20 μg p753. Cells were split 1:4 into selection media containing 0.8 mg/ml G418 (Sigma) 48 h after transfection. Clones were picked after 14 days and propagated in selection media.

2.3. *Peptide characterization*

Cells from 150 cm^2 confluent flasks were extracted in 2 ml 50% (v/v) acetic acid, boiled for 3 min, and sonicated for 15 min in a bath sonicator prior to centrifugation at 3000 $\times$ *g* for 15 min. The supernatant was dried in a vacuum centrifuge and reconstituted in assay buffer (see later). The protein content of the acetic acid extracts was determined using the Bio-Rad protein assay. The tissue culture media were either assayed directly or aliquots (usually 10 ml) were precipitated with 4 vols of acetone. The precipitate was dried under nitrogen and reconstituted in 5% (v/v) formic acid (Merck) containing 10 mg/l of bovine serum albumin. The human neuroblastoma cell line, SH-SY5Y, was used as a source of pro-NPY, as described elsewhere [15].

2.3.1. Radioimmunoassays

The recognition site for the two NPY antisera used are indicated in fig.1 and the radioimmunoassays have been described in detail previously [15]. Rabbit antiserum NPY-8999, raised against Cys-NPY (31–36)-peptide coupled to BSA with maleimidobenzoyl-*N*-hydroxysuccinimide, is highly specific for the C-terminal part of NPY, including the amide function. Rabbit antiserum NPY-337, rais-

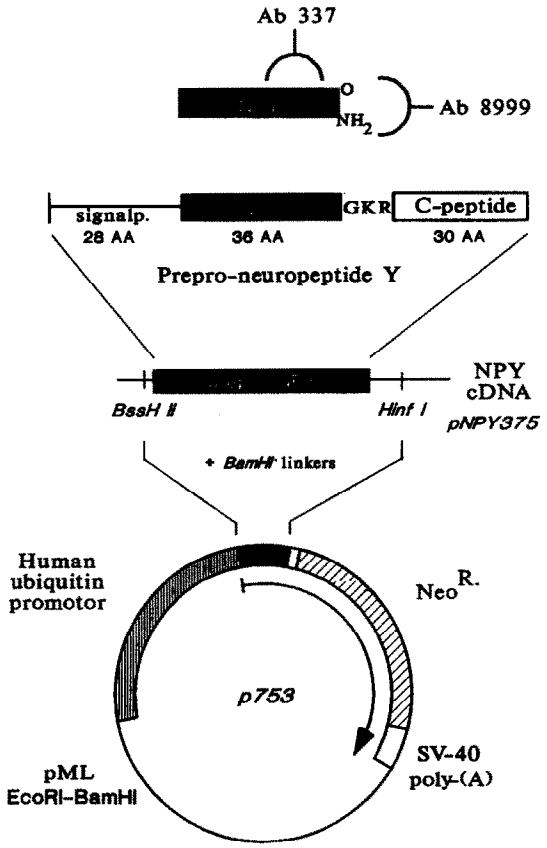

Fig.1. Diagram of the eukaryotic expression vector and of the NPY antisera. In the middle is a schematic representation of the cDNA for human NPY. Above is shown the primary translation product, prepro-NPY, in which NPY is preceded by a 28 amino acid signal-peptide and separated from a 30 amino acid C-terminal spacer-peptide, C-peptide, by a combined cleavage and amidation site, Gly-Lys-Arg (GKR). On top are shown the recognition sites of the two NPY antisera used, Ab 337 which reacts both with NPY and pro-NPY and Ab 8999 which is specific for the amidated C-terminus of NPY, and therefore does not react with precursors for NPY. At the bottom is shown the expression vector, p753, in which the NPY cDNA is placed under the transcriptional control of the human ubiquitin (UbC) promotor as described in detail in the text.

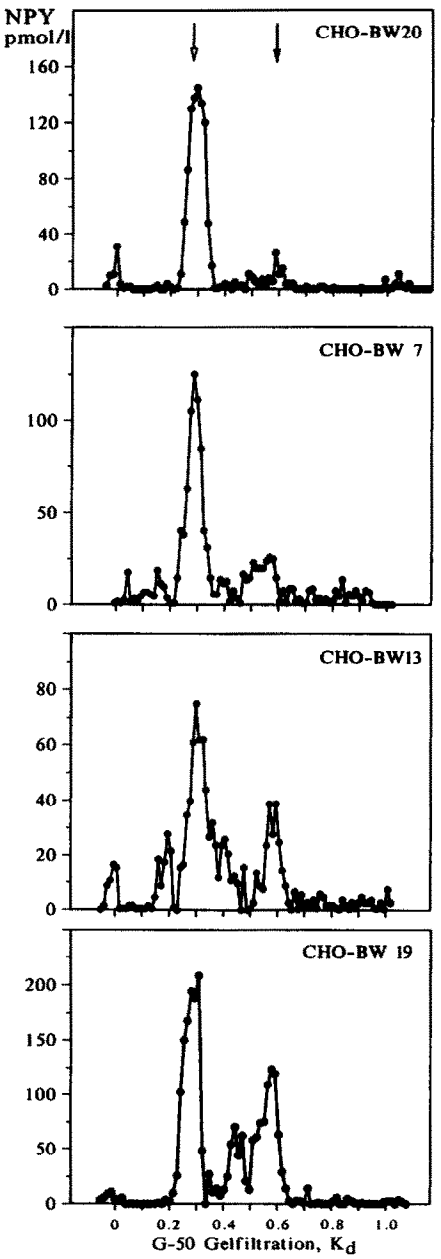

Fig.2. Gel filtration profile of NPY immunoreactivity extracted from 4 different clones of transfected CHO cells. NPY immunoreactivity was detected using Ab 337. The elution positions of pro-NPY (open arrow) and NPY (closed arrow) are indicated.

ed against NPY coupled to BSA with carbodiimide is not dependent on the amide function but does recognise the C-terminal part of NPY, as it reacts equally well with e.g. NPY (13-36)-peptide. Radioiodinated porcine NPY was prepared and purified as described previously [16]; [^{125}I-Tyr^{1}]monoiodo NPY was used as tracer in the assay employing antibody NPY-8999. The assays were performed in a total of 0.6 ml in 0.05 M sodium phosphate buffer, pH 7.4, containing 2.5 g/l of BSA and 0.5 mM thiomersalate. Incubation was performed at 4°C for 2 days without and 1 day with tracer. Antibody-bound and free tracer were separated by plasma-coated charcoal.

2.3.2. Chromatography

Cell or media extracts were applied to Sephadex G-50 superfine columns (1.6 × 95 cm) eluted with 5% (v/v) formic acid containing 10 mg/l BSA at 4°C. Fractions were dried in a vacuum centrifuge and reconstituted in assay buffer.

3. RESULTS

Stable clones of the CHO cells producing between 100 and 5000 pmol/l immunoreactive NPY per 24 h per 2.5×10^7 cells in 10 ml of medium, were selected. Chromatographic characterization of the NPY immunoreactivity extracted from 4 different clones, which contained between 180 and 475 fmol of NPY per 5×10^7 cells are shown in fig.2. As expected in these non-neuroendocrine cells, the majority of the NPY was found in the form of the unprocessed propeptide. However, some degree of intracellular cleavage of the precursor was observed in all clones. Between 5% and 45% of the NPY immunoreactive material was eluted corresponding to NPY or intermediary forms (fig.2). For a given clone, the amount of intracellular processing was constant over several passages. As shown for cell line CHO-BW19 in fig.3, a major portion of the NPY immunoreactivity, which in apparent size corresponded to NPY1-36, was amidated as judged by its reactivity with the amide-specific assay. Thus, in transfected CHO clones, limited proteolysis of pro-NPY and amidation of NPY takes place to a variable, albeit limited degree.

When the NPY immunoreactivity from the tissue culture medium of the same CHO clones was characterized, a more uniform pattern appeared. In this case the immunoreactivity was almost equally distributed between molecular forms corresponding in size to the propeptide and to a form eluting just before standard NPY (fig.4). Thus NPY immunoreactivity of an apparent size corresponding approximately to NPY precursor, e.g. CHO-BW20 and CHO-BW7 (fig.2 and 4). This discrepancy indicated that the cleavage of the pro-NPY could occur extracellularly. When pro-NPY, isolated and purified from a human neuroblastoma cell

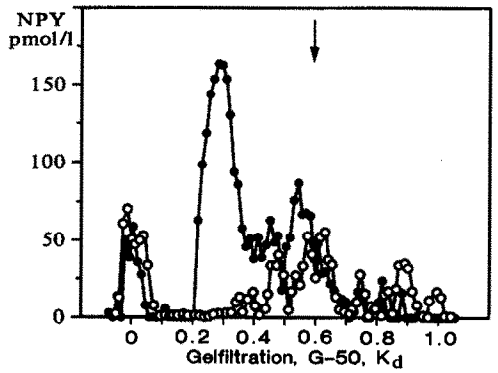

Fig.3. Gel filtration profile of NPY immunoreactivity extracted from the clone CHO-BW19. To get enough material for assaying without overloading the column, fractions from two consecutive gel filtrations were pooled and assayed using both Ab 337 (●—●) and Ab 8999 (○—○).

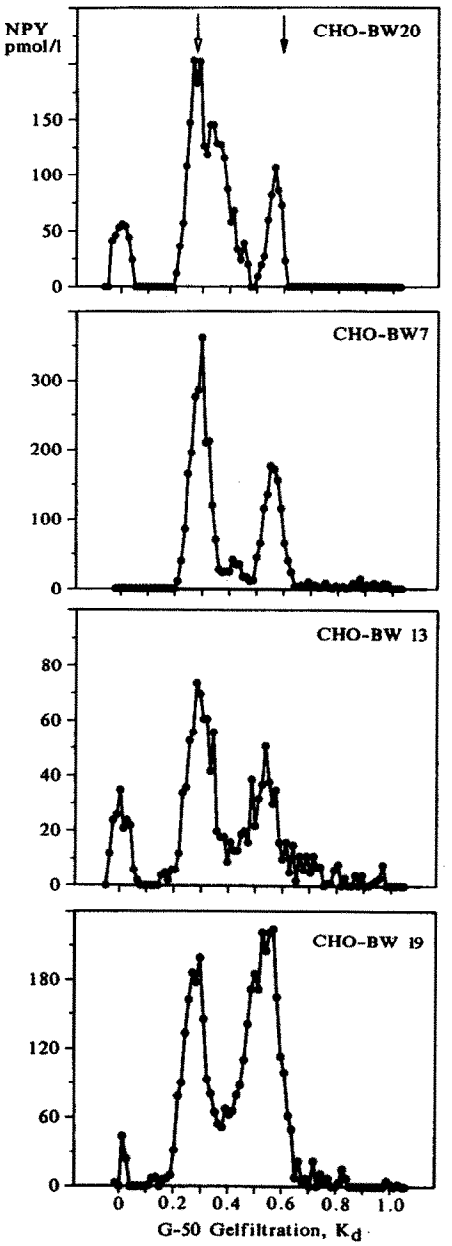

Fig.4. Gel filtration profile of NPY immunoreactivity extracted from the tissue culture medium from 4 different clones of transfected CHO cells. NPY immunoreactivity was determined with Ab 337. The elution position of pro-NPY (open arrow) and NPY (closed arrow) are indicated. Variable amounts of apparent immunoreactivity are observed in the void volume dependent on the amount of material which was used for radioimmunoassay.

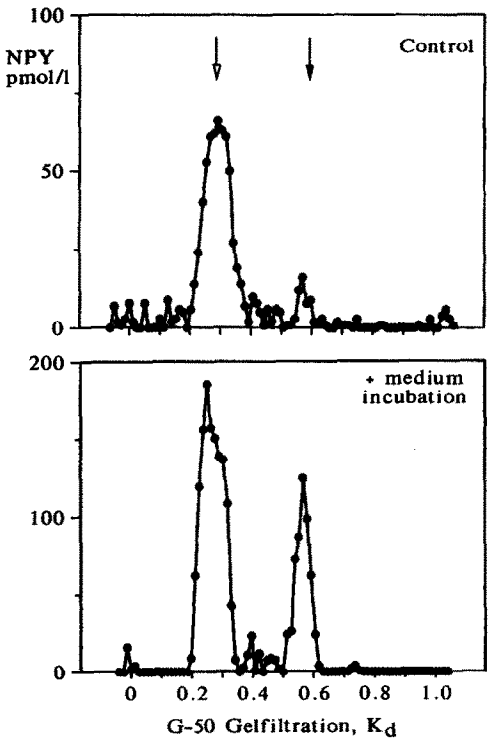

Fig.5. Degradation of pro-NPY by tissue culture medium. Gel filtered pro-NPY isolated from a human neuroblastoma cell line, SH-SY5Y, was incubated with tissue culture medium, supplemented with 15% foetal calf serum, and either analyzed immediately (control) or after 24 h of incubation (+ medium incubation).

line SH-SY5Y [15], was incubated overnight with unconditioned tissue culture medium only, it was found that the medium degraded the precursor to a moleclar form of immunoreactive NPY eluting on the G-50 columns right before NPY (fig.5). Only a minimal part of this material, which eluted as several molecular species both on HPLC and on isoelectric focusing was amidated, indicating different kinds of proteolytic degradation (data not shown).

4. DISCUSSION

In the present study, limited processing of a peptide precursor was observed in transfected non-neuroendocrine cells, both as a biological process within the cells and as a trivial problem in the tissue culture medium. Such processing and degradation should be taken into account when precursor processing is being studied, even in neuroendocrine cells.

A variable amount of the NPY immunoreactivity found in the transfected CHO cells corresponds in size to authentic NPY, and some of it even appears to be amidated. These findings indicate that precursor processing can occur in non-neuroencodrine CHO cells. It was previously thought that the amidating enzyme was expressed only in true neuroendocrine cells. However, northern blot analysis has demonstrated that mRNA for the enzyme also is found in several non-endocrine tissues and cell lines, e.g. 3T3 cells (Betty Eipper, personal communication). It should also be noted, that probably all cells in their secretory pathway, conceivably in the Golgi complex, have proteases with specificities for basic residues. Such proteases which cleave at sites of multiple basic residues are involved in e.g. the processing of the precursor for the insulin receptor [17], and in the processing of other normal membrane proteins and e.g. viral envelope proteins [18]. Recently it has been suggested that the processing of proinsulin, at least at one of the processing sites, starts already in the Golgi area of the cell [19]. It is likely that certain peptide precursors, like pro-NPY, prosomatostatin, and pro-PTH are more susceptible to such proteolysis than other precursors. In the case of pro-PTH, the small 6 amino acid propeptide is cleaved at the tri-basic processing site e.g. in transfected 3T3 fibroblasts [20]. The degree of processing of prosomatostatin in non-endocrine cells can be increased by changing the Arg-Lys sequence to Lys-Arg or Arg-Arg by site-directed mutagenesis [21].

NPY immunoreactivity of a size comparable to NPY was observed in the media from the transfected CHO cells. Most of this results from extracellular processing of pro-NPY, and at least some of the proteolytic enzymes appeared to be present in the unconditioned tissue culture medium. No attempt was made in the present study to define precisely where the cleavages of the precursor occurred. There are several possible cleavage sites in the precursor of these, the Lys^{38} and Arg^{39}, the natural processing site of pro-NPY, and the Arg^{33} and Arg^{35} in the NPY sequence itself, would all be good targets for trypsin-like enzymes. Cleavage at any of these sites could generate peptides which would elute on both gel filtration and HPLC with the unhomogeneous immunoreactivity from the transfected CHO cell media. The sequence, Arg^{35}-Tyr^{36}, for example, is known to be a very susceptible site for tryptic cleavage in vitro (unpublished observations).

Although some of the NPY precursor is processed in the CHO cells, most of the NPY remains in the form of the unprocessed prohormone. In contrast to this rather limited processing, a very efficient processing of pro-NPY to amidated NPY occurs in transfected corticotrophe cells (AtT-20 [22] and in transfected insulinoma (RIN), neuroblastoma (NB1), and medullary thyroid carcinoma cells (CA-77)*.

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