Communication

Variants of the Carboxy-terminal KDEL Sequence Direct Intracellular Retention*

(Received for publication, November 29, 1989)

Douglas A. Andres, Ian M. Dickerson, and Jack E. Dixon

From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907 and the Department of Neuroscience, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Soluble proteins which reside in the lumen of the endoplasmic reticulum share a common carboxy-terminal tetrapeptide Lys-Asp-Glu-Leu (KDEL). Addition of the tetrapeptide to a normally secreted protein is both necessary and sufficient to cause retention in the endoplasmic reticulum. In order to characterize the critical residues in the KDEL signal, cDNAs encoding proneuropeptide Y (pro-NPY) with the 4-amino acid carboxy-terminal extension KDEL or a series of KDEL variants were expressed in the AtT-20 cell line. AtT-20 cells, a mouse anterior pituitary corticotrope cell line, synthesize, process, and secrete the pro-ACTH/endorphin precursor. Since post-translational processing in AtT-20 cells has been extensively characterized, it provides a model system in which the processing of a foreign peptide precursor (pro-NPY) and the endogenous precursor (pro-ACTH/endorphin) can be compared. Altered cDNAs encoding pro-NPY with KDEL, DKEl, RDEL, KNEL, KDLQ, or KDEA at the COOH terminus were used to generate stable AtT-20 cell lines. The processing of pro-NPY to neuropeptide Y and the carboxy-terminal peptide was studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, tryptic peptide mapping, and radioactive peptide sequencing. Addition of the tetrapeptides KDEL, DKEL, RDEL, or KNEL to the COOH terminus of the propeptide Y precursor, a peptide hormone normally processed and secreted from neuronal cells, caused complete intracellular retention of the unprocessed prohormone in AtT-20 cells. However, KDLQ and KDEA-extended pro-NPY molecules were processed and secreted like wild-type pro-NPY when expressed in AtT-20 cells. The secretion of pro-NPY-derived peptides in these cell lines paralleled secretion of endogenous pro-ACTH/endorphin-derived products under both basal and stimulated conditions. These mutagenesis studies demonstrate that variants of the KDEL retention signal can direct intracellular retention.

The factors involved in protein sorting in the endoplasmic reticulum are beginning to be understood at the molecular level. Correct folding and oligomerization appear to be required for transport to the Golgi apparatus (1, 2). Improperly folded molecules or incompletely assembled protein complexes not competent for Golgi transport are retained and eventually degraded in the endoplasmic reticulum (3). Another mechanism must regulate the retention of correctly folded resident proteins (4, 5). The tetrapeptide Lys-Asp-Glu-Leu (KDEL) is found at the carboxy terminus of a set of soluble endoplasmic reticulum proteins and is essential for their retention (6, 7). Pelham et al. (8) has proposed that a "KDEL receptor" in an early Golgi compartment recognizes and selectively recycles proteins bearing the KDEL tetrapeptide. We have employed a model system to characterize the specificity of the KDEL retention signal. This system utilizes the expression of the neuropeptide Y precursor (pro-NPY)1 under the control of the mouse metallothionein I promoter (9) in AtT-20 cells.

Neuropeptide Y is a 36-amino acid neuropeptide which is synthesized as a larger precursor, prepro-NPY (10). Prepro-NPY consists of a 28-amino acid signal sequence followed by 69 residues (Fig. 1). The mature hormone is located at the amino terminus of the propeptide and is cleaved proteolytically during biosynthesis at a pair of basic amino acids (Lys38, Arg49). The precursor contains neither disulfide bonds nor asparagine-linked glycosylation sites, making it an attractive model system to examine the effects of placing KDEL or variants of this sequence at the COOH terminus of pro-NPY. When AtT-20 cells are stably transfected with a plasmid expressing prepro-NPY, the biosynthesis, processing, storage, and secretion of the "foreign" precursor protein proceeds as in neuronal cells which synthesize the peptide from the endogenous gene product (11-13).

MATERIALS AND METHODS

Construction of Expression Vector—Cloning of the cDNA for human pro-NPY has been described (14). The cDNA for pro-NPY was isolated from the plasmid p2G10 on a 5'-HindIII and 3' -BamHI restriction fragment (15) and subsequently cloned into complementary sites in M13mp18. Single-strand DNA was prepared and used as the template for oligonucleotide-directed mutagenesis (16). Two oligonucleotides were synthesized; the first created a unique XhoI site (CTCGAG) to replace the wild-type CTTGAA for Leu63-Glu4 and the second a unique BglII site (AGACTT 10 nucleotides 3' to the stop codon (Fig. 1). The introduction of these restriction sites did not alter the coding sequences for human prepro-NPY but facilitated the generation of mutants by insertion of double-stranded oligonucleotides having unique XhoI and BglII sites. The mutated NPY cDNA was then excised by a HindIII-XhoI restriction digest and ligated into a similarly treated pUC19 vector (proNPYXBPUC19). A series of double-stranded oligonucleotides was generated with 5'-XhoI and 3'-BglII sites which encoded the last 5 amino acids of pre-NPY and either the carboxy-terminal sequence KDEL or a series of KDEL variants (Fig. 3). The oligonucleotides were annealed and ligated into proNPYXBPUC19 digested with XhoI and BglII. The specific mutants were then excised and ligated between the HindIII and XhoI restriction sites of pMt.neo-1 (provided by K. Peden, Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907 and the Department of Neuroscience, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205) and ligated into proNPYXB-pUC19 digested with HindIII and XhoI.

The abbreviations used are: NPY, neuropeptide Y; CSFM, complete serum-free medium; pro-ACTH, pro-adrenocorticotropic hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; dansyl, 5-dimethylaminonaphthalene-I-sulfonyl.

* This work was supported by National Institutes of Health Grants NIDDKD 18849 (to J. E. D.) and 32948 (to Richard Mains). This is Journal Paper 12236 from the Purdue University Agriculture Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Transfection and Cell Culture—AtT-20/D-16v cells were grown in medium containing 10% horse serum, 10% fetal bovine serum, and 10% NuSerum (Collaborative Research) (11). AtT-20 cells were separately transfection with the six mutant NPY expression vectors using the calcium phosphate coprecipitation method (18). Stable cell lines were selected in medium containing 0.55 mg/ml geneticin (GIBCO) (11, 19). The most rapidly growing clones under geneticin selection were incubated with 10 μM CldC1 for 16 h and analyzed for NPY mRNA production by Northern blot analysis (19, 20). Clones showed elevated levels of pro-NPY mRNA were then analyzed for NPY peptide production by immunoprecipitation. Cell lines were maintained in 0.55 mg/ml geneticin during serial passages.

Immunoprecipitation—Each cell line was incubated in 96-well microtiter plates and preincubated in complete serum-free medium (CSFM) (21) containing 10 μM CldC1 and 100 μM ascorbate for 12–16 h. Cells were then labeled for 2–8 h in CSFM deficient in tyrosine supplemented with 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.). After the labeling period, the medium was removed (pulse) and the cells incubated in CSFM supplemented with 100 μM ascorbate for 6–10 h, after which the cell extracts and medium were harvested (chase). Samples were immunoprecipitated with affinity-purified antibodies (22) directed against either NPY (11) or β-endorphin (23). NPY immunoprecipitates were subjected to SDS-PAGE on phosphate-buffed gels (24) while β-endorphin immunoprecipitates were separated on borate-buffered tube gels (11, 25). Dansylated cytochrome c was included in each gel as an internal size marker. Tube gels were sliced, radiolabeled peptides eluted, and the samples analyzed by scintillation spectroscopy.

Radiosequencing and Tryptic Peptide Analysis—To prepare radio- labeled pro-NPY. Mt.KDEL cells were preincubated for 14 h in CSFM containing 10 μM CldC1 and 100 μM ascorbate. Cells were then labeled for 8 h in CSFM-deficient in leucine or tyrosine, supplemented with 112 μM [*H]leucine (50 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.) or 60 μM [*H]tyrosine (56 Ci/mmol, Amersham Corp.). Radiolabeled pro-NPY-KDEL peptide was immunoprecipitated from cell extracts, subjected to gel chromatography as previously described (11), and fractions containing pro-NPY-KDEL were pooled. The [*H]leucine-labeled peptide was then subjected to automated Edman degradation (11, 26). The [*H]leucine-labeled immunoprecipitated peptide was digested with tryosine-labeled KDEL was observed, suggesting that the KDEL sequence was sufficient to cause intracellular and secretion of the endogenous pro-ACTH/endorphin precursor was unaltered in the Mt.KDEL cell line (Fig. 2).

The high level of expression attained in Mt.KDEL cells allowed analysis of the biosynthetically labeled pro-NPY-KDEL peptide. Microsequencing of the [*H]tyrosine-labeled precursor following isolation by immunoprecipitation verified that attachment of the COOH-terminal KDEL sequence and prolonged retention in the cell did not alter the site of signal peptide cleavage between residues 28 and 29 of the preprohormone and demonstrated that the NPY sequence was located at the amino terminus of the prepropeptide. [*H]Tyrosine (cycles 1, 20, 21, and 27) was observed only at those amino acid residues predicted from the cDNA sequence (data not shown). In addition, [*H]leucine-labeled pro-NPY KDEL was digested with trypsin, and the peptides were separated by HPLC. A radioactive peptide with a retention time identical to an authentic sample of HEL was observed, suggesting that the pro-NPY-KDEL protein was unaltered in the Mt-T-20 cells (data not shown).

Immunoelectron microscopy studies by Schnabel et al. (27) on proopiomelanocortin processing in AtT-20 cells indicate that endopeptidase processing and ω-amination begin in the trans-Golgi cisterna and continue in the secretory granules. The experiments described in Fig. 2 suggest that the lack of dibasic processing and proteolysis of pro-NPY-KDEL is due to selective retention prior to the trans-Golgi.
Endoplasmic Reticulum Retention Signal Mutants

Fig. 2. Analysis of pro-NPY processing and secretion in Mt.NPY la and Mt.KDEL cells. Mt.NPY la (top panel), Mt.KDEL (middle panel), and wild-type AtT-20 cells (bottom panel) were preincubated for 16 h with CdCl₂ and ascorbate and labeled for 2 h with [³H]tyrosine (pulse). Medium was collected, and the cells were incubated for an additional 6 h in unlabeled complete medium and then harvested (chase). Cell extracts and pooled medium samples were immunoprecipitated with affinity-purified NPY antibody (50% of total sample) and β-endorphin antibody (10% of total, bottom panel). Immunoprecipitates were subjected to SDS-PAGE in tube gels, sliced, eluted, and scintillation-counted. [³H]-Labeled NPY eluted in slice 26 in a parallel gel. Similar results were seen in eight other incubations.

retention, we felt that pro-NPY expressed in AtT-20 cells provided a model system with which to characterize the retention signal. To accomplish this goal, a series of KDEL variants was produced. The secretory fate of pro-NPY variants with the indicated 4-amino acid carboxyl-terminal extensions is shown in Fig. 3.

The pro-NPY peptides containing COOH-terminal KDQL and KDEA extensions were processed in the same way as wild-type pro-NPY when expressed in AtT-20 cells. Pro-NPY-KDQL and pro-NPY-KDEA were efficiently processed to NPY and the corresponding COOH peptide (Fig. 4). The reduction of radiolabeled precursor protein in the cells was paralleled by the accumulation of labeled processed peptides in the medium. We were concerned that the addition of the COOH-terminal KDEL tetrapeptide might alter pro-NPY folding and cause retention in the endoplasmic reticulum. The processing and secretion of these mutants with minimal changes in the KDEL sequence imply that pro-NPY retention is not due to misfolding or inappropriate aggregation but is a consequence of the KDEL sequence.

When the mutant cell lines Mt.DKEL, Mt.RDEL, and Mt.KNEL were labeled, the propeptides were efficiently retained in an unprocessed state within the cell (Fig. 4). No labeled immunoprecipitable material was detected in the medium after chase periods of 6–10 h. Fig. 4 shows that the tetrapeptides DKEL, RDEL, and KNEL all function as intra-

Fig. 3. Carboxyl-terminal sequences of the extended pro-NPY mutants. Plasmid names are at the left, and the relevant COOH-terminal amino acid sequences are shown in one-letter code. Charged residues within the COOH-terminal tetrapeptide are indicated by + or − symbols above the sequence.

Fig. 4. Analysis of pulse-chase experiments for the KDEL mutants. Mt.NPY la, Mt.KNEL, Mt.DKEL, Mt.RDEL, Mt.KNQL, or Mt.KDEA cells were preincubated for 15 h with CdCl₂ and ascorbate and labeled for 6 h with [³H]tyrosine. Each well was incubated for an additional 6 h in unlabeled complete medium, the medium samples pooled, and the cells extracted for peptides. Cell extracts and media samples (50% of total) were immunoprecipitated with affinity-purified NPY antibody and separated by SDS-PAGE. Similar results were seen in four other experiments.

cellular retention signals. Although the subcellular location of the retained KDEL mutants has not been directly determined, the removal of the signal peptide indicates transport into the endoplasmic reticulum, and the lack of proteolysis and dibasic processing strongly suggests that the mutants are retained in a compartment prior to the trans-Golgi. These results are in agreement with the recycling model proposed by Pelham (7). Evidence for such a model was obtained by addition of the KDEL retention signal to the lysosomal protein cathepsin D (8). The addition of mannose 6-phosphate to lysosomal enzymes is one of the earliest postendoplasmic reticulum-processing events. KDEL-extended cathepsin D was retained in the endoplasmic reticulum but was still a substrate for the first of the enzymes involved in mannose 6 phosphate addition. No further enzymatic processing was observed, providing strong evidence for retrieval from an early
The regulation of "foreign peptide" secretion was also investigated in each AtT-20 cell line. Both the endogenous pro-ACTH/endorphin and pro-NPY polypeptide should be targeted to the regulated secretory pathway, the products of which are released in response to secretagogues (20, 29). Secretagogue (5 mM 8-bromo-cAMP) treatment increased the rate of endogenous pro-ACTH/endorphin- and pro-NPY-derived peptide secretion 2-2.5-fold in Mt.KDQL, Mt.KDEA, and Mt.NPY1α cells (data not shown). The molar ratio of pro-NPY-derived peptides to pro-ACTH/endorphin-derived peptides in these cell lines was constant, showing parallel regulation of secretion and implying a common secretory pathway. Secretagogue treatment increased endogenous but not pro-NPY-derived peptide secretion in Mt.KDEL, Mt.KNEL, Mt.RDEL, and Mt.DKEL cells. The sorting of regulated proteins appears to take place in the trans-Golgi (30), suggesting that intracellularly retained pro-NPY molecules never reach this intracellular compartment.

The results of the amino acid substitution experiments allow some predictions regarding the nature of the retention signal. For the purpose of discussion K has been assigned as residue 1 with L as residue 4 (within the KDEL sequence). The ability of Lys to be functionally substituted by Arg and Asp at the first position, and Asp to be replaced by Glu and Lys in position 2 implies that the structural requirements for the initial two positions of the retention signal are not rigid. Interestingly, Pelham et al. (31) have shown that the COOH-terminal sequence HDEL is not an efficient retention signal in COS cells so the initial two positions of the tetrapeptide are not completely elastic. In contrast, the negatively charged Glu at position 3 cannot be substituted with Gln, a residue with a similar size but no charge, suggesting that an acidic residue might be important at this position for signal recognition. Also, the inability of Ala to functionally replace Leu implies that leucine or a branched nonpolar side chain might be needed in position 4. Further mutations will be needed before a consensus is reached on the minimal retention signal. However, the results of our model studies employing pro-NPY-KDEL mutants emphasize the role of the terminal two amino acids in the functioning of the endoplasmic reticulum retention signal.

In summary, we have created a series of KDEL mutant COOH-terminal extensions to the model protein pro-NPY to investigate the nature of the KDEL retention signal. The mutant proteins were tested for their ability to be intracellularly retained when expressed in AtT-20 cells. We show that a number of mutants (DKEL, RDEL, KNEL) to the initial two amino acids of the tetrapeptide signal can be made without affecting intracellular retention. These results suggest that soluble proteins in the endoplasmic reticulum may carry sequences other than KDEL at their COOH termini. These mutants will be important in characterizing the cellular machinery involved in KDEL retention.
Variants of the carboxyl-terminal KDEL sequence direct intracellular retention.

D A Andres, I M Dickerson and J E Dixon

*J. Biol. Chem.* 1990, 265:5952-5955.

Access the most updated version of this article at [http://www.jbc.org/content/265/11/5952](http://www.jbc.org/content/265/11/5952)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/11/5952.full.html#ref-list-1](http://www.jbc.org/content/265/11/5952.full.html#ref-list-1)