PROEXENDIN-4 PROCESSING *IN VITRO* AND *IN VIVO*

by

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Abstract

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Glucagon-like peptide 1 (GLP-1) regulates blood glucose principally through stimulation of glucose-dependent insulin secretion. The peptide exendin-4 (Ex-4), isolated from the venom of the Gila monster *H. suspectum*, is a potent agonist of the GLP-1 receptor *in vivo* and *in vitro*. It is not known however whether proexendin-4 (proEx-4) can be expressed and post-translationally processed in mammalian cells *in vivo*. Given the amino acid residues that flank mature Ex-4, we hypothesised that Ex-4 could be generated by both furin- and prohormone convertase (PC)-mediated cleavage events. The processing profile of proEx-4 was therefore examined *in vitro* in heterologous mammalian cell-lines stably expressing the proEx-4 cDNA, as well as *in vivo* in transgenic mice expressing proEx-4. The cell lines analyzed included the fibroblast baby hamster kidney (BHK) cells containing the ubiquitous convertase Furin, mouse pituitary corticotroph AtT-20 cells which contain high levels of PC1, and islet A InR1-G9 cells which contain high levels of PC2. Radioimmunoassay and reverse-phase high pressure liquid chromatography were employed to determine the proEx-4 processing profile for each cell-line and in selected tissues and plasma from transgenic mice. All of the cell lines demonstrated the presence of a peak in the elution position of synthetic Ex-4. Trangenic mice, under the control of an inducible metallothionein promoter, were also shown to express and process proEx-4 to bioactive Ex-4 in both endocrine and non-endocrine tissues and to release Ex-4 into the circulation. Thus, these results demonstrate that lizard proEx-4 can be expressed and processed to Ex-4 in a variety of different mammalian cells.

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List of Abbreviations

ANOVA	analysis of variance
AtT-20	mouse pituitary corticotroph (cells)
BHK	Baby Hamster Kidney (cells)
C	carboxy
C18	carbon-18
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CPE	carboxypeptidase E
CRE	cAMP-response element
DPP-IV	dipeptidyl peptidase-IV
ENTP	end N-terminal peptide
ER	endoplasmic reticulum
Ex-4	exendin-4
ExLI	exendin-4-like immunoreactivity
FBA	fetal bovine serum
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
GLP-1R	glucagon-like peptide-1 receptor
GLP-1R-/-	glucagon-like peptide-1 receptor knock-out
GI	gastro-intestinal
G protein	GTP-binding protein

GRPP	glicentin-related pancreatic peptide
HbA_{1c}	hemoglobin A_{1c}
hGH	human growth hormone
HPLC	high pressure liquid chromatography
ICV	intracerebroventricular
InR1-G9	hamster islet A (cells)
IP	intervening peptide
i.v.	intravenous
kb	kilobase
kDa	kilodalton
L	line of transgenic mice
LP	lizard proglucagon
LPH	lipotrophic hormone
ma-CPE	membrane-associated-carboxypeptidase E
min	minutes
MPGF	major proglucagon fragment
mRNA	messenger ribonucleic acid
MT	metallothionein
N	amino
NEP	neutral endopeptidase 24.11
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
P	position

PAM	peptidylglycine α-amidating monooxygenase
PBS	phosphate-buffered saline
PC	prohormone convertase
PC1	PC1/3
PDGPs	proglucagon-derived peptides
PKA	protein kinase A
POMC	proopiomelanocortin
ProEx-4	proexendin-4
ProG	proglucagon
rER	rough endoplasmic reticulum
RIA	radioimmunoassay
RSP	regulated secretory pathway
RT-PCR	reverse-transcriptase-polymerase chain reaction
SEM	standard error of the mean
SG	secretory granules
TFA	trifluoroacetic acid
TG	transgenic
TGN	*trans*-Golgi network
TM	transmembrane
U	units
UTR	untranslated regions
UV	ultra violet
v	volume

VIP	vasoactive intestinal peptide

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Chapter 1. Introduction

Introductory Statement

Cell-specific post-translational processing of proglucagon (proG) leads to the production of several peptides, including glucagon-like peptide-1 (GLP-1), in the dispersed enteroendocrine L-cells of the small and large intestine. GLP-1 is released into the blood stream after feeding and acts as an important hormonal mediator of glucose homeostasis through integrated actions to stimulate glucose-dependent insulin secretion and inhibit glucagon secretion and gastric emptying [1,2].

Exendin-4 (Ex-4), a GLP-1 agonist, was originally isolated from the venom of the Gila monster lizard *H. suspectum* [3]. Ex-4 displays 52% identity to mammalian GLP-1 at the amino acid level [4] and has been demonstrated to be a super GLP-1 receptor (GLP-1R) agonist both *in vitro* and *in vivo* [5-7]. The major limitation of mammalian GLP-1 is that it is rapidly inactivated [8] by the enzyme dipeptidyl peptidase IV (DPP-IV) through cleavage at the N-terminal penultimate alanine [9]. A glycine, instead of alanine, at position 2 of Ex-4 raises the possibility that Ex-4 is comparatively more resistant to DPP-IV degradation than GLP-1. Taken together, the enhanced bioactivity and resistance of Ex-4 to catabolism as compared to GLP-1 highlights its pharmaceutical potential as a super-GLP-1 agonist in the treatment of diabetes.

In the lizard, proEx-4 (proexendin-4) and proG are encoded by distinct genes [4,10]. To date the proG gene, but not the Ex-4 gene, has been found in mammals. The possibility that other species, and perhaps humans, may also contain distinct exendin genes, and the potential therapeutic use of Ex-4, makes the elucidation of the tissue-processing mechanisms of proEx-4 of interest. As this peptide was originally isolated from lizard

salivary glands [3], it is currently not known whether the proEx-4 precursor can be expressed and post-translationally processed in mammalian cells *in vivo*. However, proEx-4 does contain classic consensus sites for cleavage by the processing enzymes furin and the prohormone convertases. Insight into the processing of this hormone may be of value in future gene therapy approaches to the use of Ex-4 in the treatment of diabetes.

1.1 Glucagon-like Peptide 1

1.1.1 Synthesis of the Proglucagon-derived Peptides

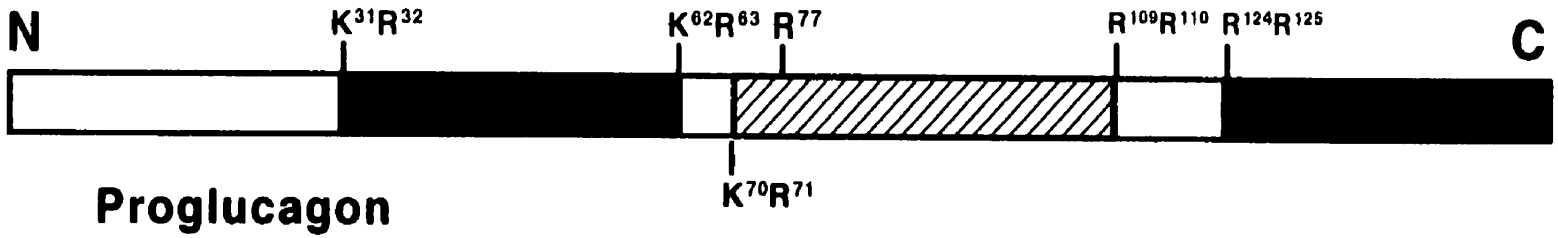
The mammalian proG gene is composed of 6 exons and 5 introns [11] which form a single identical transcript in the pancreas, intestine and areas of the brain including the hypothalamus [12-14]. Cell-specific post-translational processing of the proG prohormone is responsible for the tissue-specific expression of the proG-derived peptides (PGDPs). Specifically, differential expression of processing enzymes or prohormone convertases (PCs) in pancreatic A and intestinal L cells results in the distinct profile of PGDPs (see section 1.3.3 for details). In the intestinal L cell, glicentin, oxytomodulin, GLP-1 and GLP-2 are liberated, whereas in the pancreatic A cell, proG is processed to glicentin-related pancreatic peptide (GRPP), glucagon and the major proG fragment (MPGF) (Fig. 1).

1.1.2 Biological Activities of the PGDPs

In the pancreas, glucagon is released in response to hypoglycemia, with receptors located in the liver and fat. The role of glucagon in the control of glycemia, acting as the major

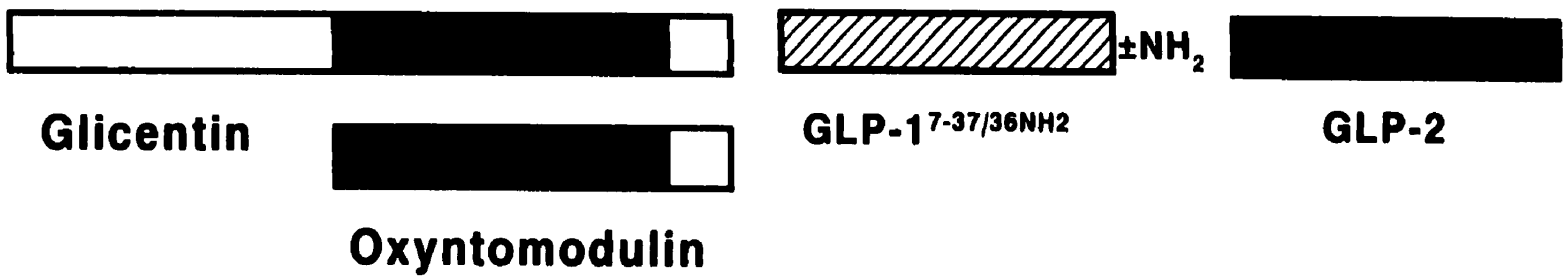
Fig. 1

Schematic representation of the post-translational processing of mammalian proG. Glicentin-related pancreatic peptide (GRPP), glucagon and the major progulcagon fragment (MPGF) are produced in the pancreas, and glicentin, oxyntomodulin, GLP-1 and GLP-2 are produced in the intestine. Consensus sequences for cleavage by prohormone convertases are shown for proG.



Pancreas

Intestine



counter-regulator of insulin's actions are well established [15]. GRPP and the MPGF have no known function.

The best characterized of the intestinal PGDPs is GLP-1. The enteroendocrine L cell is subject to regulation by luminal intestinal components, such as glucose and long-chain monounsaturated fatty acids, at its apical membrane, circulating hormonal factors at its basolateral membrane, and neural inputs, all of which have been implicated in the regulation of secretion of GLP-1 (and the other intestinal PDGPs) [1,2,16]. The GLP-1R is a seven transmembrane G-protein-coupled receptor and GLP-1R mRNA has been detected by Northern blotting in several rodent tissues including the islets, stomach, lung, kidney and brain [17,18] and by RNAse protection analysis in human pancreas, stomach, lung, kidney, heart, and brain [19]. Of note, is that the GLP-1R has been localized on the pancreatic insulin secreting β-cell [20], somatostatin secreting δ-cells [21], glucagon secreting α-cell [22] and in hypothalamic nuclei implicated in appetite regulation [23]. Receptor activation in primary islet cultures, β-cell lines and heterologous cell lines transfected with the GLP-1R cDNA have demonstrated signalling through both the adenylyl cyclase and phospholipase C pathways [17,18,24-26].

GLP-1 is released into the blood stream after feeding and acts as an important hormonal mediator of glucose homeostasis. It is an *incretin*, defined as a gut hormone released during food intake which stimulates glucose-dependent insulin secretion [27]. The glucose dependency of GLP-1 action has been well established both *in vitro* and *in vivo*. In addition, GLP-1 stimulates insulin gene transcription [24,28], presumably by increasing intracellular cAMP and effecting the cAMP-responsive element located in the 5'

regulatory region of the insulin gene [29]. Thus, enhanced insulin secretion is paralleled with replenishment of β-cell insulin stores. In concert with its insulinotropic actions, GLP-1 inhibits glucagon secretion [30], either directly through actions on the α-cell or indirectly through stimulation of the glucagonistic hormones, insulin or somatostatin [31]. GLP-1 also inhibits gastric emptying [32], limiting further transfer to and digestion of foodstuff in the intestine until the load already present has been absorbed. As well, gastric acid secretion is inhibited [33]. In contrast, although the highest levels of GLP-1R mRNA are found in the lung [17], the physiological role for pulmonary actions by GLP-1 remain to be determined. A potential role for GLP-1 in the central control of feeding has also been suggested, as intracerebroventricular (ICV) injection of GLP-1 induces *c-fos* expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala while inhibiting feeding in fasted rats [34]. Furthermore, ICV administration of the GLP-1R antagonist exendin$^{9-39}$ doubles food intake in satieted rats [34]. However, GLP-1R knockout mice (GLP-1-/-) exhibit no abnormal body weight or feeding behaviour suggesting redundancy in these mechanisms. In contrast, consistent with its physiological importance in glucose regulation, GLP-1R-/- display mild fasting hyperglycemia and both glucose intolerance and decreased insulin levels after an oral glucose load although circulating glucagon levels are normal [35,36]. Thus, the major role of GLP-1 in physiology appears to be in the regulation of glucose homeostasis through insulin secretion. This biological activity has led to the suggestion that GLP-1 might be useful as a treatment for type II diabetes, a condition characterized by a lack of sufficient insulin secretion to compensate for the peripheral insulin resistance [37]. Since GLP-1's

actions on insulin secretion are glucose-dependent, it is also hoped that GLP-1 treatment might reduce the incidence of hypoglycemia, an undesirable side effect often seen with sulfonylurea treatment of patients with type II diabetes.

Although a recent report has demonstrated beneficial effects of GLP-1 on insulin sensitivity in depancreatized dogs [38], previous studies in both healthy man [39] and patients with type II diabetes [40] demonstrated no such response. Thus, the peripheral effects of GLP-1 remain controversial and further studies are needed.

Much less is known about the actions of the other PGDPs, however, GLP-2 has recently been characterized as an intestinotrophin, stimulating crypt cell proliferation and decreasing the number of apoptotic cells in the villus tip [41]. It also stimulates intestinal glucose transport, reduces intestinal epithelial permeability and inhibits gastric emptying [41]. Thus, GLP-2 has gained a lot of attention as a potential treatment for short bowel syndrome. Finally, oxyntomodulin has been shown to inhibit gastric acid secretion [42], while the physiological actions of glicentin remain obscure [41].

1.1.3 Degradation and Clearance of GLP-1

1.1.3.1 Dipeptidyl Peptidase-IV

Dipeptidyl Pedptidase-IV (DPP-IV, E.C. 3.4.14.5), a highly specialized aminopeptidase, is the main enzyme responsible for the degradation of GLP-1 [43,44]. DPP-IV has a widespread distribution, being present both in the plasma as well as in numerous tissues, and cleaves dipeptides from peptides or proteins with N-terminal penultimate proline or alanine residues (e.g. NH_2-P_1-P_2; P_1 = X, P_2 = Pro/Ala). *In vitro* studies using human plasma [43,44] or purified DPP-IV [44,45], and *in vivo* studies in Wistar rats [45], DPP-IV deficient

rats [45] and pigs [8] have demonstrated the principal role for this enzyme in the degradation of GLP-1$^{7\text{-}37/7\text{-}36NH_2}$; DPP-IV activity results in an *in vivo* half-life for GLP-1 of only 0.9 minutes (i.v. bolus) [8] and the production [9] of the weak GLP-1R antagonist, GLP-1$^{9\text{-}37}$.

1.1.3.2 Neutral Endopeptidase 24.11

GLP-1 has also been shown to undergo endoproteolysis *in vitro* by neutral endopeptidase (NEP), with cleavage occurring at ten sites [46,47]. The specificity of NEP is such that it cleaves on the amino sides of hydrophobic amino acids residues such as Phe, Tyr, Leu, Ile, Val [48]. The importance of NEP in GLP-1 degradation *in vivo* remains to be established.

1.1.3.3 Clearance of GLP-1

The kidney is the primary site for the clearance of GLP-1, and uremic patients have been reported to have high levels of circulating immunoreactive GLP-1 as compared to controls (154 ± 33 vs. 41 ± 13 pmol/L in the fasted state) [49]. Direct evidence that the kidneys are involved in the clearance of GLP-1 has been obtained in several experiments. Bilaterally nephrectomy and ureteral-ligation of rats led to slower half-life disappearance times for i.v. I^{125}-GLP-1 as compared to sham-operated controls [50]. Furthermore, in isolated perfused rat kidney experiments *in vitro*, again using I^{125}-GLP-1, the values for organ clearance were similar to the glomerular filtration rate [50], while *in vivo* in anesthetized pigs, measurements of removal across different organs by radioimmunoassay (RIA) also demonstrated that the kidney is the major source of

endogenous GLP-1 elimination [51].

In summary, GLP-1 is an important hormonal mediator of glucose homeostasis with potential therapeutic effectiveness in the treatment of type II diabetes. However, its short half-life severely limits its effectiveness *in vivo*. As a result, the DPP-IV resistant, GLP-1R agonist, lizard peptide Ex-4, has recently become a focus of attention.

1.2 Exendin-4

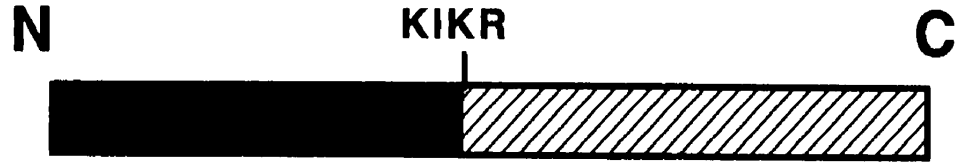
1.2.1 Discovery of Exendin-4

A common biochemical feature of biologically active peptides in the brain-gut axis is the presence of amino-terminal histidine residues in peptides that activate intracellular adenylyl cyclase. Many members of the glucagon super-family of peptides, including glucagon, GLP-1, GLP-2, vasoactive intestinal peptide (VIP) and secretin, have been shown to contain an amino-terminal histidine residue as well as a phenylalanine residue at position six [52]. Thus, Ex-4 was identified using an assay in which samples were screened for His^1-containing peptides by performing a single cycle of amino acid sequencing. These experiments led to the purification of Ex-4 from the venom of the lizard *Heloderma suspectum* [3] (Fig. 2). The name exendin is derived from the fact that this peptide is found in exocrine secretions, yet has endocrine activity, as discussed below [53].

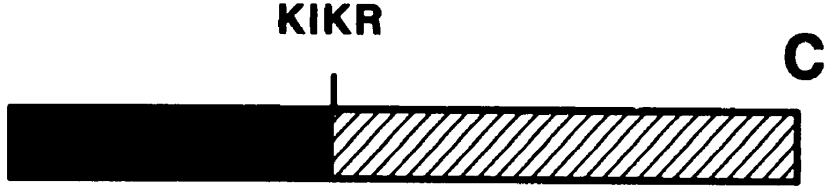
Initial studies had demonstrated that Ex-4 bound to the GLP-1R mimicking several of the biological actions of GLP-1 [54]. However, in 1997, Chen and Drucker [4], demonstrated that Ex-4 is not simply the reptilian equivalent of GLP-1, but rather that

Fig. 2.

Schematic representation of the post-translational processing of lizard pre-proEx-4. End N-terminal peptide (ENTP) and Ex-4 are produced in the salivary glands. Consensus sequences for cleavage by prohormone convertases are shown for proEx-4.



Pre-proexendin-4



Proexendin-4

Salivary Glands

ENTP Exendin-4

Ex-4 is encoded by a gene which is separate from that of proG in the lizard. To clone lizard proG, Chen and Drucker used degenerate oligonucleotide primers corresponding to conserved sequences of proG in RT-PCR reactions, thus generating partial lizard proG cDNAs from lizard pancreatic and intestinal RNA. However, no cDNA product was generated when these same primers were used in RT-PCR reactions with lizard salivary gland RNA. To further characterize the lizard proG mRNAs, the lizard pancreatic proG RT-PCR product was used as a probe to screen a *H. Suspectum* lizard pancreatic cDNA library. Two different proG cDNAs were isolated from this library, lizard proG (LP)-I and LP-II, with unique 3' untranslated regions (UTR). The LP-I sequence contains the coding regions for GRPP, glucagon, IP-1 and GLP-1, whereas the LP-II mRNA corresponds to the mammalian proG cDNA containing the coding regions for the above peptides as well as for IP-2 and GLP-2. Northern blot analysis of RNA from lizard intestine, pancreas, and salivary glands was then carried out using various lizard proG cDNA probes. It was determined that, in the pancreas, mRNA transcripts of ~1.1, 1.6 and 2.1kb are found, whereas in the intestine a single ~1.1kb mRNA is present. Furthermore, using a cDNA probe specific for the LP-I 3' UTR only the 1.6 and 2.1kb mRNA transcripts are detected, whereas a probe specific for the LP-II UTR generated only the 1.1kb transcript. In contrast, no transcripts corresponding to proG could be detected in lizard salivary glands [4] suggesting that Ex-4 and proG are encoded by different genes.

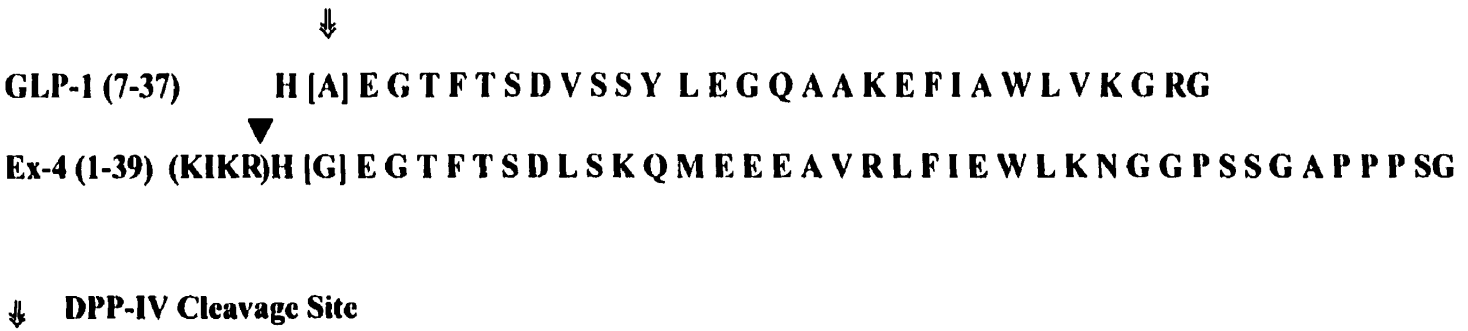
In order to clone lizard proEx-4, degenerate oligonucleotide primers corresponding to the known amino acid sequence of Ex-4 were used in RT-PCR reactions with lizard salivary

gland RNA. Cloning and sequencing of the RT-PCR product from this reaction demonstrated that it encoded a partial Ex-4 cDNA. Using this partial cDNA sequence as a probe, the full-length Ex-4 cDNA was subsequently isolated from a lizard salivary gland cDNA library. Northern blot analysis using lizard RNA and the full-length Ex-4 cDNA probe demonstrated the presence of a single mRNA transcript in RNA from lizard salivary glands. However, no transcripts were detected in RNA from lizard liver, pancreas or intestine [4]. Additionally, Pohl and Wank, using Ex-4 cDNA specific primers and RT-PCR, detected products of the expected size only in the salivary glands [10]. Furthermore, using southern blotting techniques with either the lizard full-length Ex-4 probe or proG cDNA probes common to both LP-I and LP-II, distinct bands are generated [4], which are also seen when the coding regions for Ex-4 or GLP-1 are used [10]. Chen and Drucker further characterized the lizard Ex-4 cDNA and determined that lizard Ex-4 is encoded within a larger, proEx-4 precursor mRNA transcript of ~550bp. Lizard proEx-4 encodes a 45 amino acid peptide of unknown function, which has been designated End N-terminal peptide (ENTP), followed by a consensus cleavage site of basic residues (KIKR) and then the 40 amino acid sequence corresponding to Ex-4 (Fig.3).

The demonstration that, in the lizard, proEx-4 and proG are encoded by distinct genes [4,10], prompted the search for a mammalian homolog of the proEx-4 gene. Work done by Ms. L. Baggio, using the full-length proEx-4 cDNA as a probe to screen several cDNA libraries (including: mouse heart, pancreas, spleen, testis, as well as chicken genomic DNA and human skeletal muscle cDNA) has not resulted in the isolation of a mammalian

Fig. 3

Comparison of the GLP-1 and Ex-4 amino acid sequences. N-terminal to the Ex-4 sequence are both putative Furin and PC cleavage sites. Ex-4 displays 52% identity to mammalian GLP-1 at the amino acid level. A penultimate N-terminal glycine [G], instead of alanine [A], results in the loss of the cleavage site for DPP-IV, the major enzyme responsible for the inactivation of GLP-1.



⇓ **DPP-IV Cleavage Site**

▼ **PC Cleavage Site**

▼ **Furin Cleavage Site**

Ex-4 gene (Ms. L Baggio personal communication). As well, Pohl and Wank demonstrated that no transcripts could be detected when the lizard Ex-4 cDNA was used as a probe to screen Northern blots containing RNA from various rat and human tissues, including salivary gland, pancreas, small intestine and brain [10]. However, these results do not preclude transient developmental expression of a mammalian Ex-4 gene, or perhaps expression at low levels. A precedent for the discovery of a non-mammalian peptide and later matching to a mammalian homolog is evident with amphibian bombesin and mammalian gastrin-releasing peptide [55]. As well, other secretagogues isolated from the venom of the Gila monster, helospectin (exendin-1) and helodermin (exendin-2), which interact with the mammalian receptors for VIP and secretin, have been immunolocalized to mammalian neural tissues [56]. These results are controversial [56] and indeed a mammalian gene homolog to helodermin was not found in an extensive search [10].

1.2.2 Biological Activities of Exendin-4

Several lines of evidence have demonstrated that Ex-4 is a super-agonist at the GLP-1R. First, Ex-4 has been shown to bind with greater affinity to the GLP-1R than GLP-$1^{7\text{-}37/7\text{-}36NH2}$, in the rat insulinoma-derived RINm5F cell line [54], in chinese hamster fibroblasts expressing the GLP-1R [57] and in rat lung membranes [54].

Furthermore, both *in vitro* and *in vivo*, Ex-4 has been shown to be a more potent GLP-1R agonist than GLP-1. In isolated Islets of Langerhan harvested from Wistar rats, Ex-4 induces increases in cAMP that are approximately threefold higher than the maximal response of equimolar GLP-1 [5]. As well, in Wistar rats, acute i.v. administration of Ex-4 produces an approximate doubling of the maximal insulin response of equimolar GLP-1 [5].

A preliminary study in Sprague-Dawley rats has also shown, by intraveneous (i.v.) glucose challenge and peptide administration, that the 60 minute insulin area-under-the-curve is 2.8 fold greater with Ex-4 treatment as compared to saline, whereas only a 20% increase was seen with GLP-1 infusion [7].

Ex-4 has also been shown to be a potent insulinotropic agent in models of type II diabetes. Acute administration of Ex-4 to *db/db* mice at concentrations as low as 0.01μg resulted in significantly lowered plasma glucose levels, at 1 hour post injection, whereas this effect was seen only with GLP-1 concentrations of 100μg or higher [6]. As well, plasma glucose levels were significantly lower even at 4 hours after Ex-4 administration at doses of 0.1μg or higher, but not with any dose of GLP-1. As GLP-1 doses as low as 1μg significantly lowered plasma glucose at 30 min post injection, a waning of the GLP-1 response is seen at these later time points [6]. In both *db/db* and *ob/ob* mice, using the percent fall from preinjection glucose concentration after 1 hour, Ex. 4 was approximately 5,500-fold more effective than GLP-1 [6]. These effects were glucose-dependent, as the greatest fall was seen to be a function of initial hyperglycemia. Recently Egan et al. have also shown that chronic, once-daily intraperitoneal (i.p.) Ex-4 administration for 12-13 weeks in *db/db* mice has a prolonged lowering effect on glycemia, lowering $\%HbA_{1c}$ concentrations and increasing plasma insulin levels, as compared to NaCl-treated *db/db* mice [5]. Although Ex-4 did not maintain blood glucose in the euglycemic range, it greatly reduced, though did not prevent, the rise in HbA_{1c} as compared to non-diabetic littermates [5]. Similarly, chronic administration of Ex-4 (twice-daily for six weeks) to diabetic *fa/fa* Zucker rats demonstrated a dose-dependent

reduction in food intake, body weight, and $\%HbA_{1c}$ levels [6]. Improved insulin sensitivity with Ex-4 treatment was also noted as a 49% increase in the glucose infusion rate was seen with a hyperinsulinemic euglycemic clamp [6].

1.2.3 Degradation and Clearance of Exendin-4

1.2.3.1 Dipeptidyl Peptidase IV

A single amino acid substitution at position 2 in Ex-4, as compared with GLP-1, (Ala to Gly) results in the loss of a DPP-IV cleavage site (Fig. 3). Since DPP-IV is the major enzyme responsible for inactivating GLP-1, it can be assumed that this is the major contributor to the extended half-life of Ex-4 (18-41 minutes after i.v. bolus [58]) as compared to GLP-1 (0.9 minutes after i.v. bolus [8]). This improved half-life could account for the prolonged bioactivity [6], and enhanced effectiveness of once-daily treatments [5] of Ex-4 as compared to GLP-1.

1.2.3.2 Neutral Endopeptidase

Using the recombinant human form of neutral endopeptidase (NEP) 24.11, it has been demonstrated that Ex-4 is a very poor substrate as compared to GLP-1 [47]. As NEP cleaves at the amino-terminal of hydrophobic residues, some of the target bonds for NEP are absent in Ex-4 as compared to GLP-1. Since NEP prefers smaller peptides, Ex-4's larger size may also contribute to its being a poor substrate. As well, certain undefined conformational differences between the two peptides may account for differences in their susceptibility to endoproteolysis by NEP [47].

1.2.3.3 Clearance of Exendin-4

In a preliminary study, it has been suggested that Ex-4 is cleared from the plasma predominantly by glomerular filtration, as the rate of Ex-4 clearance (man 120-130ml/min, 350g rats ~3.2-4.4ml/min) is similar to the glomerular filtration rate (man ~125ml/min, 350g rat 4.2ml/min) [7]. Thus, to examine the role of the kidney as the site of Ex-4 clearance, a study was done in control and nephrectomized rats. Ex-4 clearance in sham-operated control rats was 4.3ml/min, similar to values seen in unoperated rats, whereas clearance decreased to 0.86ml/min in nephrectomized rats [7]. This study thus showed that the kidney is the major site of Ex-4 clearance, accounting for at least 80% of the elimination in rats. Furthermore, as no immunoreactive Ex-4 is seen in the urine, it has been suggested that brush-border enzymes within the nephron degrade the circulating peptide [7].

In summary, therefore in the lizard, proEx-4 and proG are encoded by distinct genes [4]. To date the proG gene, but not the proEx-4 gene, has been found in mammals. The possibility that other species, and perhaps humans, may also contain distinct exendin genes, and the potential therapeutic use of Ex-4, makes the elucidation of the tissue-processing mechanisms of proEx-4 of interest.

1.3 The Constitutive and Regulated Secretory Pathways

There are two main pathways in which proproteins are processed, (i) the constitutive and the (ii) regulated secretory pathways. Prohormone (proprotein) convertases (PCs) act in these pathways to produce bioactive proteins and peptides.

(i) All cells possess a constitutive or default secretory pathway. This pathway is characterized by rapid secretion via vesicles from the Golgi-complex to the membrane. In the constitutive pathway, there are no storage compartments and release is insensitive to secretagogues. The PCs Furin, PACE4, PC5/6B and PC7/8/LPC are known to process proproteins that traverse the constitutive secretory pathway [59,60].

(ii) In the neuroendocrine cells of the nervous and endocrine system there is also a regulated secretory pathway (RSP), in which proproteins are targeted from the *trans*-Golgi to secretory granules for storage and subsequent release by secretagogues. PC1/3 and PC2 are the major PCs in the RSP [61]. PC4, which is only expressed in the testes [62], and the PC5/6A isoform, which lacks a transmembrane (TM) domain, also act in this pathway [63].

1.3.1 Proprotein Sorting

Proprotein/prohormones are all synthesized in the endoplasmic reticulum (ER). They are then transported to the *trans*-Golgi network (TGN), where proteins destined for the regulated pathway are separated from constitutively-secreted proteins [64,65]. A sorting mechanism which includes a sorting signal in the N-terminal region of propeptides, with membrane-associated carboxypeptidase E (ma-CPE) being a putative sorting receptor, has been proposed [66,67]. The amphipathic loop at the N-terminus of proopiomelanocortin (POMC) contains two acidic residues (Asp_{10} and Glu_{14}) and two aliphatic hydrophobic residues exposed on the surface. These acidic residues were shown to form ionic bonds with the basic Arg_{255} and Lys_{260} residues present in a loop that is unique to CPE (compared to other carboxypeptidases) and which is also independent of the active site of

CPE. Proinsulin and proenkephalin, which also contain this putative sorting signal, have also been shown to bind to ma-CPE [67]. Furthermore, depletion of CPE in Neuro2A cells by overexpression of an antisense CPE construct results in missorting of POMC [66], proenkephalin and proinsulin [68] to the constitutive pathway. In addition CPE-mutant Cpe^{fat}/ Cpe^{fat} mice, exhibit constitutive secretion of POMC from both the anterior and intermediate lobes of the pituitary gland [66,69]. Binding to the sorting receptor in the lumen of the TGN is postulated to cause the protein-receptor complex to be packaged within the budding, immature secretory granules for transfer to the RSP. CPE does not act as a sorting receptor for chromogranin A [68,70], thus other mechanisms must also be responsible for sorting of specific proproteins. In fact, the C-terminal regions of the PCs and other prohormones have also been suggested as having sequences important in their sorting with loss of these regions resulting in mis-sorting to the constitutive secretory pathway (elaborated in 1.3.2.1)[63,71].

1.3.2 Processing Enzymes

Post-translational endoproteolytic cleavage of inactive precursors results in the generation of bioactive proteins and peptides. In the last twelve years great advances have been made in our understanding of these complex mechanisms. In 1987, yeast KEX2 endoprotease was shown to cleave proalbumin *in vitro* [72] and coexpression of POMC with KEX2 in mammalian cells results in the production of the bioactive β-lipotrophic hormone (LPH), γ-LPH and β-endorphin products [73]. Thus, speculation about a mammalian counterpart to this yeast enzyme, grew. In 1989, KEX2 was shown to be

related to the bacterial subtilisin [74]. That same year, the first mammalian homolog, the *fur* gene, was also found by a search of sequence data bases [75]. The use of degenerate oligonucleotide primers corresponding to conserved sequences in the catalytic domains of these enzymes ultimately led to the initial isolation of the PCs, PC2 [76], PC1/3 [77,78], PACE4 [79], PC5/6 [80,81] and PC7/8/LPC [82-84], from cDNA, and PC4, from genomic DNA [85]. Of these seven subtilisin-related serine proteases, PACE4 [79,86-88], PC4 [62,89] and PC5/6 [90] also have isoforms generated by alternative splicing of their primary transcripts (termed PACE4A/B/C/CS/D/E which differ in their N- or C- termini, and PC4A/B/C and PC5/6/A/B which differ in their C-termini) [60].

1.3.2.1 The Sequences of the PCs

All of the post-translational processing enzymes described to date contain a signal peptide, a pro-segment, a catalytic subunit, and a P-domain. The signal peptide targets the nascent protein to the rough endoplasmic reticulum (rER). The subtilisin-like catalytic domain is highly conserved, containing the catalytic triad Asp, His, and Ser, as well as an Asn which has been shown to stabilize the oxyanion hole during the transition state [91,92] in all PCs except for PC2 in which this is an Asp residue [76]. The P domain, also referred to as the middle or Homo B domain, is well conserved in the eukaryotic convertases and plays a role in folding as well as being essential for catalytic activity [87,93,94]. Within this region is a conserved Arg-Gly-Asp motif which is a recognition sequence for cell surface integrins implicated in cell-cell adhesion (except PC7/8/LPC in which it is a Arg-Gly-Ser [82]). The role of this recognition sequence has not been determined [60], however, mutation of this sequence in PC1/3 results in loss of catalytic

activity and mis-sorting to the constitutive secretory pathway [71]. The C-terminus is the region with the greatest variation in the PCs. PC1/3 and PC2 contain a stretch of charged amino acids and a putative amphipathic alpha-helix loop [78], a feature shared with carboxypeptidase E (CPE) [95]; it is unclear if this shared feature is a sorting mechanism [96]. Furin [97], PACE4 [79] and PC5/6B [80,81,98] also have a cysteine rich region which are well conserved [98], although its function is currently not known. Furin, PC5/6B and PC7/8/LPC also have a transmembrane domain near the C-terminus important for intracellular localization [60]. Finally, the unique C-terminal 38aa of PC5/6A seems to be important for sorting, as mutants lacking this segment are sorted to the constitutive pathway [63].

1.3.2.2 Furin

Fur gene's transcription is regulated by at least 3 distinct promoters. One may be a regulated promoter, while the other two have characteristics of housekeeping genes [99]. Northern-blot analysis revealed that the furin transcript is present in all tissues and cell lines examined, with high levels of expression in the liver and kidney [59]. Furin is initially synthesised as a 100kDa protein, with a 104kDa core-glycosylated pro-furin form, which are rapidly and autocatalytically converted to 94 and 98kDa proteins, respectively, in the ER [100-102]. Even after this cleavage, the propeptide remains associated with the furin protein acting as a potent autoinhibitor. *In vitro* conditions mimicking those in the TGN, (e.g. acidic and low Ca^{2+}), cause release of the propeptide with a second cleavage in the middle of this propeptide, ending this inhibition and allowing furin to be fully active [103]. Under steady-state conditions, furin is localized in the TGN [104-106] and in immature

secretory granules of neuroendocrine and endocrine cells [107], with a proportion of furin molecules cycling between the TGN and the cell surface. Two determinants which have been localized to the cytoplasmic domain of furin are responsible for its TGN localization and recycling from the cell surface. (i) A serine-containing acid cluster, SDSEEDE, which fits the consensus sequence for phosphorylation by casein kinase II is present in furin. It has been suggested that, in the dephosphoryated state, furin is located within the TGN, with phosphorylation causing furin to remain at the cell surface or in endosomes. A putative furin phosphatase would thus enhance movement back to the TGN [108]. (ii) A YKGL sequence which serves mainly as a signal for internalization from the plasma membrane [101,105,109,110], is also present in furin, and may be involved in cycling furin back to the TGN *via* endosomes . The YXXØ motif, where X = any amino acid and Ø = an amino acid with a bulky hydrophobic side chain, has been shown to be involved in direct interaction of proteins with the clathrin adaptor complexes AP-1 and AP-2 [111], although it is not known whether this is the role of this sequence in furin.

Consistent with its localization in the TGN, Furin is active in a broad pH range between 6.0 and 8.5, with an optimum activity at 7.0 and a Ca^{2+} requirement of 1-2mM [112].

The following sequence rules have been generated governing the sites at which furin cleaves proproteins [113-115].

1. An Arg is essential at the P_1 position, being the position directly N-terminal to the protein-product.

2. At least 2 out of the 3 residues at P_2, P_4 and P_6 are required to be basic for efficient cleavage ($P_6PP_4PP_2P_1$-protein).

3. An amino acid with a hydrophobic aliphatic side chain is not suitable at the P_{+1}

position.

Furin's need for a minimum of 3 basic residues (rule 1 and 2) allows it to selectively cleave products without acting on the paired basic amino acid sites present in most prohormones, which are cleaved only after they enter the secretory granules. Some authors have concluded that the minimal recognition site for furin is RXXR [116,117], however, this is contradicted by the fact that the human proinsulin-like growth factor I which, has a (PA)KSAR sequence [118,119], is also cleaved by furin.

Although furin is the only ubiquitous convertase, PACE4, PC5/6 and PC7/8/LPC can also cleave furin substrates [119] and are expressed in a broad range of tissues and cell lines.

1.3.2.3 PC1/3 and PC2

Most prohormones and neuroendocrine precursors are processed by the PCs in the dense core granules of the regulated secretory pathway. A similar mechanism of activation to that of furin has been demonstrated for PC1/3 (PC1) [61] with rapid autocatalytic cleavage from the 87kDa proenzyme to an 84kDa active form in the ER [120,121]; the active enzyme is then transported to the Golgi. In the TGN, PC1 is active at a pH below neutrality. PC1 undergoes C-terminal truncation in the TGN or the immature secretory granules [122], resulting in a 66kDa species with a relatively acidic pH optimum of 5.0-6.5 and requiring concentrations of Ca^{2+} above 10mM for full activity [123]. This truncation greatly enhances the activity of the convertase towards a number of substrates [121,124-127].

PC2 is unique in that it requires the acidic conditions of the late post-Golgi compartment for autoactivation. In the ER, proPC2 interacts with 7B2, a 27kDa neuroendocrine secretory protein [128-130]. In the absence of 7B2, autocleavage of the PC2 prodomain

occurs, however, this gives rise to only an inactive enzyme [131]. 7B2 interacts with the prodomain [128] and the oxyanion 'hole' Asp [129], binding to proPC2 after it has folded and facilitating its transport and activation [130]. 7B2 undergoes cleavage at a multiple-basic site near the C-terminus, most likely by furin or another TGN protease [132], resulting in a N-terminal 21kDa fragment and a C-terminal 6kDa inhibitory peptide [133,134]. Mutagenesis studies indicate that the inhibitory activity of this peptide is mediated by the ability of PC2 to recognize a paired-basic cleavage site (Lys-Lys) within its sequence but not cleave it readily [133]. This inhibitory peptide is ultimately cleaved by PC2 within the secretory granules [135]. Thus PC2, unlike PC1, is not active in the Golgi apparatus, since it requires a greater length of time for activation, as well as the higher acidity and greater calcium concentration present in the post-TGN organelles for activity.

It has been well established that both PC1 and PC2 are able to cleave proteins at pairs of basic amino acids. However, it has also been demonstrated that PC1 cleaves proteins at selected single basic amino acids [136].

Proneuropeptide Y (proNPY) contains a pair of basic amino acids which can be cleaved *in vitro* by both PC1 and PC2 [137-140]. Thus ProNPY cleavage has been an effective model to demonstrate the biochemical conditions necessary for the activity of PC1 and PC2. Temperature-blockade (20°C incubation), which arrests secretory pathway transport at the TGN [124,141,142], and the use of weak-bases (such as chloroquine, methylamine, and ammonium chloride) to eradicate intracellular pH gradients have been used with pulse-chase techniques to study processing activity [143].

In stably transfected, PC1-containing, AtT-20 cells proNPY processing proceeds efficiently with temperature-blockade whereas it is fully blocked in PC2-containing GH3

cells transfected with proNPY [144]. Furthermore, treatment with the weak-base chloroquine, at non-toxic concentrations, has only a minor effect on proNPY processing in AtT-20 cells, whereas similar treatment of GH3 cells leads to an almost complete inhibition of processing [144].

Similarly, endogenous POMC is processed in AtT-20 cells to ACTH and β-lipotropin, and neither temperature-blockade nor weak-base treatment change this processing profile [124,143]. However, in AtT-20 cells, stably transfected with PC2, PC2-dependent cleavage of β-lipotropin to β-endorphin was largely prevented by both temperature-blockade [145] and weak base application [144]. The same selectivity is demonstrated in GH3 cells, in which temperature-blockade and weak-base application block PC2 cleavage of prosomatostatin [146,147] and cleavage of proinsulin at the C-peptide/A-chain junction [148]. When taken together, temperature-blockade demonstrates that PC1 is active within the TGN, while weak-base administration demonstates that PC1 is active at a pH below neutrality.

Thus, although PC1 and PC2 may have overlapping substrate cleavage sites, their subcellular areas of activity are different, with PC1 active earlier in the RSP and both PCs being active in the secretory granules (SG). The fact that PC1 and PC2 do not form the same products in the RSP, indicates that the primary sequence itself is not always sufficient to determine the cleavage pattern of a prohormone. Possibilities include each enzyme having its own unique preferential cleavage site which is determined, in part, by residues surrounding the cleavage site. As well, specific conformational information may be necessary in addition to the presence of the consensus cleavage sequence, such as cysteine residues or glycosylation sites providing a suitable conformation for cleavage. Thus secondary and tertiary structure of a prohormone may be important in the

determination of its cleavage products. Finally, kinetic factors such as the rate of release from PC active sites, may also contribute to specific PC processing.

1.3.3 Proglucagon Processing

PC1 has been demonstrated to be both necessary and sufficient for the processing of proG in the L cell to the intestinal PGDPs, glicentin, oxyntomodulin, GLP-1^{7-37} and GLP-2 [136,149-153]. These studies have utilized various methods including: stable transfections [149,151] transient transfections [153], *in vitro* assays [152], antisense ablation [149,153], vaccina virus infection [136], and the examination of processing in normal islet and islet cell lines [150]. Furthermore, immunohistochemical analysis has confirmed the presence of PC1 in the L cell [154].

PC2 has been suggested to be responsible for the production of glucagon in the pancreatic A cell, however, conflicting evidence is present. Some studies have implicated PC2 as a critical enzyme in the processing of proG to glucagon [155,156]. Furthermore, PC2, not PC1, has been colocalized in the glucagon-containing α-cells [157] and mice lacking active PC2 have impaired processing of proG to glucagon with chronic fasting hypoglycemia and hyperplasia of α-cells [158]. However, other studies have only shown processing of proG to glicentin via the actions of PC2 [136,149,152]. In light of this discrepancy, further studies are needed before any definitive conclusions on the role of PC2 in glucagon biosynthesis can be made.

In summary, it is known that prohormone convertase enzymes are responsible for the cleavage of prohormones at specific sites to generate bioactive peptides. The most well

characterized of these cleavage enzymes are furin, which is expressed ubiquitously, and PC1 and PC2, which are localized exclusively in endocrine and neuroendocrine cells. It has been demonstrated that PC1 is responsible for the tissue-specific processing of mammalian proG to produce GLP-1 [136]. However, the GLP-1-related peptide, Ex-4, is a distinct peptide, encoded by a different gene in the lizard [4]. Furthermore, unlike GLP-1, Ex-4 is found in exocrine rather than endocrine or neuroendocrine cells. It is therefore currently not known whether the proEx-4 prohormone can be expressed and posttranslationally processed in mammalian cells *in vivo*. However, proEx-4 does contain classic consensus sites for furin- and PC-mediated cleavage. Insight into the processing of this hormone may be of value in future gene therapy approaches to the use of Ex-4.

1.4 Hypothesis

Given the amino acid residues that flank mature Ex-4, we hypothesised that proEx-4 is a substrate for processing in both classical endocrine and non-endocrine cells.

This hypothesis will be examined in both transformed cells *in vitro* and in mouse tissues *in vivo*.

Chapter 2. Materials and Methods

2.1 Samples

2.1.1 Cell-Lines

2.1.1.1 Culture Techniques

The non-endocrine baby hamster kidney (BHK) fibroblast cells, and the endocrine AtT-20 (F-2 subclone) mouse pituitary corticotrophs and InR1-G9 hamster islet A cell-lines were obtained from Dr. D.J. Drucker (Toronto, Canada). BHK fibroblasts and InR1G9 islet cells were grown in monolayers in Dulbecco's modified Eagle's medium containing 4.5g/L glucose (Life Technologies Inc., Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100μg/ml streptomycin. The AtT-20 corticotroph cell line was grown as above, except with 10% (v/v) FBS. All cells were maintained at 37°C under an atmosphere of 5% CO_2/95% air with constant humidity.

2.1.1.2 Plasmid Constructs

The pcDNA3.1 expression vector was employed for constitutive expression in the mammalian cell lines (Invitrogen, Carlsbad, CA). This vector contains a cytomegalovirus enhancer-promoter for high-level expression, a bovine growth hormone polyadenylation signal and transcription termination sequence to enhance mRNA stability, and a Neomycin resistance gene to allow for the selection of positive clones.

An ~0.5kb fragment which was flanked by EcoR1 sites and contained the full-length lizard proEx-4 cDNA [4] was inserted into the EcoR1 site of pcDNA 3.1 in both the sense and antisense (control) orientation by Mrs F. Wang.

2.1.1.3 Transfection Procedure

Cells were transfected with the recombinant pcDNA3.1 vector using the calcium phosphate method [159]. Cells at approximately 60% confluency in 5-cm dishes were used for transfection. Cells were incubated with 5μg plasmid DNA (or buffer only in mock transfections) in 500μL with 2X HEPES-buffered saline and 2M $CaCl_2$ for 4 h at 37°C. Cells were then subjected to a 4-min glycerol shock [15% (v/v) glycerol in HEPES-buffered saline], washed twice in PBS, and incubated overnight in culture medium at 37°C. The cells were maintained in culture medium containing 200μg/ml G418 (Sigma Chemical Co., Oakville, Ontario, Canada) for 48 h, and then subjected to increasing concentrations of G418. The final concentrations of G418 employed were: 600μg/ml for the BHK cell-line, and 1000μg/ml for both the InR1-G9 and AtT-20 cell-lines. Complete death of mock transfected plates was used as an index for the amount of G418 to be added to the cell lines. Although mixed cell populations were used for BHK (3'), InR1-G9 (5' and 3') and AtT-20 (5' and 3') cell lines, death was almost complete allowing for the growth of colonies from one to a few cells. Upon complete death of mock transfected plates, cells were maintained in culture medium at 500μg/ml G418. The BHK 5' cells were transfected by Dr. N. Wine and individual cell clones were obtained by limited dilution cloning, expanded for further characterisation and maintained in culture medium at 500μg/ml G418.

2.1.2 Mouse Tissues and Plasma

2.1.2.1 Generation of the Transgenic Mouse

An ~500bp fragment containing the entire lizard proEx-4 cDNA was cloned by Ms. Laurie Baggio in the lab of Dr. Daniel J. Drucker (Toronto, On) into the Bgl II site of the pEV142 expression vector [160]. This vector contains an 800bp fragment of the mouse metallothionein-1 (MT-1) gene (encompassing the MT 5' flanking sequences, promoter and the first 64bp of exon 1) [161], as well as a 625bp fragment of the human growth hormone (hGH) gene (encompassing the 3' untranslated region of exon 5, the polyadenylation signal (polyA) and the 3' flanking sequences) [162].

The mouse MT-1 promoter is an inducible promoter which can be upregulated by the supplementation of drinking water with a heavy metal such as zinc or cadmium [163,164]. A 1.9kb fragment containing the mouse MT-1 promoter, the lizard proEx-4 cDNA and the hGH sequences was purified and used for microinjection. The transgenic mice were generated by DNX Transgenic Sciences (Princeton, NJ). Two founder transgenic animals were generated by DNX and used by Ms. L. Baggio to produce two lines (18 and 19) of transgenic mice.

2.1.2.2 Tissue and Plasma Collection Techniques

Adult male and female transgenic mice as well as age-matched non-transgenic mice were used to determine tissue and plasma levels of exendin-like immunoreactivity (ExLI). Briefly, ExLI levels was assayed in two lines of transgenic mice fed *ad libitum* (basal expression) and in one line fed *ad libitum* and supplemented with 25 mM $ZnSO_4$ in their

drinking water (induced expression) for 3 days.

Mice were sacrificed by inhalation of CO_2 and tissues [1. Brain (Cerebral Cortex, Cerebellum, Hypothalamus, Pituitary), 2. Peripheral Tissues (Muscle, Fat), 3. Internal Organs (Gonads, Kidney, Liver, Heart, Lung, Adrenals, Spleen), 4. GI Tract (Stomach, Duodenum, Jejunum, Ileum, Colon, Pancreas)] were removed and either homogenized immediately in 2-5ml of ice cold extraction medium [1 M HCl, containing 5% (v/v) formic acid, 1% (v/v) trifluoroacetic acid [TFA; (Fischer Scientific, Fair Lawn, NT)] and 1% (v/v) NaCl] and stored, or frozen on dry ice and stored at - 70°C before peptide extraction (see 2.2.1). Blood was obtained by cardiac puncture and mixed with 10% (v/v) of TED solution [Trasylol (500KIU/ml), EDTA (1.2mg/ml), Diprotin A (0.1mM)]. Plasma was collected after centrifugation at 4°C, and stored at -70°C.

2.2 Sample Analysis

2.2.1 Peptide Extraction

Nearly confluent cells were washed twice with PBS and maintained in fresh media containing 200μg/ml G418 for 24 h at 37°C. Culture media was collected and centrifuged at 4°C for 10 minutes to remove any floating cells. A volume of 1% (v/v) TFA corresponding to 10% (v/v) of the media volume was subsequently added. Cells were washed twice with PBS before the cells were scraped and homogenized in 10ml of ice cold extraction medium.

Two volumes of 1% (v/v) TFA, pH adjusted to 2.5 with diethylamine [165], was added to the plasma. Tissue and cell homogenates, and media and plasma were separately passed

twice through a C18 silica cartridge (C18 Sep-pak, Waters Associates, Milford, MA), pre-rinsed with 10ml of 80% (v/v) isopropanol (Caledon, ON) in 1% (v/v) TFA and then with 10ml of 1% (v/v) TFA. Adsorbed peptides were eluted with 3-4ml of 80% (v/v) isopropanol/0.1% (v/v) TFA and stored at -20 or -70°C. Recovery of spiked peptide in plasma was 49 ± 5% using this method (n=3).

2.2.2 High Pressure Liquid Chromatography (HPLC)

Extracted peptides were separated on the basis of their relative hydrophobicity using a Waters Associates Liquid Chromatography System (Waters Associates). The system consisted of two model 510 pumps, two UV detectors (214 and 280nm), a C18 Guard Pak and a C18 μBondapak HPLC column. The column was pre-equilibrated at 15% (v/v) solvent B [solvent A: 0.1% (v/v) TFA; solvent B: 80% (v/v) acetonitrile (HPLC grade; Mallinckrodt, Miss, ON)/ 0.1% (v/v) TFA] and the gradient used for separation of the peptides ran from 30-80% of solvent B over 72 minutes followed by a 10 minute purge at 99% (v/v) solvent B. The flow rate was 1.5ml/ minute and fractions were collected every minute. Samples containing the extracted peptides were diluted 1/6 with solvent A prior to being loaded onto the column. I^{125}-GLP-2 was used as an internal standard at the beginning of each day.

2.2.3 Radioimmunoassay (RIA)

Aliquots of peptide extracts and HPLC fractions were dried *in vacuo* prior to RIA. Antisera for Ex-4 were generated by serial bleeds of rabbits immunized with Ex-4 by

Cocalico Biologicals Inc. (Reamstown, PA). Two antisera were generated (#17 & 18) and their specificity was determined in part, by Dr. Q. Xiao in our laboratory and Ms. L. Baggio. The present study contributed to the determination of their antigenicity (see Results). The RIAs utilized synthetic Ex-4 (California Peptide Research Inc., Napa, CA) as the standard, and I^{125}-Ex-4 as the tracer [prepared by the chloramine T method [166]] and involved a 5 day incubation followed by the adsorption of unbound peptides to dextran-coated charcoal and analysis of the free counts per minute. The detection limits of the assays were 5-2000pg/tube. Tissue protein was assayed using the Bradford method (Bio-Rad, Richmond, CA) with bovine γ-globulin as a standard.

2.3 Data Analysis

All data are expressed as mean ± SEM. Some data were $\log_{10}$ transformed to normalize variances prior to statistical analysis. Data analyses were performed by ANOVA using 'n-1' post hoc custom hypotheses tests, on a Statistical Analysis System program (Cary, NC).

Chapter 3. Results

In order to determine the specificity of two rabbit antisera generated against Ex-4, Ms. L. Baggio and Dr. Q. Xiao in our laboratories demonstrated that both antisera #17 and 18 did not cross-react with glucagon, glicentin, oxyntomodulin, GIP, VIP, and GLP-2. Antisera #17 showed significant displacement at 100-fold excess concentrations of GLP-1, whereas antisera #18 did not show significant displacement with any of the four forms of GLP-$1^{1\text{-}37/1\text{-}36NH2/7\text{-}37/7\text{-}36NH2}$ (data not shown). To determine if the free N-terminus of Ex-4 was necessary for antigenicity, binding curves with increasing concentrations of synthetic Ex-$4^{1\text{-}39}$ and Ex-$4^{9\text{-}39}$ with both antisera were established (Fig. 4 and 5). As I^{125}-Ex-4 binding to antisera #17 (Fig. 4) and #18 (Fig. 5) was dose-dependently displaced by both the 1-39 and 9-39 forms of synthetic Ex-4 it was concluded that the antigenicity of these antisera are determined by the regions carboxyterminal to amino acid 9 of the peptide. Since the free N-terminus of the peptide is not necessary for antibody binding, these results also suggest that both antisera would be effective in the recognition of proEx-4. Since it was previously determined that antiserum #18 did not cross react with GLP-1, even at high concentrations, this antiserum was therefore used for the detection of ExLI in all further studies.

In order to determine if the non-endocrine fibroblast BHK cell-line, known to contain the processing enzyme furin [167], could process proEx-4 to Ex-4, wild type cells were transfected with both the sense and anti-sense full-length proEx-4 constructs. Selection of a single cell clone expressing the 5' cDNA was carried out by Dr. N. Wine. Cells transfected with the 3' cDNA were analysed as mixed cell populations. BHK cells and 24 hour media were extracted to isolate peptides. RIA of the extracts indicated that

Fig. 4.

Antiserum #17 binding to synthetic Ex-4^{1-39} and Ex-4^{9-39} (n=3).

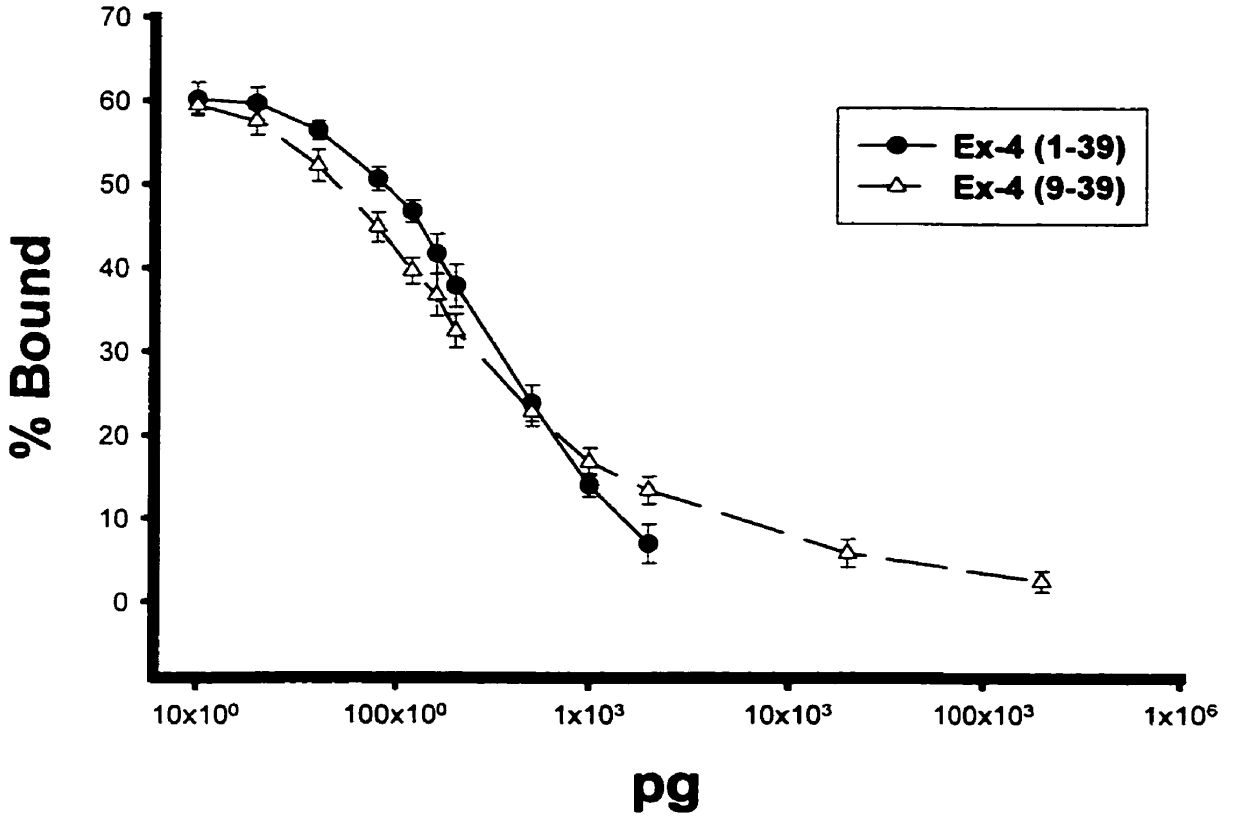
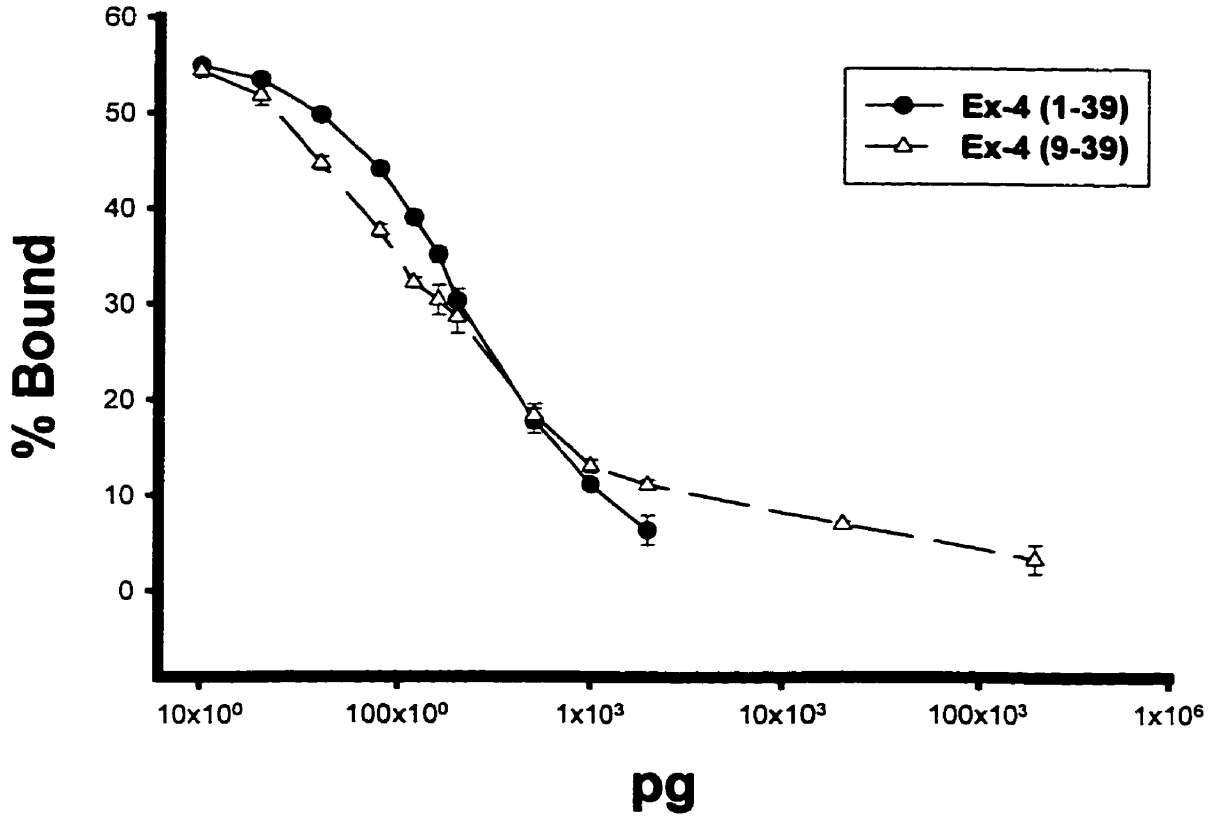

70
60
50
40
30
20
10
0
% Bound
Ex-4 (1-39)
Ex-4 (9-39)
10x10⁰
100x10⁰
1x10³
10x10³
100x10³
1x10⁶
pg

Fig. 5.

Antiserum #18 binding to synthetic Ex-4^{1-39} and Ex-4^{9-39} (n=3).


60
50
40
30
20
10
0
% Bound
Ex-4 (1-39)
Ex-4 (9-39)
10x10⁰
100x10⁰
1x10³
10x10³
100x10³
1x10⁶
pg

approximately 95% of the ExLI was secreted into the media, consistent with the non-endocrine, constitutive nature of the secretory pathway of these cells (Fig. 6). Controls with the antisense gene did not have any ExLI.

To determine the molecular form of ExLI in BHK media, HPLC separation of the peptides contained in BHK 5' Ex-4 media extract was done. RIA analysis consistently indicated the presence of a peak in the elution position of synthetic Ex-4 (fraction 36, Fig. 7). In 2 of the analyses, a larger more hydrophilic peak, potentially representing proEx-4, was detected in fractions 27 to 30. In the 3rd profile, from a sample which had been stored at -20°C for over 3 months, there was a disappearance of the more hydrophilic peak with the appearance of a peak with greater hydrophobicity (fractions 42-44).

In order to determine if the endocrine corticotroph AtT-20 cell-line, known to contain high levels of PC1 [125,168], could process proEx-4 to Ex-4, wild type cells were transfected with both the sense and anti-sense full-length proEx-4 construct. Transfected cells were analysed as mixed cell populations. AtT-20 cells and 24 hour media were extracted to isolate peptides. The AtT-20 cell line transfected with the sense proEx-4 cDNA both contained and secreted peptide with ExLI (Fig. 8). Approximately equivalent amounts of the immunoreactivity was contained within the cells and the media, consistent with the regulated secretory pathway of these cells. Controls with the antisense gene did not demonstrate any ExLI.

After HPLC separation of the extracted peptides, RIA analysis of AtT-20 5' Ex-4 cells indicated the presence of a peak in the elution position of synthetic Ex-4 in two of the extracts, with the 3rd having a peak slightly shifted leftward (Fig. 9). In two of the

Fig. 6.

BHK 5' & 3' Ex-4 cell and media binding curves. BHK 5' & 3' cells and 24 hour media were extracted to isolate peptides. Increasing volumes of the extracts were assayed for their ability to bind antiserum #18 as compared to known amounts of the synthetic standard (n=3).

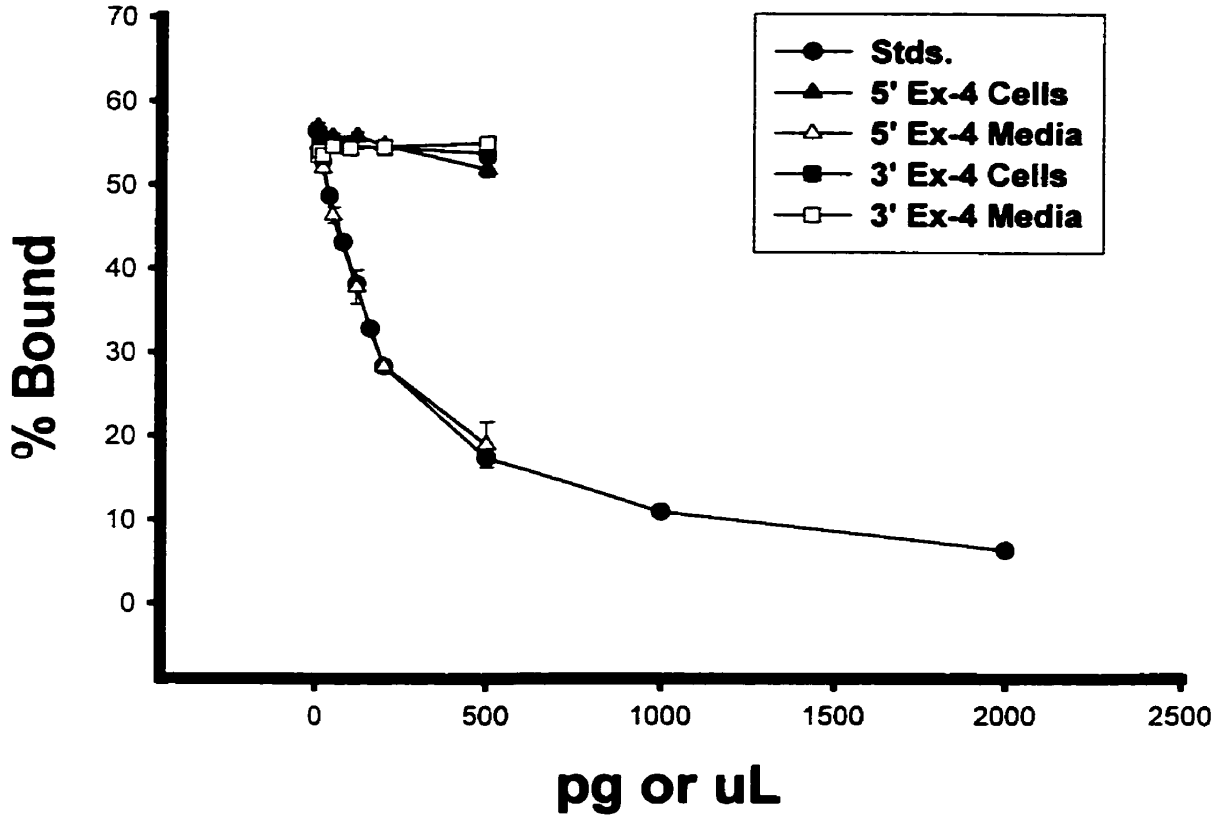

70
60
50
40
30
20
10
0
% Bound
0
500
1000
1500
2000
2500
pg or uL
Stds.
5' Ex-4 Cells
5' Ex-4 Media
3' Ex-4 Cells
3' Ex-4 Media

Fig. 7.

HPLC analysis of ExLI in BHK 5' Ex-4 media extracts. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the media extracts (n=3).

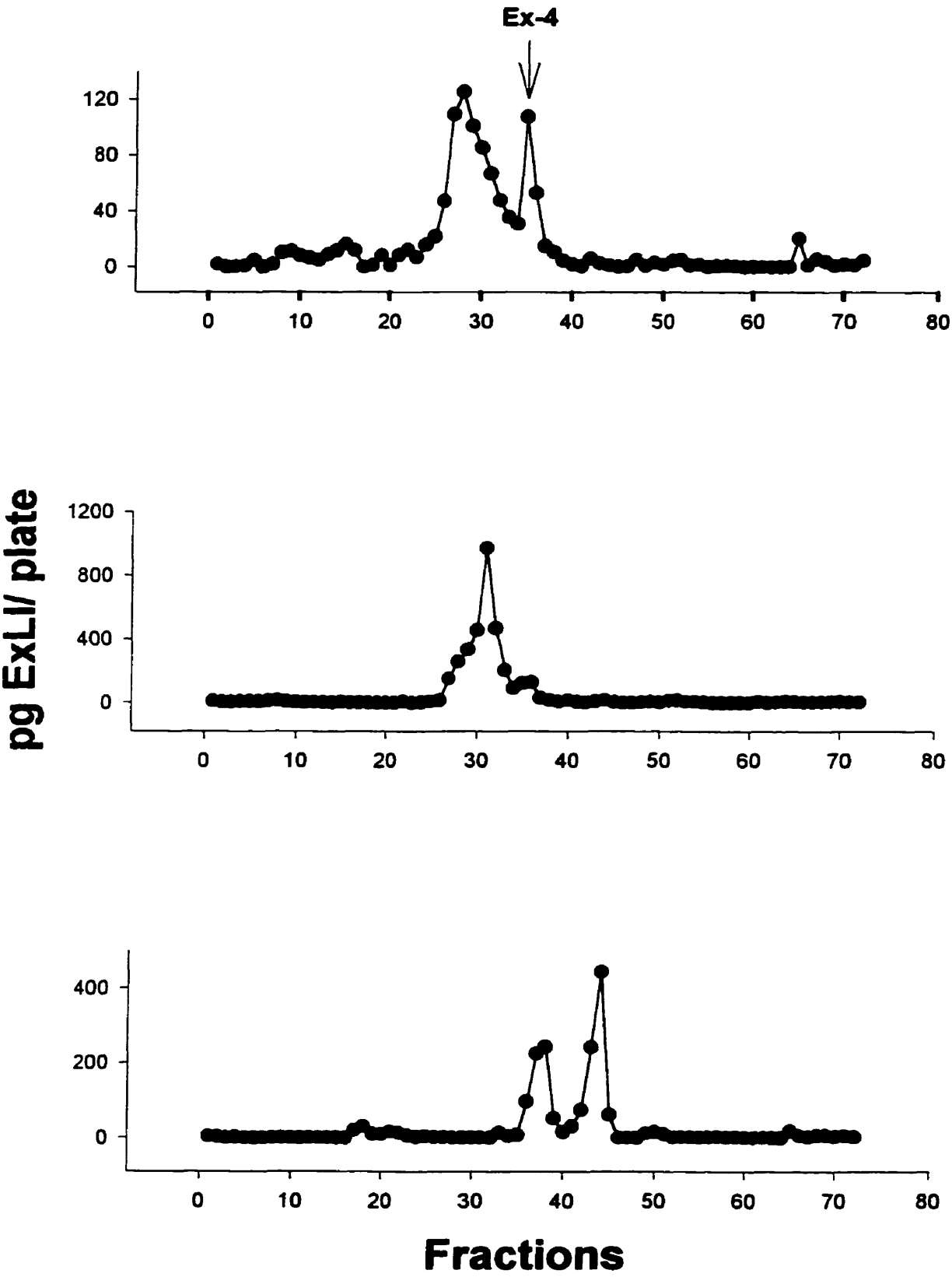

Ex-4
120
80
40
0
0
10
20
30
40
50
60
70
80
pg ExLI/ plate
1200
800
400
0
0
10
20
30
40
50
60
70
80
400
200
0
0
10
20
30
40
50
60
70
80
Fractions

Fig. 8.

AtT-20 5' & 3' Ex-4 cell and media binding curves. AtT-20 5' & 3' cells and 24 hour media were extracted to isolate peptides. Increasing volumes of the extracts were assayed for their ability to bind antiserum #18 as compared to known amounts of the synthetic standard (n=3).

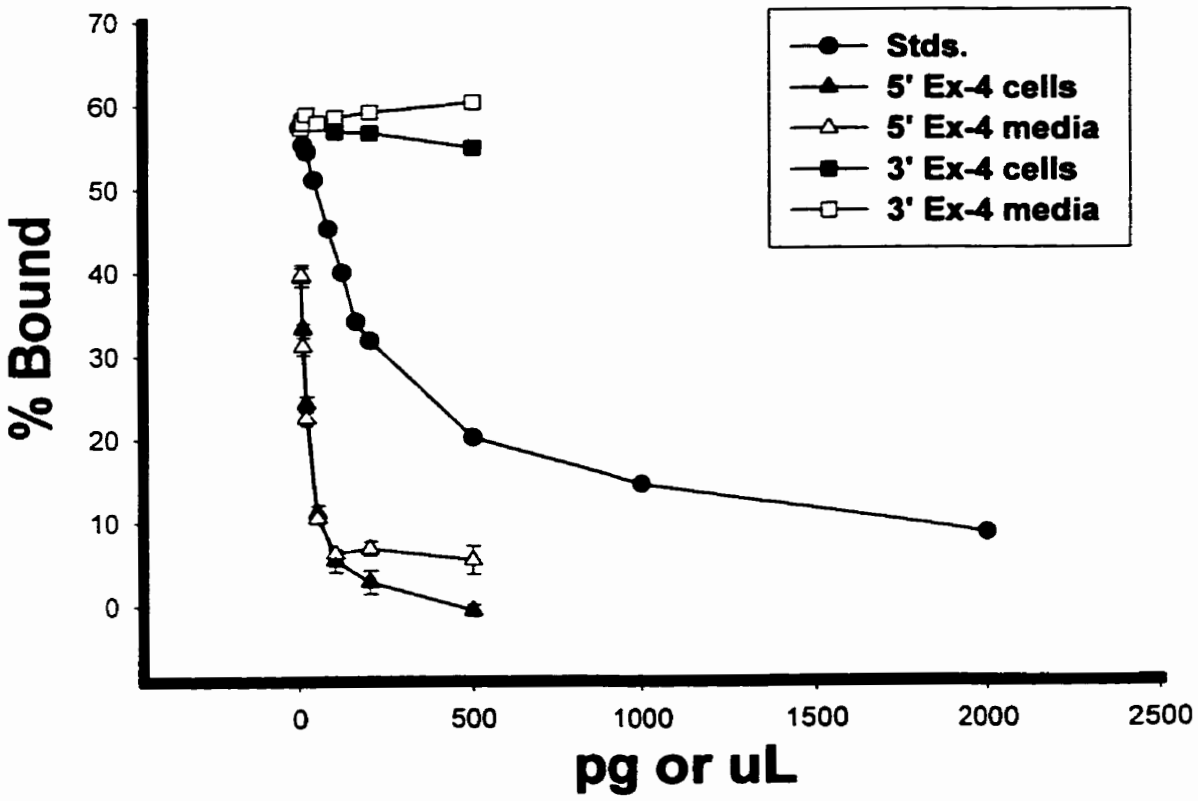
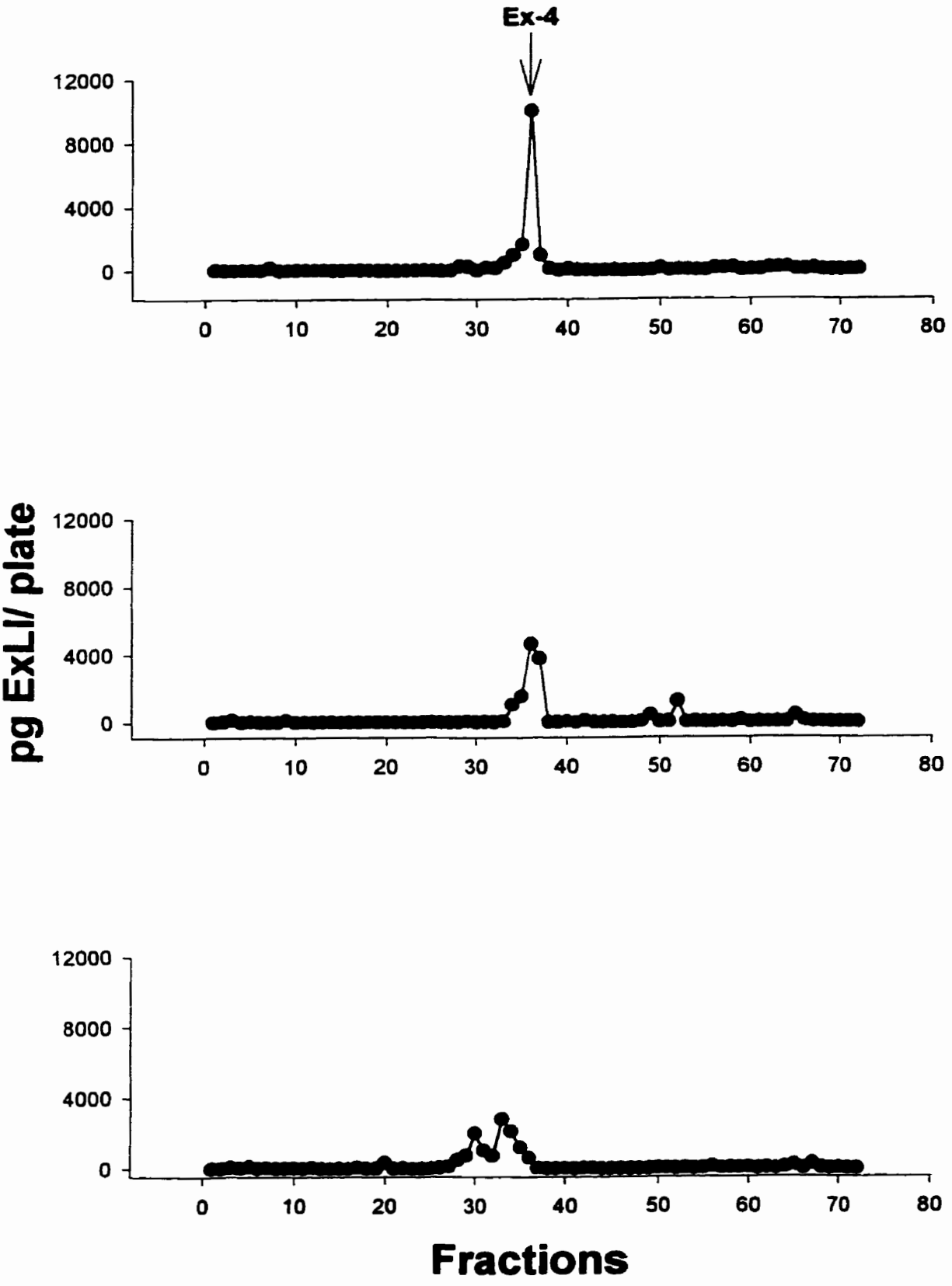

70
60
50
40
30
20
10
0
% Bound
0
500
1000
1500
2000
2500
pg or uL
Stds.
5' Ex-4 cells
5' Ex-4 media
3' Ex-4 cells
3' Ex-4 media

Fig. 9.

HPLC analysis of ExLI in AtT-20 5' Ex-4 cell extracts. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the cell extracts (n=3).


Ex-4
12000
8000
4000
0
0
10
20
30
40
50
60
70
80
pg ExLI/ plate
Fractions

profiles, a small peak was detected in fractions 27-30, potentially representing proEx-4. RIA analysis of the AtT-20 5' Ex-4 media indicated the presence of a peak in the elution position of synthetic Ex-4 in all 3 profiles (Fig. 10). In the 1st profile (Fig. 10, top), the only sample analysed immediately after extraction, a small peak at elution position 27-30 was observed. In the 2nd analyses, a sample stored at -20°C for over 5 weeks, a more hydrophobic peak was observed at position 44.

In order to determine if the endocrine islet InR1-G9 cell-line, known to contain high levels of PC2 [149,169], could process proEx-4 to Ex-4, wild type cells were transfected with both the sense and anti-sense full-length proEx-4 construct. Transfected cells were analysed as mixed cell populations. InR1-G9 cells and 24 hour media were extracted to isolate peptides. The InR1-G9 5' Ex-4 cell line both contained and secreted peptide with ExLI (Fig. 11). Approximately 73% of the immunoreactivity was contained within the cells, consistent with the regulated secretory pathway of these cells. Controls with the antisense gene displayed low levels of ExLI.

After HPLC separation of the extracted peptides, RIA analysis of InR1-G9 5' Ex-4 cells indicated the presence of a peak in the elution position of synthetic Ex-4 in all 3 profiles (Fig. 12). In the 1st profile (Fig. 12, top), the only sample analysed immediately after extraction, a peak was detected at position 27, potentially representing proEx-4, and a small peak of intermediate hydrophobicity was observed at position 30. In the 2nd and 3rd profiles from samples stored for approximately 3 months, no identifiable peak was seen in position 27-30; this was paralleled by the presence of a more hydrophobic peak at position 39-41. A small, third peak at position 36-38 was also seen in these profiles. RIA analysis of the InR1-G9 5' Ex-4 media indicated the presence of a

Fig. 10.

HPLC analysis of ExLI in AtT-20 5' Ex-4 media extracts. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the media extracts (n=3).

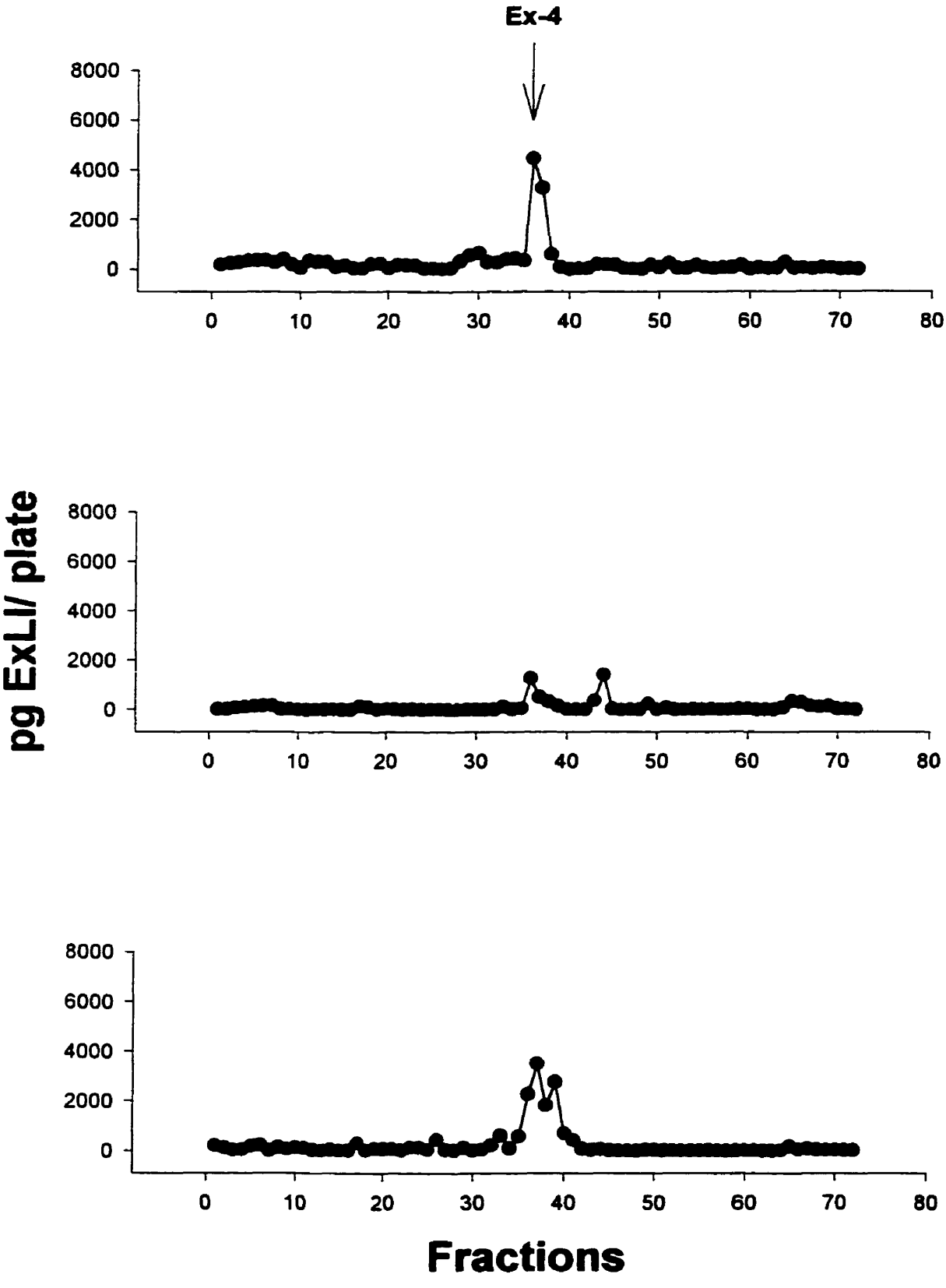

Ex-4
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
80
pg ExLI/ plate
Fractions

Fig. 11.

InR1-G9 5' & 3' Ex-4 cell and media binding curves. InR1-G9 5' & 3' cells and 24 hour media were extracted to isolate peptides. Increasing volumes of the extracts were assayed for their ability to bind antiserum #18 as compared to known amounts of the synthetic standard (n=3).

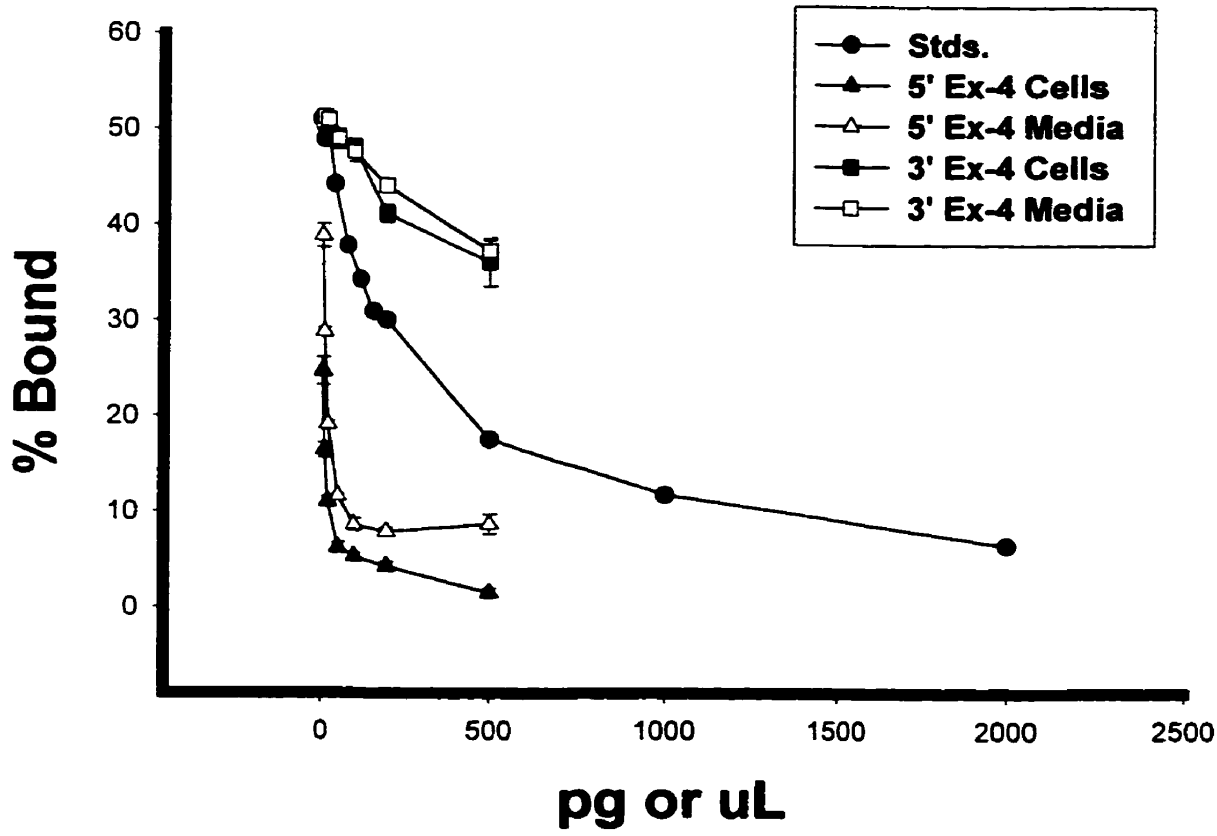
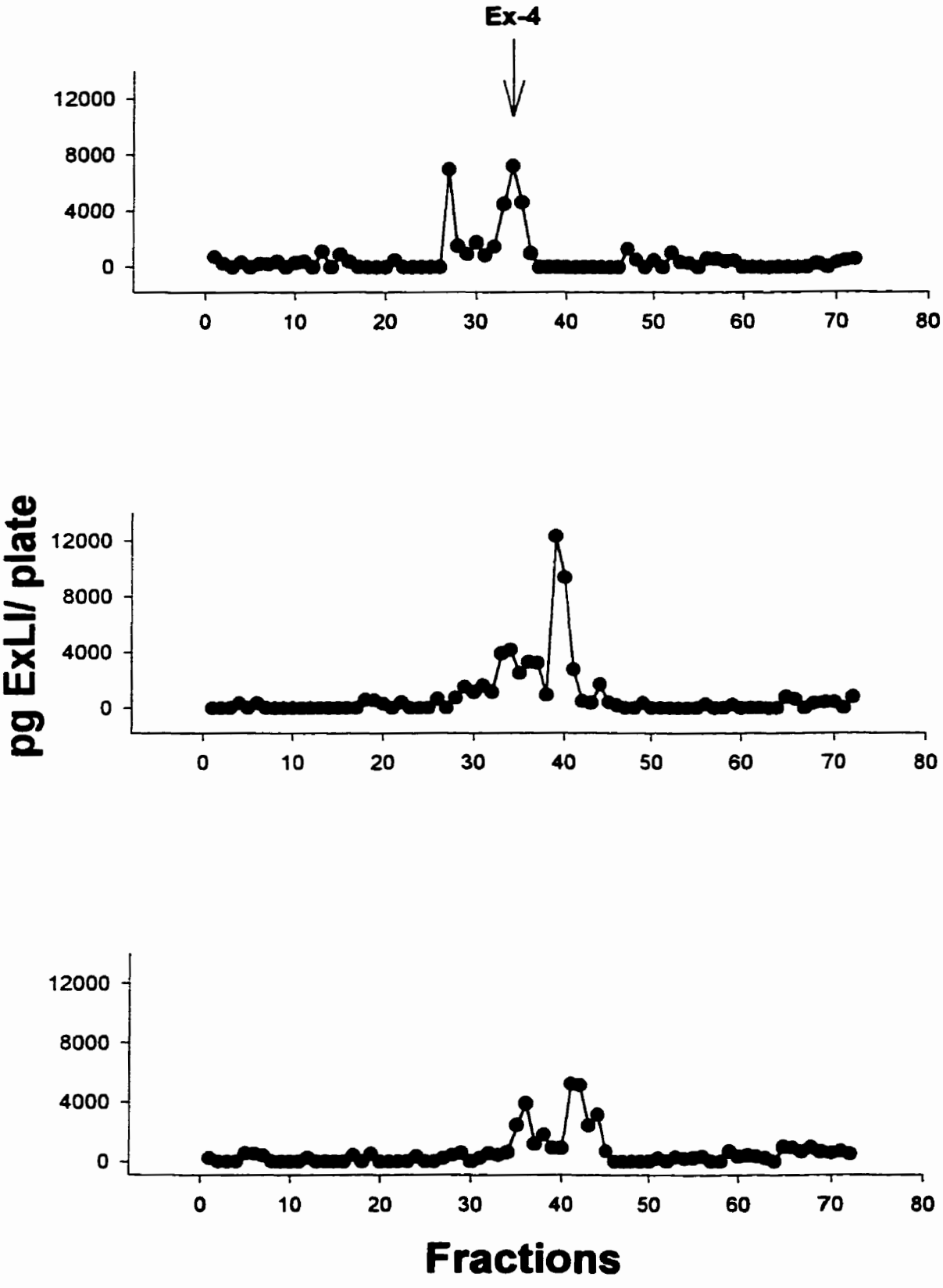

Stds.
5' Ex-4 Cells
5' Ex-4 Media
3' Ex-4 Cells
3' Ex-4 Media
% Bound
60
50
40
30
20
10
0
0
500
1000
1500
2000
2500
pg or uL

Fig. 12.

HPLC analysis of ExLI in InR1-G9 5' Ex-4 cell extracts. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the cells extract (n=3).


Ex-4
12000
8000
4000
0
0
10
20
30
40
50
60
70
80
pg ExLI/ plate
Fractions

peak in the elution position of synthetic Ex-4 as well as a peak in fractions 27-30 in all 3 profiles (Fig. 13). In the 1st two analysis, a peak of intermediate hydrophobicity was also observed. Of note is the fact that the size of the Ex-4-like peak was larger in the 3rd profile than that seen in the 1st two.

To determine if the full-length Ex-4 cDNA was translated *in vivo*, tissues from 3-6 males and 3-6 females from each of two lines of transgenic mice (L18 and L19) as well as from age-matched non-TG mice were extracted to isolate peptides. Very low levels of ExLI were observed in all tissues from non-TG male and female mice (Fig. 14 and 15). In the males from L18 significantly increased levels of ExLI were observed in fat and duodenum ($p<0.05$) as compared to non-TG mice, while lower levels were detected in the ileum ($p<0.05$) (Fig. 14). In the males from L19, higher levels of ExLI were seen in the pituitary ($p<0.001$) and fat ($p<0.01$). Males from L19 also expressed low, but significant concentrations of ExLI in the kidney ($p<0.05$), heart ($p<0.01$) and colon ($p<0.01$).

In the females from L18, no increase in ExLI was seen in any of the tissues examined (Fig. 15). In contrast, the females from L19 exhibited high levels of ExLI in the pituitary ($p<0.05$). Low but significant levels of ExLI were also detected in skeletal muscle ($p<0.05$), adrenal glands ($p<0.01$), spleen ($p<0.05$), ileum ($p<0.05$) and pancreas ($p<0.01$).

To determine the concentration of circulating ExLI, as well as the change in these levels with induction of the promoter, plasma from mice of both sexes from both lines was examined with and without prior zinc treatment of the mice. Low levels of ExLI were detected in plasma from non-TG mice with and without zinc treatment (Fig. 16). Although there was a tendency towards increased levels of ExLI in mice from line 18,

Fig. 13.

HPLC analysis of ExLI in InR1-G9 5' Ex-4 media extracts. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the media extracts (n=3).

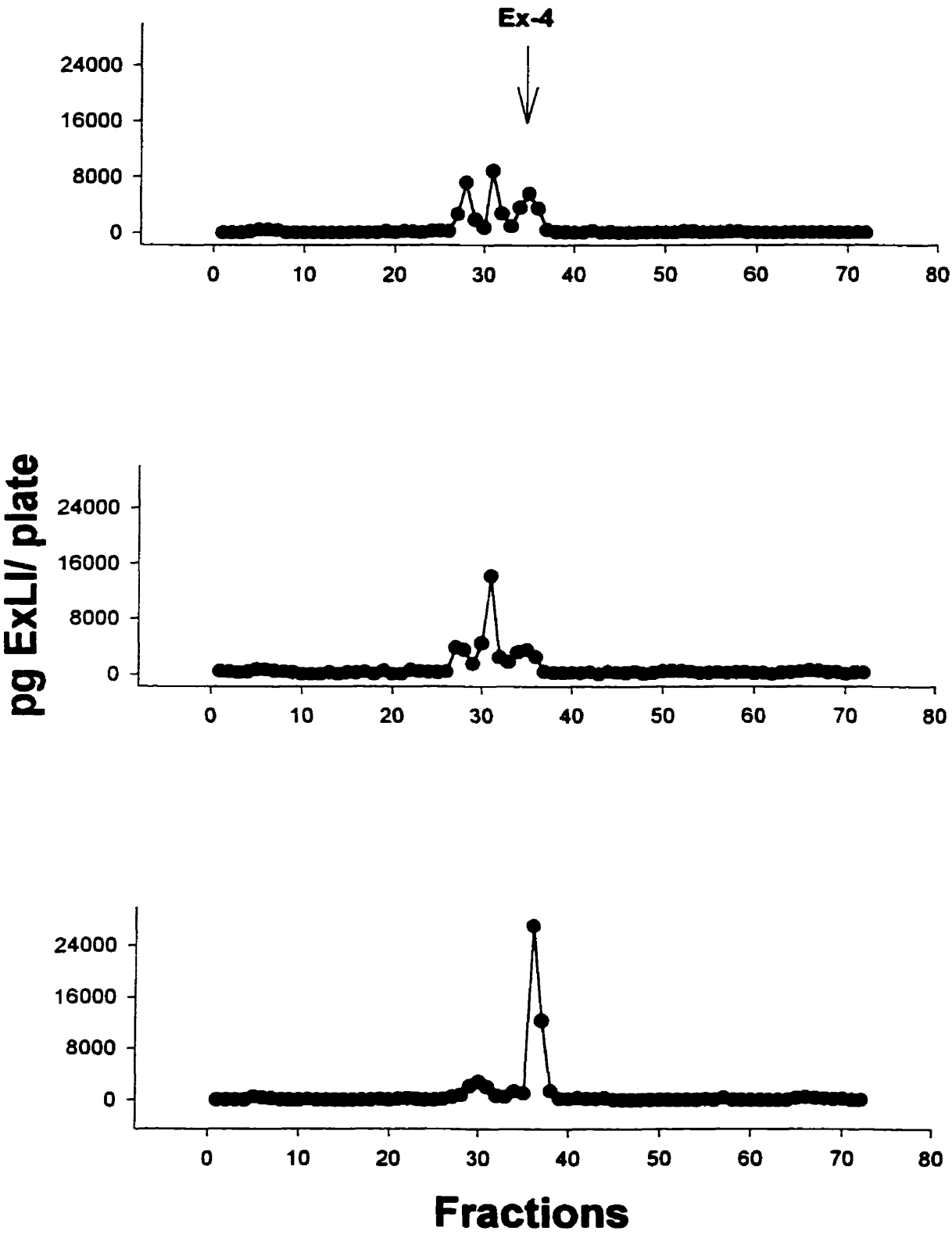

Ex-4
24000
16000
8000
0
0
10
20
30
40
50
60
70
80
pg ExLI/ plate
Fractions

Fig. 14.

ExLI in tissues from L18 and L19 transgenic male mice. Tissues from 3-6 males from each of 2 lines of transgenic mice (L18 and L19) and from age-matched non-transgenic (non-TG) mice were extracted to isolate peptides. ExLI was detected in the tissues by RIA. (CC = cerebral cortex, CBM = cerebellum, HYP = hypothalamus, PIT = pituitary, MUS = skeletal muscle, FAT = fat, TEST = testis, KID = kidneys, LIV = liver, HRT = heart, LUNG = lungs, ADR = adrenal glands, SPLN = spleen, STOM = stomach, DUO = duodenum, JEJ = jejunum, ILM = ileum, CLN =colon, PANC = pancreas; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ as compared to non-TG).

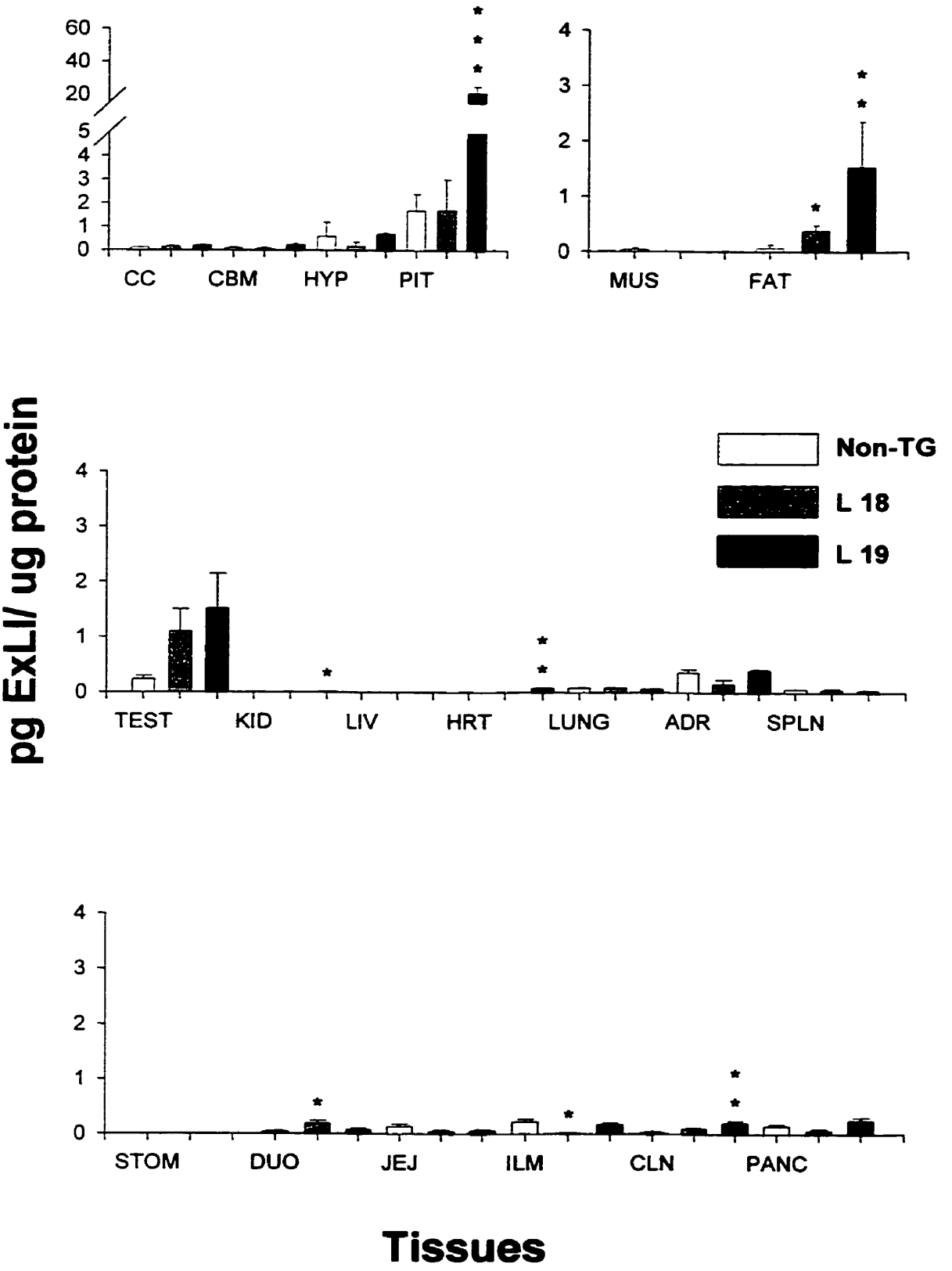

60
40
20
5
4
3
2
1
0
CC
CBM
HYP
PIT
4
3
2
1
0
MUS
FAT
pg ExLI/ ug protein
Non-TG
L 18
L 19
4
3
2
1
0
TEST
KID
LIV
HRT
LUNG
ADR
SPLN
4
3
2
1
0
STOM
DUO
JEJ
ILM
CLN
PANC
Tissues

Fig. 15.

ExLI in tissues from L18 and L19 transgenic female mice. Tissues from 3-6 males from each of 2 lines of transgenic mice (L18 and L19) and from age-matched non-transgenic (non-TG) mice were extracted to isolate peptides. ExLI was detected in the tissues by RIA. (CC = cerebral cortex, CBM = cerebellum, HYP = hypothalamus, PIT = pituitary, MUS = skeletal muscle, FAT = fat, OV = ovaries, KID = kidneys, LIV = liver, HRT = heart, LUNG = lungs, ADR = adrenal glands, SPLN = spleen, STOM = stomach, DUO = duodenum, JEJ = jejunum, ILM = ileum, CLN =colon, PANC = pancreas; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ as compared to non-TG).

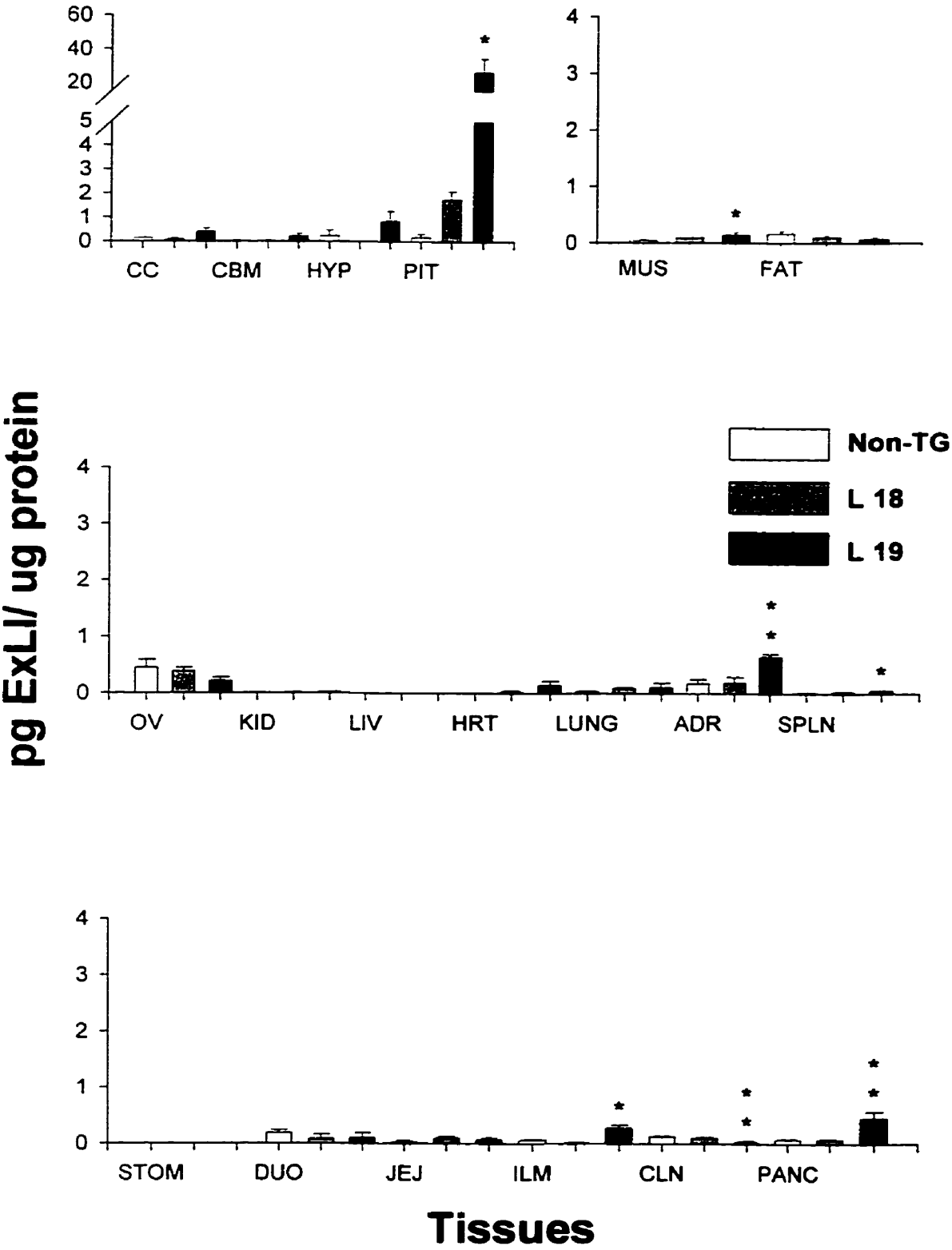
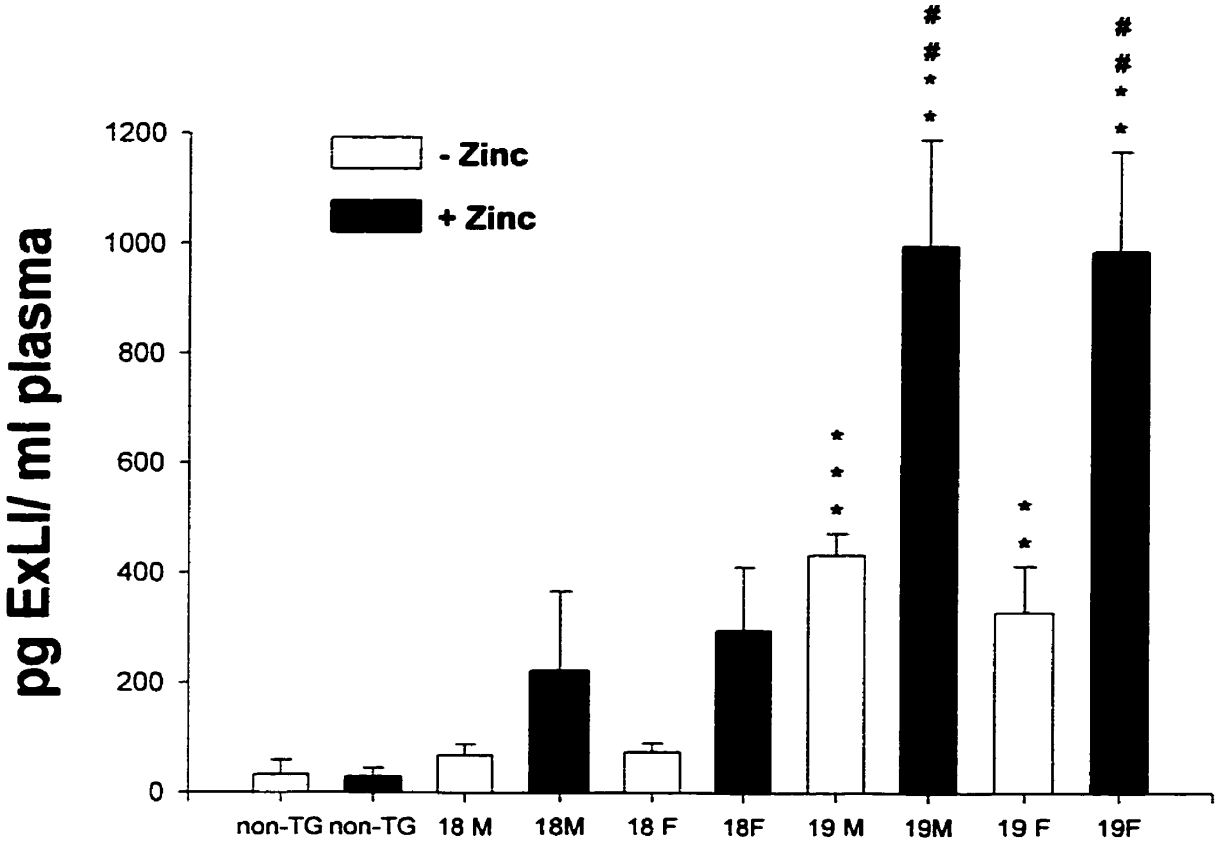

60
40
20
5
4
3
2
1
0
CC
CBM
HYP
PIT
4
3
2
1
0
MUS
FAT
pg ExLI/ ug protein
Non-TG
L 18
L 19
4
3
2
1
0
OV
KID
LIV
HRT
LUNG
ADR
SPLN
4
3
2
1
0
STOM
DUO
JEJ
ILM
CLN
PANC
Tissues

Fig. 16.

ExLI in plasma from L18 and L19 transgenic mice with/without zinc treatment. Plasma from 3-8 mice of both sexes from each of 2 lines of transgenic mice (L18 and L19) and from age-matched non-transgenic (non-TG) mice was extracted to isolate peptides. Mice received either normal (- zinc) or zinc-supplemented (+ zinc) drinking water for 3 days prior to plasma collection. ExLI was detected in the plasma by RIA. (M = males, F = females, non-TG = males and females; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ as compared to non-TG; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to non-treated mice).

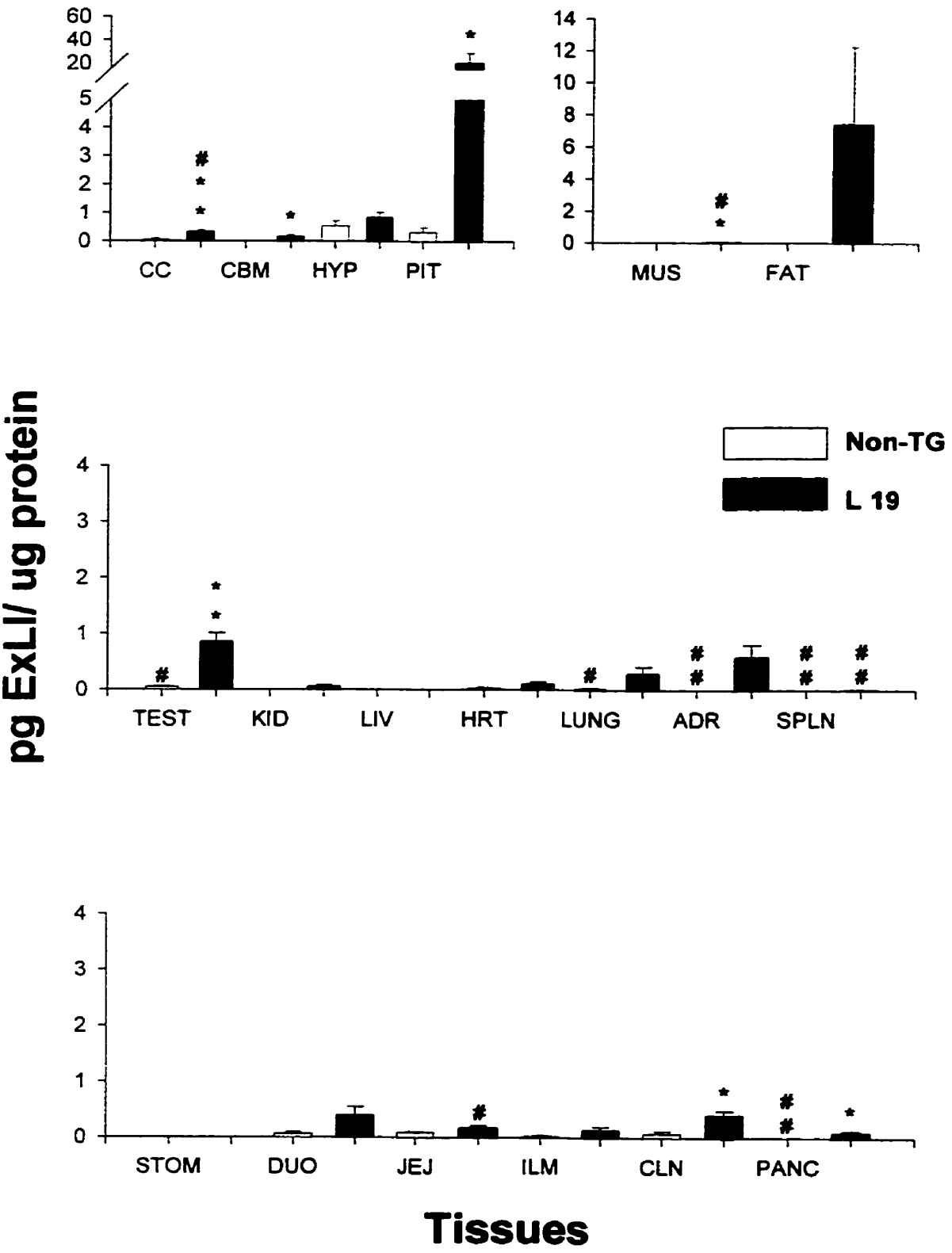

pg ExLI/ ml plasma
1200
1000
800
600
400
200
0
- Zinc
+ Zinc
non-TG
non-TG
18 M
18M
18 F
18F
19 M
19M
19 F
19F

this did not reach significance, even with zinc induction. In contrast, significant levels of circulating ExLI were detected in L19 mice without zinc treatment, (males $p<0.001$, females $p<0.01$) as compared to non-TG mice. Induction of the promoter caused an approximate doubling of the plasma ExLI levels in both sexes ($p<0.01$).

As both tissue and plasma data indicated higher levels of ExLI (as compared to non-TG mice) in L19 than L18, it was decided that tissue ExLI levels should be determined in mice from L19 following induction of the promoter with zinc. Three male and 3 female mice from L19, as well as age-matched non-TG mice, were therefore treated for three days with zinc and tissues were extracted to isolate peptides. In male mice, concentrations of ExLI were again elevated in the pituitary ($p<0.05$) as compared to treated non-TG mice (Fig. 17), however, these levels were not significantly different from the un-induced state (Fig. 14). In fat, the mean ExLI levels increased close to five fold as compared to the levels seen in the un-induced state, however, due to a low value in one mouse, the levels were not significant as compared to zinc treated non-TG mice or to non-induced L19 mice. However, ExLI levels in testes were significantly increased ($p<0.01$), contributed to in part by a statistically significant decrease in the levels seen in the non-TG treated mice as compared to the non-TG non-treated animals ($p<0.05$). Lower levels of ExLI were also seen in the cerebral cortex ($p<0.01$ as compared to treated non-TG and $p<0.05$ as compared to non-treated L19), cerebellum ($p<0.05$), muscle ($p<0.05$ as compared to treated non-TG and $p<0.05$ as compared to non-treated L19), colon ($p<0.05$) and pancreas ($p<0.05$ as compared to treated non-TG, with treated non-TG being statistically different from the non-treated non-TG $p<0.01$). Significant

Fig. 17.

ExLI in tissues from L19 transgenic male mice with zinc treatment. Tissues from 3 L19 transgenic males mice and from age-matched non-transgenic (non-TG) mice were extracted to isolate peptides. All mice received zinc-supplemented drinking water for 3 days prior to tissue collection. ExLI was detected in the tissues by RIA. (CC = cerebral cortex, CBM = cerebellum, HYP = hypothalamus, PIT = pituitary, MUS = skeletal muscle, FAT = fat, TEST = testis, KID = kidneys, LIV = liver, HRT = heart, LUNG = lungs, ADR = adrenal glands, SPLN = spleen, STOM = stomach, DUO = duodenum, JEJ = jejunum, ILM = ileum, CLN =colon, PANC = pancreas; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ as compared to non-TG; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to non-treated L19 transgenic male mice Fig. 14).


60
40
20
5
4
3
2
1
0
CC
CBM
HYP
PIT
14
12
10
8
6
4
2
0
MUS
FAT
pg ExLI/ ug protein
Non-TG
L 19
4
3
2
1
0
TEST
KID
LIV
HRT
LUNG
ADR
SPLN
4
3
2
1
0
STOM
DUO
JEJ
ILM
CLN
PANC
Tissues

changes were seen in various other tissues in comparing the non-treated to treated state (lung, adrenal, spleen, jejunum), however, the absolute levels of ExLI were negligible.

In female mice from L19, high levels of ExLI were also seen in pituitary ($p<0.05$) as compared to treated non-TG mice (Fig. 18), however, as in the males, these levels were not significantly different from the un-induced state (Fig. 15). In the adrenal glands, significant increases in ExLI were seen ($p<0.05$) as compared to both treated non-TG mice (Fig. 18) and untreated L19 mice (Fig. 15). Lower levels of ExLI were also detected in the cerebral cortex ($p<0.001$), heart ($p<0.05$), duodenum ($p<0.05$), ileum ($p<0.05$), colon ($p<0.05$) and pancreas ($p<0.05$). In various other tissues, significant changes between treated and non-treated mice were seen (ovaries, kidneys, ileum, colon, pancreas), which in some cases may have contributed to the significance observed in some tissues expressing low levels of ExLI (e.g. ileum, colon and pancreas).

Since it was determined that, in zinc-fed mice from L19, high levels of ExLI were circulating in the plasma (both sexes), and were also seen in the pituitary (both sexes), fat (males), testes (males), and adrenals (females), HPLC analysis was carried out to determine the forms of ExLI in plasma and in these tissues. Specifically, the processing profile in the plasma, fat and testes of zinc-treated males, and in the pituitary and adrenal glands of zinc-treated females, was examined.

In the plasma, all three analyses demonstrated a clear peak of ExLI in the elution position of synthetic Ex-4 (fraction 36, Fig. 19). In two of the profiles, a peak in fractions 27-30 was also present, however, this peak was minor compared to that at position 36. The third profile also displayed two small peaks of intermediate hydrophobicity.

Fig. 18.

ExLI in tissues from L19 transgenic female mice with zinc treatment. Tissues from 3 L19 transgenic females mice and from age-matched non-transgenic (non-TG) mice were extracted to isolate peptides. All mice received zinc-supplemented drinking water for 3 days prior to tissue collection. ExLI was detected in the tissues by RIA. (CC = cerebral cortex, CBM = cerebellum, HYP = hypothalamus, PIT = pituitary, MUS = skeletal muscle, FAT = fat, OV = ovaries, KID = kidneys, LIV = liver, HRT = heart, LUNG = lungs, ADR = adrenal glands, SPLN = spleen, STOM = stomach, DUO = duodenum, JEJ = jejunum, ILM = ileum, CLN =colon, PANC = pancreas; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ as compared to non-TG; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ as compared to non-treated L19 transgenic female mice Fig. 15).

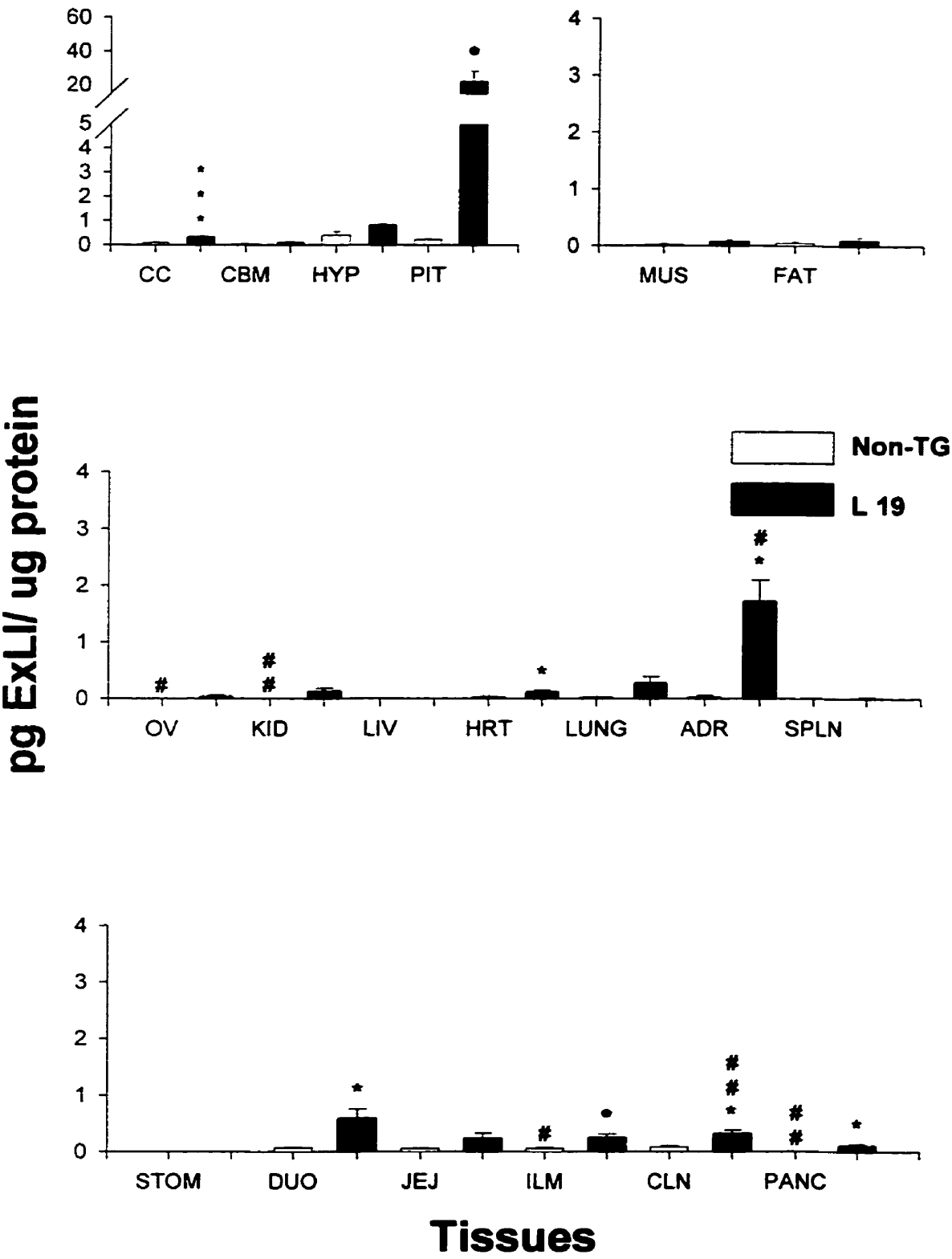
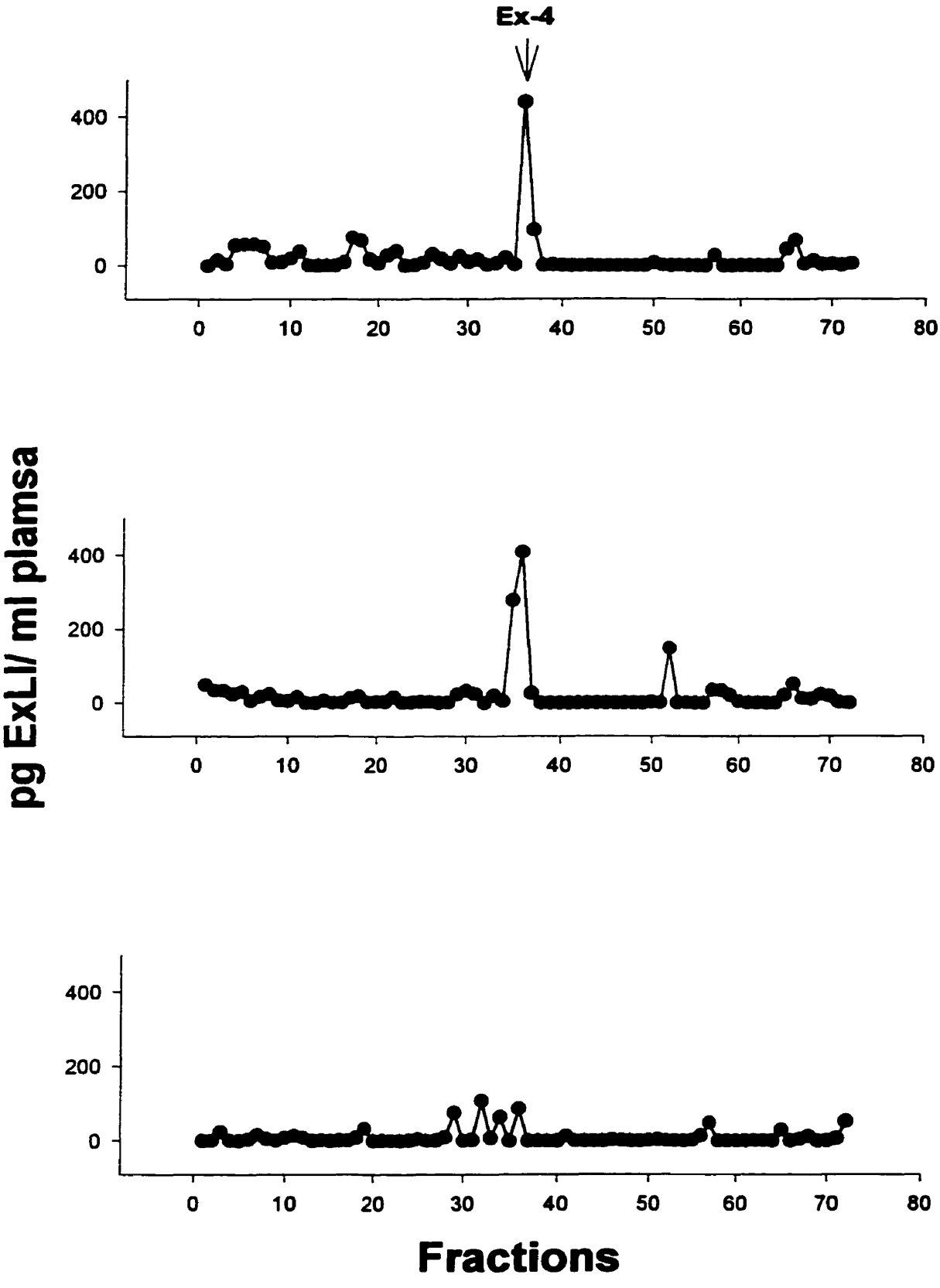

60
40
20
5
4
3
2
1
0
CC
CBM
HYP
PIT
4
3
2
1
0
MUS
FAT
pg ExLI/ ug protein
Non-TG
L 19
4
3
2
1
0
OV
KID
LIV
HRT
LUNG
ADR
SPLN
4
3
2
1
0
STOM
DUO
JEJ
ILM
CLN
PANC
Tissues

Fig. 19.

HPLC analysis of ExLI in plasma extracts of zinc-treated L19 males. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the plasma extracts (n=3).


Ex-4
400
200
0
0
10
20
30
40
50
60
70
80
pg ExLI/ ml plamsa
Fractions

In the pituitary, no clear peak of ExLI (above background) was detected at fraction 36 (Fig. 20). All three profiles did demonstrate a peak in fractions 27-30, as well as two additional, hydrophilic peaks (best seen in the first two profiles).

In extracts of fat, a very minor peak (just above background levels) was consistently seen in fraction 36, with a larger peak detected in fractions 27-30 (Fig. 21).

In the testes, a clear peak in the elution position of synthetic Ex-4 as well as a peak in fractions 27-30, was seen in all 3 profiles (Fig. 22). A smaller peak/shoulder of intermediate hydrophobicity was also detected in all profiles.

Only small peaks at approximately background levels were observed in fraction 36 of adrenal extracts (Fig. 23). Background levels of immunoreactivity in fractions 27-30 were also observed in the 1st and 3rd profiles.

Fig. 20.

HPLC analysis of ExLI in pituitary extracts of zinc-treated L19 females. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the pituitary extracts (n=3).

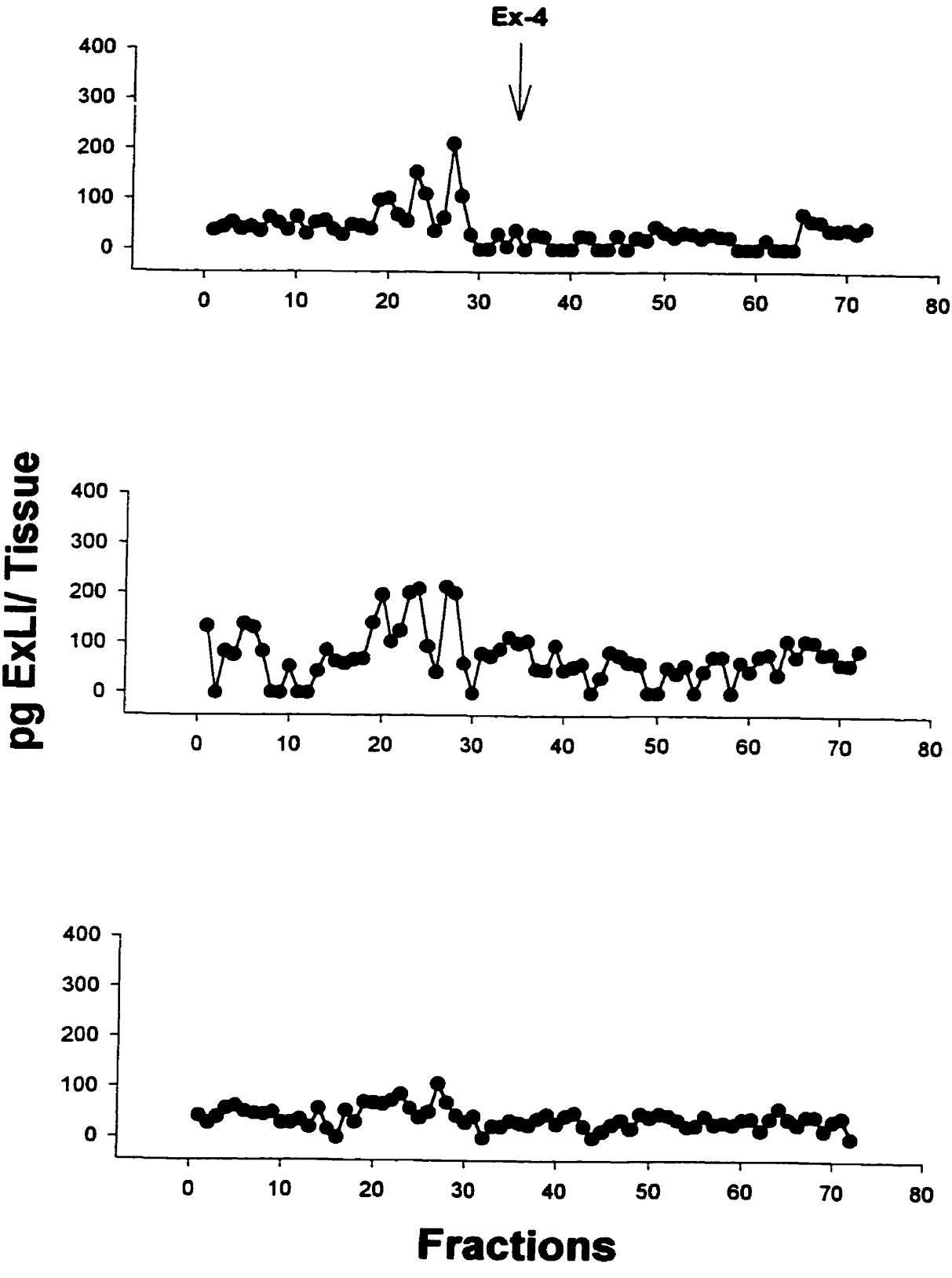

Ex-4
400
300
200
100
0
0
10
20
30
40
50
60
70
80
pg ExLI/ Tissue
Fractions

Fig. 21.

HPLC analysis of ExLI in fat extracts of zinc-treated L19 males. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the fat extracts (n=3).

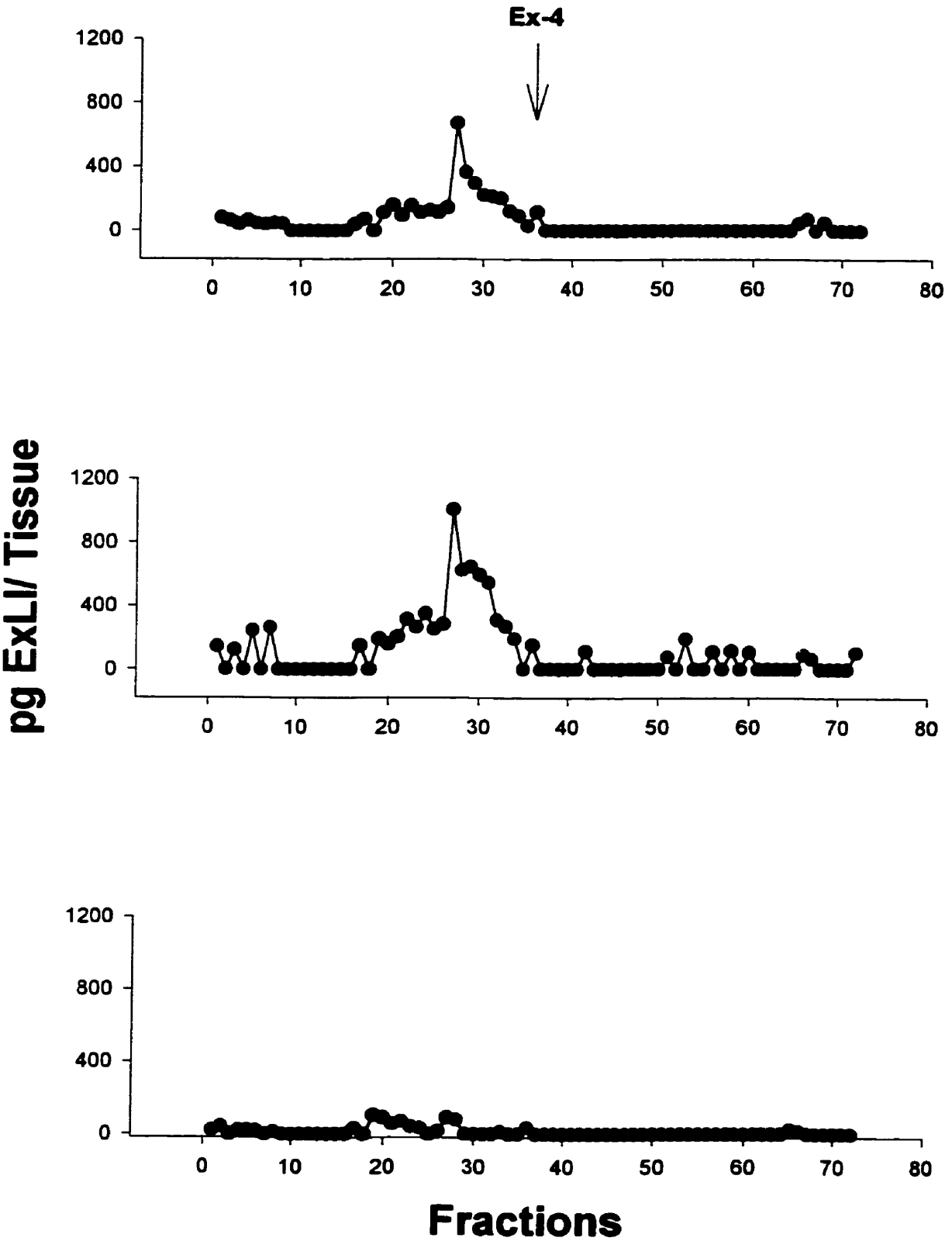

Ex-4
1200
800
400
0
0
10
20
30
40
50
60
70
80
pg ExLI/ Tissue
Fractions

Fig. 22.

HPLC analysis of ExLI in testes extracts of zinc-treated L19 males. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the testes extracts (n=3).

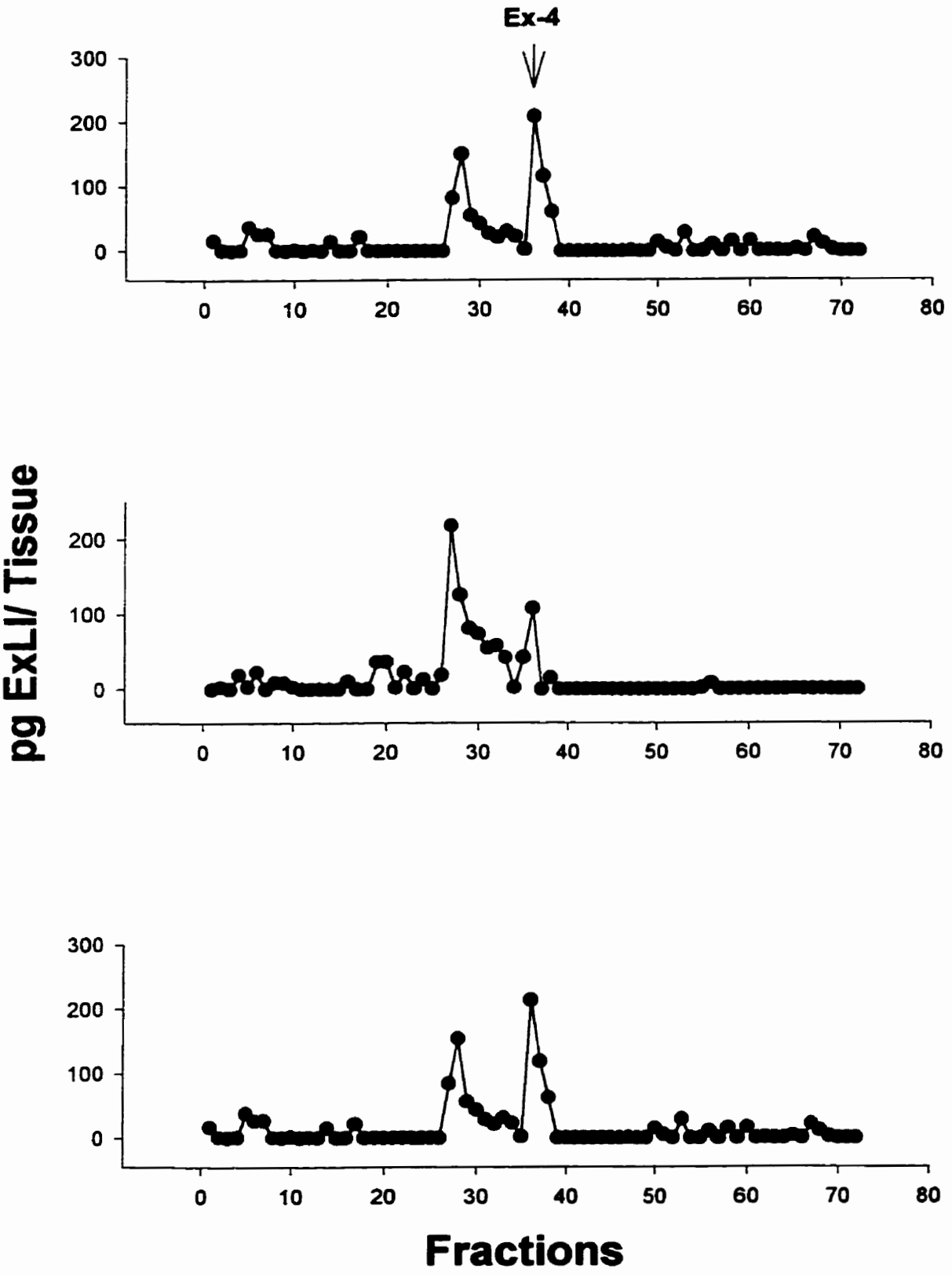
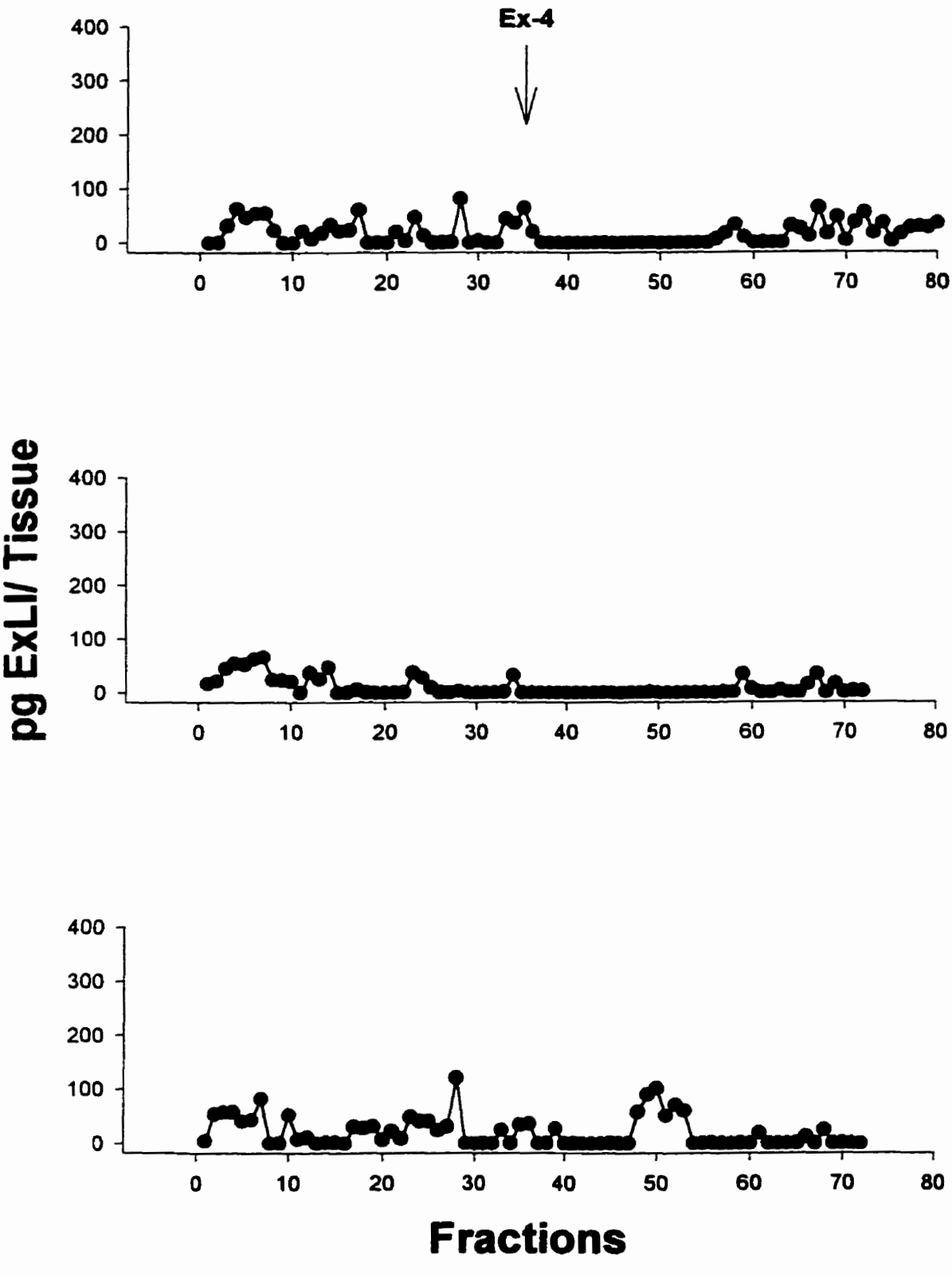

Ex-4
300
200
100
0
0
10
20
30
40
50
60
70
80
pg ExLI/ Tissue
200
100
0
0
10
20
30
40
50
60
70
80
300
200
100
0
0
10
20
30
40
50
60
70
80
Fractions

Fig. 23.

HPLC analysis of ExLI in adrenal extracts of zinc-treated L19 females. The elution position of synthetic Ex-4 is shown as compared to the ExLI contained in the adrenal extracts (n=3).


Ex-4
400
300
200
100
0
0
10
20
30
40
50
60
70
80
pg ExLI/ Tissue
Fractions

Chapter 4: Discussion

Ex-4 is a potent and long-acting GLP-1R agonist. Intriguingly, although this peptide is found in lizard exocrine secretions, it displays endocrine activity. To understand the synthesis of Ex-4, the processing of proEx-4 was examined *in vitro* and *in vivo* in both endocrine and non-endocrine cells.

4.1 Post-translational Processing of ProEx-4 *in vitro*

Antisera raised against Ex-4 may or may not allow for detection of Ex-4 as well as proEx-4 and various related peptides. However, the free N-terminus of Ex-4 (amino acids 1-8) was demonstrated to not be necessary for antigenicity with our antisera (Fig.4 and 5), suggesting that our RIA allows for the detection of proEx-4, bioactive Ex-4 and related peptides, including processing intermediates and degradation products.

In all of the extracts from all cell lines, there was the clear demonstration of a peak in the elution position of synthetic Ex-4 (fraction 36). Thus, it can be concluded that the non-endocrine BHK cells, known to contain furin [167], the AtT-20 cells, known to contain high levels of PC1 [125,168], and the InR1-G9 cells, known to contain high levels of PC2 [149,169], are all able to produce peptide with the elution position of synthetic Ex-4, although to varying degrees.

In the HPLC analyses of ExLI in a variety of cell and media extracts, two peaks of ExLI were detected in almost all profiles, one seen in the elution position of synthetic Ex-4 (fraction 36), consistent with the detection of Ex-4, and the other, a more hydrophilic putative N-terminally-extended prohormone (fraction 27-30). Although HPLC elution position of peptides can be predicted on the basis of their amino acid content, when

secondary structure is not a factor, this is impossible to do for proEx-4 as the site of cleavage between the pre and pro forms is not known. Therefore it cannot be established with certainty whether this earlier peak is in fact proEx-4. Also, there was the occasional detection of a peak of intermediate hydrophobicity; currently it is completely unknown whether this is a processing intermediate or a degradation product.

Interestingly, variability in the location of the putative proEx-4 peak was seen in some HPLC analyses. In BHK 5' Ex-4 media extracts, for example, the 3rd profile, from a sample which had been stored at -20°C for over 3 months, demonstrated the disappearance of the more hydrophilic peak of putative proEx-4 (fractions 27-30) concomitant with the appearance of a peak with greater hydrophobicity (fractions 42-44) (Fig. 7). As well in the HPLC analysis of ExLI in AtT-20 5' Ex-4 media extracts, the 1st profile, the only sample analysed immediately after extraction, demonstrated a very small peak at elution position 27-30, whereas in the 2nd analysis, of a sample stored at -20°C for over 5 weeks, only the more hydrophobic peak was observed at position 44 (Fig.10). Furthermore, in HPLC analysis of InR1-G9 5' Ex-4 cell extracts, the 1st profile, the only sample analysed immediately after extraction, a peak was detected at position 27, whereas in the 2nd and 3rd profiles from samples stored at -20°C for approximately 3 months, no identifiable peak was seen in position 27-30, paralleled by the presence of a more hydrophobic peak at position 39-41 (Fig. 12). Taken together, there was a clear trend in all cell lines of a shift in the putative proEx-4 peak to a position of greater hydrophobicity with extract storage. One suggestion as to a mechanism that might account for such a shift in elution position is a change in secondary structure with storage. This change may cause the exposure of additional hydrophobic residues which were not

exposed in the native conformation, thus causing greater hydrophobic binding to the column and a later elution time. As the proEx-4 sequence contains 3 methionine residues (two 5' to the Ex-4 sequence and one within the Ex-4 sequence), oxidation at these residues may occur upon storage of samples. This oxidation may then induce a change in the secondary structure of proEx-4, resulting in exposure of additional hydrophobic residues. In fact, oxidation and a shift in the elution position is known to occur for proG, thus, there is a precedent for such findings [152].

Two additional points with regards to the HPLC analyses of ExLI in the cell lines warrant discussion. First, in the BHK 5' Ex-4 media extract profiles (Fig.6), large variations in the levels of ExLI were detected, whereas little variation was seen in any of the other cell line extract profiles (Fig. 9,10,12,13). This can be explained by the fact that the BHK extracts were the only samples run on the HPLC that were not previously assessed for their total ExLI by RIA; as less ExLI was contained in the BHK cell-line extracts, samples were run in their entirety on the HPLC. Thus, in contrast to the other cell types for which equivalent and known amounts of ExLI were loaded, this was not the case with the BHK extracts. Thus, differences in the total number of cells extracted may account for these variations.

Secondly, in the InR1-G9 cells, an additional third peak of intermediate hydrophobicity was detected (Fig. 12,13). There are several possible explanations that may be suggested for this result. First, this may be an Ex-4 degradation product. Although being degradation-resistant, perhaps some cleavage may occur *via* DPP-IV during this 24 hour incubation; it has been noted that such cleavage of glucose-dependent insulinotropic peptide results in an earlier elution position (while cleavage of GLP-1 results in a later

elution position) [45]. In fact, DPP-IV has recently been co-localized within islet A cells [170]. Additional PC-mediated processing at one of the many single basic sites of proEx-4 may also be the cause of this intermediate peak. Alternatively, this may be an amidated form of Ex-4 as this peptide does contain a C-terminal glycine. InR1-G9 cells do possess the peptide amidating enzyme peptidylglycine α-amidating monooxygenase (PAM), which cleaves C-terminal glycine residues to produce amidated peptides (e.g. GLP-$1^{7\text{-}36NH2}$) [150]. This seems unlikely, however, as AtT-20 cells which also contain PAM [171], did not produce this peptide of intermediate hydrophobicity.

In summary, both the non-endocrine BHK cells possessing a constitutive secretory pathway and the endocrine AtT-20 and InR1-G9 cells possessing regulated secretory pathways processed proEx-4 to a peptide with the elution position of synthetic Ex-4.

4.1 Post-translational Processing of ProEx-4 *in vivo*

In transgenic mice containing the full length proEx-4 cDNA under the control of metalothionein-1 (MT) promoter/enhancer sequences and flanked by human growth hormone (hGH) 3'-flanking sequences, ExLI was detected under basal, non-induced conditions. In both sexes of line 19, higher levels of ExLI were detected as compared to line 18 (Fig. 14 and 15). In the males, highest levels of ExLI were detected in the pituitary, fat and testes (Fig. 14). Zinc induction of this promoter did not increase the levels of expression in the pituitary or testes, but caused an approximate five-fold increase in the ExLI in the fat (Fig. 17). In the females of line 19, the pituitary and adrenal gland expressed the highest levels of ExLI (Fig. 15) and again zinc induction did

not increase the levels detected in the pituitary, but caused slight elevations in the levels seen in the adrenal glands (Fig. 18). It must be noted, however, that it is assumed that the ExLI detected in these various tissues indicates synthesis and not uptake. Thus, line-, tissue- and sex-specific differences were seen in the levels of ExLI. One can speculate that differences in the site of integration of the transgene in the host genome may effect transgene transcription and explain the differences seen in the expression of ExLI in these two lines of mice. Additionally, the number of sites of transgene incorporation and whether the mice tested are hetero- or homozygous may play a role; these factors are unknown for the mice used in the present study (personal communication, Ms. L Baggio). It is not surprising that the pituitary was found to be the predominant site of expression of the MT-proEx-4 transgene. Low et al. have demonstrated that cryptic human growth hormone gene sequences found in the 3' sequence of this construct direct gonadotrophin-specific expression [172,173]. Furthermore, the pituitary has been previously demonstrated to be the site of greatest tissue expression of 5'-MT-prosomatostatin-hGH-3' and 5'-MT-growth hormone-hGH-3' constructs [160,172]. Although zinc is known to induce the transcriptional rate of metallothionein genes by inducing binding of the metal transcription factor-1 to a metal-responsive element in the MT-1 promoter [174-176], no increase in levels were seen in the pituitary and testes of zinc-fed mice. However, endogenous metal ions in hypothalamic extracts have been suggested as having pituitary regulatory actions as zinc at physiological concentrations reduces prolactin secretion from the pituitary *in vitro* [177,178]. Thus, maximal induction, through endogenous levels of this trace element, may already be present in the pituitary. Maximal endogenous induction of the MT promoter may also be present in the testes at basal zinc levels as testicular

functions are adversely and reversibly affected as a result of zinc deficiency in both humans and experimental animals [177]. Finally, sex-differences and expression in fat have not been reported for the 5'-MT-prosomatostatin-hGH-3' and 5'-MT-growth hormone-hGH-3'transgenes and thus, the possible effects of hormonal differences on transgene expression can only be postulated.

In plasma it was seen that both sexes of line 19 had significant levels of ExLI (Fig. 16), with non-significant levels in line 18, under basal transgene expression. Zinc induction more than doubled ExLI in line 19 with only a tendency towards increase in line 18.

Determination of the molecular forms of circulating ExLI in males from line 19 demonstrated only minor peaks in the elution position of the putative proEx-4, with a clear peak of ExLI in the elution position of synthetic Ex-4 (Fig.19). The presence of circulating bioactive Ex-4, a GLP-1R agonist, is also corroborated by the phenotype of these mice (personal communication, Ms. L Baggio). Preliminary studies indicate lower fasting blood glucose levels in the males of line 19 with significantly lower blood glucose levels following an oral glucose tolerance test (OGTT) both with and without zinc treatment. Similarly, low basal and zinc-induced levels of ExLI detected in plasma from male mice in line 18 are reflected by only a tendency towards lowered blood glucose following an OGTT, with no effect of zinc treatment. In the females, preliminary results are less clear, however, a tendency for lowered blood glucose was also seen in the females of line 19, with no effect from those of line 18 (Ms. L Baggio, personal communication). These studies are currently being verified, however, they do reflect the data presented in this study.

In contrast to the high levels of bioactive Ex-4 observed in the plasma of male mice in

line 19, none of the tissues expressing high levels of ExLI (e.g. pituitary, fat, testes, adrenal) mimicked this processing profile. In the pituitary, no peak in the elution position of synthetic Ex-4 was detected, however, a peak was present in the elution position of putative proEx-4 as well as two additional more hydrophilic peaks (Fig. 20). It is possible that, within the pituitary, proEx-4 may be oxidized as also seen for proG, which elutes as a trio of peaks when oxidized [152]. This seems unlikely however, as if correct this oxidation must occur without the secondary structure changes described above for the cell lines studied. Alternatively, phosphorylation (at consensus Ser-X-acidic) may also occur, as four phosphorylation sites are present N-terminal to the Ex-4 peptide. Finally, additional or alternative post-translational cleavage at one of the many single basic sites of proEx-4 may be occurring in the pituitary. It should be noted that the gonadotrophs, the cells demonstrated by others to be the site of metallothionein-fusion gene expression [172,173], contain both PC1 and PC2 by immunohistochemical analysis [179]. Thus, either processing of proEx-4 does not occur by these PCs *in vivo* in the pituitary or, alternatively, their products are secreted and not detected by analysis of the tissue extracts.

Similarly, in the fat, only a minor peak in the elution position of synthetic Ex-4 was detected, with a much larger peak of putative proEx-4 (Fig. 21). Fat may be considered an endocrine tissue because its secretion of the endocrine hormone leptin. However, on the basis of its secretory pathway, fat more closely resembles a non-endocrine tissue, releasing leptin in a constitutive manner. Although fat has not been assessed for PC localization, it would likely contain the ubiquitious convertase furin [167] and perhaps the widely expressed PACE4, PC5/6 and/or PC7/8/LPC.

In contrast to both the pituitary and fat, the testes contained a modest peak of peptide in the elution position of synthetic Ex-4, as well as a peak of putative proEx-4 (Fig. 22). Testes are unique in that they are the only site of PC4 localization [62], and it is perhaps this cell-specific PC which allowed for the synthesis of Ex-4 *in vivo*, which was not seen in tissues with PC1 and PC2. It is noted, however, that PC5 and furin are also seen in the testes by northern blot [167]. Additionally, the testes were observed to contain a smaller peak/shoulder of ExLI with intermediate hydrophobicity, reminiscent of what was seen in the InR1-G9 cell line.

Finally, although the adrenal glands contain PC5 as well as PC1 and PC2 by northern blotting [167], this tissue was found to express only background levels of both Ex-4 and putative proEx-4, making any conclusions about processing in this tissue premature (Fig. 23).

As none of the tissues studied contained a profile of Ex-4-like peptides that mimic that seen in the plasma, it must be concluded that other tissues are responsible for the circulating Ex-4, likely via constitutive secretion. Internally consistent lines of evidence support this hypothesis. First, the BHK cells containing a constitutive secretory pathway were shown to produce and release peptide with the same elution position as synthetic Ex-4; 95% of this ExLI was detected in the media of this cell culture model consistent with unregulated secretion. Furthermore, the most consistent site of expression of genes linked to the MT promoter is the liver [160], a site shown to contain negligible ExLI in the present study. Expression of the transgene in the liver and other tissues without detection of ExLI is consistent with processing and release *via* the constitutive secretory pathway. In fact, although the pituitary was the site with the highest concentrations of ExLI, it has

been suggested by others that the liver, kidney and spleen may account for most of the circulating immunoreactive peptide in MT-reporter transgenic mice because of their large mass as compared to that of the pituitary [160]. Indeed, these tissues represent a processing profile consistent with that seen in the plasma in such mice [160].

In conclusion, the results of these studies have demonstrated that both a non-endocrine cell line containing furin, as well as endocrine cell lines containing either high levels of PC1 or PC2, process proEx-4 to peptide with the elution position of synthetic Ex-4, albeit to varying degrees. As well transgenic mice expressing the proEx-4 cDNA under the control of a MT promoter process proEx-4 to bioactive Ex-4 in both endocrine (testes) and non-endocrine (fat) tissues, and Ex-4 in the circulation of these mice likely contribute to their phenotype of lower glycemia. Thus, lizard proEx-4 can be expressed and processed to Ex-4 in a variety of different mammalian cells.

4.3 Future Studies and Significance

Although it is tempting to make a conclusion about the contribution of specific PCs to proEx-4 processing, this is premature, as all cell lines contain furin [167] and other PCs may also be present in the models utilized. Similarly, it may also be suggested that, in the transgenic mouse, the constitutive pathway is involved in Ex-4 biosynthesis. However, it would also be premature to conclude that furin is responsible, as PACE4, PC5/6 and PC7/8/LPC can also cleave furin substrates [119] and are expressed in a broad range of tissues. To specifically demonstrate which PC is able to process proEx-4 to Ex-4, *in vitro* test tube cleavage assays could be carried out with recombinant PCs. Alternatively, antisense deletion of specific PCs could be carried out in the cell lines under

consideration.

One limitation of the present study was that the tissue(s) responsible for secretion of Ex-4 into the plasma was not established. Current studies determining the Ex-4 mRNA expression sites (Ms. L. Baggio) may give insight into which tissues may synthesize the Ex-4 peptide. Arterial-venous assessment of ExLI from these tissues would then allow for the assessment of their contribution of Ex-4 to the plasma through constitutive peptide secretion. Additionally, primary cultures of selected tissues may prove to be valuable models for future study of Ex-4 synthesis and secretion.

When taken together, the results of the present study have made a number of novel contributions to the literature. First, insights into the metallothionein fusion gene expression system have been made. Basal expression of this promoter, shown to be significant, has not previously been reported. Furthermore, sex differences and the expression of the promoter in fat have not been described.

Finally, given that Ex-4 is a potential treatment for type II diabetes, the results of the present studies have implications for the application of gene therapy to cell-based delivery of Ex-4. It can be concluded that both endocrine and non-endocrine cell lines can be bioengineered to produce bioactive Ex-4.

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