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Studies on teleost corpuscles of Stannius: Physiological and biochemical aspects of synthesis and release of hypocalcin in trout, goldfish and eel

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Abstract

Hypercalcemia (induced by $CaCl_2$-injection or seawater exposure of the fish) reduced the hypocalcin content of corpuscles of Stannius (CS) in trout, goldfish and eel; concomitantly the synthetic activity of CS of hypercalcemic fish, as determined *in vitro*, was enhanced. The monomeric forms of prohypocalcin and of hypocalcin of trout and goldfish are 32 and 28 kDA M_r glycoprotein species respectively; those of the eel are 2 kDa bigger, *viz*. 34 and 30 kDa respectively. Moreover, eel CS produce *in vitro* an enigmatic 70 kDa glycoprotein with affinity for concanavalin-A. It is concluded that plasma calcium levels control storage and synthesis rates of hypocalcin in the CS.

Introduction

The corpuscles of Stannius (CS) produce what is probably the major hypocalcemic calcitropic hormone in fish (Fenwick 1982; Wendelaar Bonga and Pang 1986). The hormone, variously referred to as hypocalcin or teleocalcin, is a glycoprotein (Wagner *et al*. 1986; Butkus *et al*. 1987; Lafeber *et al*. 1988). Only recently, reports have appeared in the literature on the isolation and N-terminal amino acid sequences of hypocalcins from sockeye salmon, *Oncorhynchus nerka* (Wagner *et al*. 1986), and rainbow trout, *Salmo gairdneri* (Lafeber *et al*. 1988). For the Australian eel, *Anguilla australis*, the presumptive total amino acid sequence of the protein core of the hypocalcin molecule was elucidated by cDNA techniques (Butkus *et al*. 1987). A considerable homology was observed among N-terminal amino acid sequences of the isolated salmon, eel and trout hypocalcins and this explains the cross-reactivity of hypocalcin samples in heterologous bioassays (Pang *et al*. 1974; Wendelaar Bonga *et al*. 1986; Lafeber *et al*. 1988). Conversely, significant variation exists in the molecular size of the hypocalcin molecules isolated from different species with reported molecular radii ranging from 39 kDa in salmon (Wagner *et al*. 1986) to approximately 54 kDa in rainbow trout (Lafeber *et al*. 1988). Both Wagner and colleagues and Lafeber and colleagues concluded from their studies that the native hypocalcin molecule is likely to be a homodimer. More recently, evidence has been given from studies on *in vitro* synthesis of hypocalcin by trout CS that hypocalcin is a 56 kDa dimeric glycoprotein, which is processed from a 64 kDa dimeric prohormone. These dimers are highly susceptible to reducing agents such as dithiothreitol and 2-mercapto-ethanol (the use of these agents is

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a requirement in many biochemical analyses) and appear as 28 and 32 kDa monomers in the presence of these agents (Flik, unpublished data).

The studies presented here focus on the effects of experimentally induced variations in plasma calcium concentrations on the storage and rate of synthesis of hypocalcin by CS of rainbow trout, *Salmo gairdneri*, goldfish, *Carassius auratus*, and North American eel, *Anguilla rostrata* LeSueur. Further studies on molecular characteristics of the respective hypocalcins are also reported.

Materials and methods

Animals

Rainbow trout, *Salmo gairdneri*, of both sexes ranging in body weight from 200 to 700 g, were kept indoors under a photoperiod of 16h light alternating with 8h darkness in 1000 l tanks supplied with well-aerated, dechlorinated and filtered City of Edmonton tapwater or in artificially prepared seawater (Wimex sea salt) for at least 6 weeks. The water temperature was controlled at 10 ± 1°C, the water pH was 7.4 and the Ca content of the freshwater was 0.85 ± 0.12 $mmol.l^{-1}$ and of the seawater 10 ± 0.5 $mmol.l^{-1}$. The trout were fed daily with Purina trout pellets. Goldfish, *Carassius auratus*, weighing around 50 g, were kept in 100 l aquaria supplied with a continuous flow of City of Edmonton tapwater at 22°C and fed daily with Tetramin tropical fish food. Eels, *Anguilla rostrata* Le Sueur, weighing around 100 g were housed in 500 l basins supplied with tapwater of 18°C. The eels were not fed.

Trishydroxymethyl aminomethane (Tris)-buffered (pH 7.4) 3-aminobenzoic acid ethyl ester (MS 222, Sigma; 0.5 $g.l^{-1}$) was used as an anesthetic medium. The fish were killed by transection of the spinal cord.

Calcium injection

Fish were injected intraperitoneally with 0.34 $mol.l^{-1}$ $CaCl_2$ solution (100 μl/100g fish per day) for three consecutive days. Injections of NaCl solution of identical osmolarity served as controls. Four hours after the last injection, a blood sample was taken by puncture of the vessels of the caudal peduncle using a heparinized syringe with a 21-G needle. Cells were separated from plasma by centrifugation (15 s, 9000 × g) and the plasma stored at −20°C until further analysis. The CS were removed and prepared for incubation (see below).

Analytical procedures

Plasma total calcium was determined with a commercial calcium kit (Sigma); combined calcium/phosphate standards were used as a reference. Protein was estimated with a commercial reagent kit (Biorad) using bovine serum albumin (BSA, Biorad) as reference.

Labeling incubations

Immediately after killing the fish the CS were removed and placed in Hanks' balanced salt solution (HBSS; Sigma, H13387). Adhering renal tissue was carefully removed using a binocular microscope, and the connective tissue capsule of the CS incised. CS tissue was weighed and pre-incubated for 60 min in 500 μl HBSS at 22°C. The tissue was then transferred to 100 μl HBSS containing 1.85 to 3.70 MBq of the radiolabeled [^{35}S]-cysteine (specific activity 4.58 GBq.mmol; New England Nuclear). At the end of the incubation the tissue was carefully rinsed in HBSS (three times 500 μl). The tissue was homogenized in 1 ml of 50 mM acetic acid (HAc) in a tight all-glass homogenizer and the homogenate was centrifuged in an Eppendorf minifuge at 9000 × g for 10 min. An aliquot of the supernatant (hereafter called 'extract') was used to determine protein content and the remainder lyophilized and stored at −80°C until further analysis.

Immunoprecipitation and concanavaline-A absorption

Indirect immunoprecipitation of hypocalcin anti-

gen was carried out by the procedure of Anderson and Blobel (1983) using a well-characterized antiserum for trout hypocalcin (RADH I; Kaneko *et al.* 1988) and Protein A Sepharose CL-4B (Pharmacia) as a solid-phase immunoadsorbent. Lyophilized CS extract was reconstituted with distilled water and sodium dodecyl sulphate (SDS) was used as detergent in the immunoprecipitation procedure.

Glycoprotein isolation was carried out using Concanavaline-A sepharose (Pharmacia) as a solid-phase adsorbent. Reconstituted CS extract was mixed overnight with 10 volumes Con-A sepharose in a 'Con-A buffer' (50% vol/vol) consisting of 15 mM Tris-HCl (pH 7.4), 1 $mmol.l^{-1}$ each $MnCl_2$, $MgCl_2$ and $CaCl_2$, and 1 $mol.l^{-1}$ NaCl (Roelfzema and Van Erp 1983). Products not bound to Con-A were removed with 5 washes of Con-A buffer and precipitating the Con-A sepharose by short centrifugation (15 s, 9000 × g). The material bound to Con-A was dissociated from the lectin by Con-A buffer made 0.3 $mol.l^{-1}$ in α-methyl-D-glucoside and precipitated with trichloroacetic acid (10% wt/vol, final concentration).

Separation techniques

To estimate the relative molecular weight of the CS products, samples were separated by SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) using a Biorad Protean II or Mini Protean II Slab Cell, following the protocol of Laemmli (1970). The markers used for molecular weight were prestained SDS-PAGE standards (Biorad; lysozyme, 17 kDA, soybean trypsin inhibitor, 27 kDa, carbonic anhydrase, 39 kDa, ovalbumin, 50 kDa, bovine serum albumin, 75 kDa, and phosphorylase B, 130 kDa) that were mixed with [^{14}C]-methylated protein markers (Amersham plc., CFA.626; lysozyme, 14.3 kDa, carbonic anhydrase, 30 kDa, ovalbumin, 46 kDa, bovine serum albumin, 69 kDa, phosphorylase B, 92.5 kDa, and myosin, 200 kDa).

Gels were fixed in 40% (vol/vol) methanol:10% (vol/vol) acetic acid (HAc) in water (1h), 5% (vol/vol) methanol:7% (vol/vol) HAc in water (1h) and 10% (vol/vol) glutaraldehyde (BDH) in water (30 min) and rinsed for at least two hours in flowing demineralized water. Staining was performed in the first fixative, which contained 2 $g.l^{-1}$ Coomassie Blue R-250 (Biorad). The gels were destained in the same aqueous solution of methanol and HAc. For autoradiography the fixed gels were impregnated with PVO/POPOP according to the method of Bonner and Laskey (1974). Before drying (Biorad slab dryer), the gels were dehydrated for 8 to 12h in 50% (vol/vol) methanol in water containing 3% (vol/vol) glycerol. Preflashed Kodak XAR-5 X-ray film was used for autoradiography; exposure time was 2 to 72h at −80°C. In some cases gels were sliced (2 mm) and the radioactivity in the gel determined by liquid scintillation counting. To this end, 0.5 ml H_2O_2 was added to the gel slice, followed by incubation for 12h at 60°C. Next 4 ml of Scintiverse (Fisher) was added and the radioactivity determined in a LKB Rackbeta LSC equipped with a dpm program. Stained gels and autoradiographs were scanned densitometrically with a LKB 2202 Ultroscan Laser Densitometer equipped with a LKB 2220 Recording Integrator.

Enzyme-linked immunosorbent assay (ELISA)

To determine total hypocalcin antigenic activity in CS extracts an ELISA was used that has been described in detail (Kaneko *et al.* 1988). Microtitration plates were coated with 200 μl of serial dilutions of purified trout hypocalcin or tissue extract. Alkaline phosphatase conjugated IgG was the second antibody; alkaline phosphatase activity was determined on the basis of para-nitrophenyl phosphate hydrolysis, determined spectrophotometrically as the change in A_{405}.

Statistics

Significance of differences ($p < 0.025$) between treatment groups was assessed by the Mann-Whitney U-test.

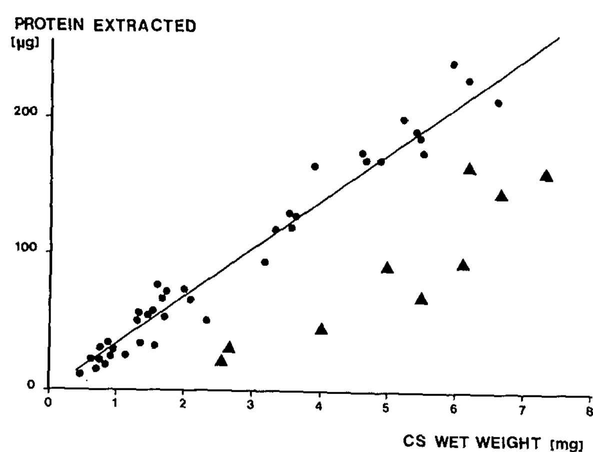

Fig. 1. Protein extracted from trout corpuscles of Stannius (CS) as a function of the wet weight of the CS. $CaCl_2$-treatment (values represented by triangles) resulted in significantly lower (Mann-Whitney-test, $p < 0.001$) protein content of the CS compared with fresh water controls (dots). The regression is defined as: extracted protein = 36.14 × (wet weight CS, in mg) − 1.94 µg ($p < 0.001$).

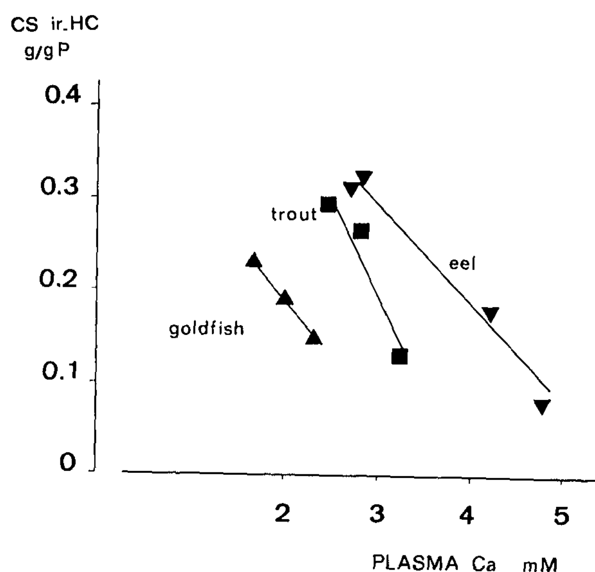

Fig. 2. Corpuscle of Stannius (CS) immunoreactive hypocalcin (ir-HC, determined by ELISA) content and plasma calcium levels in freshwater rainbow trout, goldfish and eel. The ir-HC content of CS extracts is negatively correlated with the plasma calcium level of the fish. Hypercalcemia was induced by $CaCl_2$-injections (see Materials and Methods section).

Results

As shown in Fig. 1, significantly less protein was extracted from CS of trout injected with $CaCl_2$. Mac-

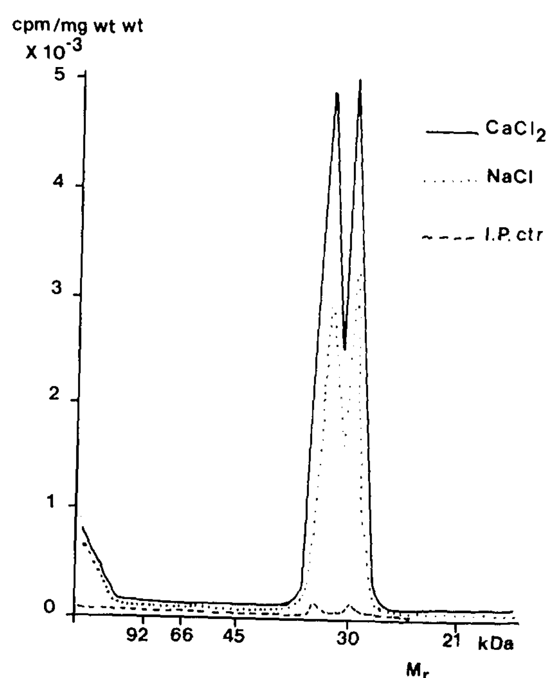

Fig. 3. SDS-PAGE analysis (gels were sliced in 2 mm samples (of immunoprecipitated products synthesized by CS of $CaCl_2$-injected (solid line) and NaCl-injected (dotted line) trout, formed during a 4h incubation with [^{35}S]-Cys in HBSS. The interrupted line represents the radioactivity recovered from the supernatant by TCA-precipitation after immunoprecipitation. Notice the increased labeling brought about by $CaCl_2$ injections. For comparison the data have been normalized to the wet weight of the CS samples. Positions of marker proteins are indicated on the X-axis.

roscopically, the glands of $CaCl_2$-injected fish appeared blue and translucent compared to the opaque white of the glands in the controls.

The CS immunoreactive hypocalcin content determined by ELISA is inversely related to the plasma calcium concentration in goldfish, eel and trout (Fig. 2).

After a 4h labeling of CS from NaCl-injected control fish or $CaCl_2$-injected fish with [^{35}S]-cysteine, both 32 and 28 kDa, RADH-I-immunoprecipitable M_r species are formed. $CaCl_2$-treatment of the fish more than doubled (207 ± 21%, n = 6, $p < 0.001$) the amount of immunoprecipitable products formed during such an incubation (comparable amounts of CS wet weight of $CaCl_2$-injected and NaCl-injected fish were incubated under identical conditions). Analysis of the supernatant remaining after immuno-precipitation indicated

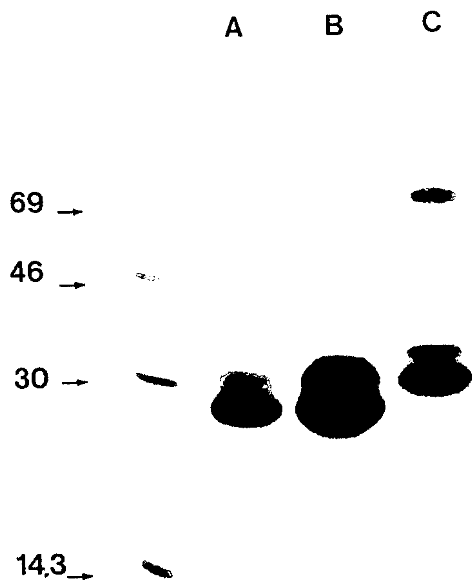

Fig. 4. SDS-PAGE analysis (autoradiograph) of concanavaline-A adsorbed CS products formed after labeling of 4h with [^{35}S]-Cys in HBSS. Lane A goldfish CS, lane B trout CS, lane C eel CS. M_r values of marker proteins are indicated on the left.

that virtually all newly-synthetized products were precipitated (IP ctr; Fig. 3).

Fig. 4 shows the concanavalin-A adsorbable [^{35}S]-cysteine labeled products formed during a 4h labeling of CS of trout, goldfish and eel. For reasons of comparison, similar amounts of wet weight tissue were incubated under identical conditions. After extraction of the products from the CS, equivalent amounts of protein were subjected to concanavalin-A adsorption. For trout and goldfish 32 and 28 kDa M_r species were recovered. The eel yielded 70, 34 and 30 kDa M_r species.

In Fig. 5, CS synthetic activities of freshwater and of seawater trout are compared. Plasma calcium levels in the seawater trout were significantly elevated compared with freshwater trout (2.67 ± 0.12 and 3.05 ± 0.08 for freshwater and seawater trout, respectively; $n = 6$, $p < 0.01$). In both groups 32 and 28 kDa M_r species are the conspicuous products labeled by [^{35}S]-cysteine during a 4h incubation. Macroscopically, the CS of seawater trout appeared translucent and resembled those of $CaCl_2$-injected trout. The synthetic activity of seawater trout CS exceeded that of freshwater trout by a factor 2.1 ± 0.2 ($n = 5$, $p < 0.001$; comparison of the total radioactivity in the 32 and 28 kDa peaks). Remarkable differences in peak height were consistently observed with the 32 kDa peak of seawater trout CS surpassing that of freshwater trout CS by 34% and the 28 kDa peak of seawater trout surpassing that of freshwater trout CS by 60%. Together these observations indicate enhanced processing of hypocalcin in hypercalcemic trout (comparison with freshwater trout).

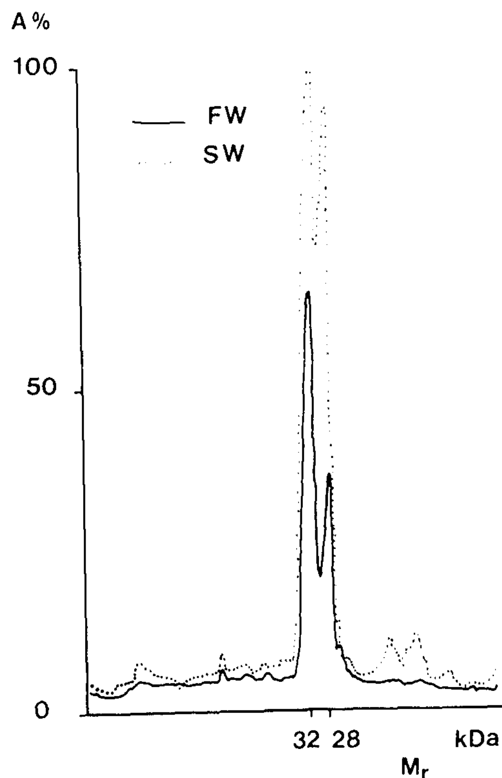

Fig. 5. SDS-PAGE analysis (scan of autoradiograph) of CS products labeled during 4h incubation with [^{35}S]-Cys in HBSS. CS of freshwater fish (solid line) were compared with CS of seawater fish (dotted line). Y-axis: relative absorbance (A%); the height of the 32 kDa peak was designated 100% A. M_r values of the major products formed are indicated on the X-axis.

Discussion

From the data presented in this paper it is concluded that stored hypocalcin is reduced in response to experimentally induced, mild hypercalcemia in freshwater trout, goldfish and eel. CS taken from hypercalcemic fish showed increased synthetic activity *in vitro*, as the amounts of newly-synthetized, immu-

noprecipitable hypocalcin produced during 4h labeling doubled compared with control freshwater fish. Trout and goldfish prohypocalcin and hypocalcin have comparable molecular radii (32 and 28 kDa, respectively, for the reduced, monomeric form of the molecules). The molecules of eel differ from those of trout and goldfish, having a 34 and 30 kDa molecular radius respectively. Also eel CS produce significant amounts of a 70 kDa M_r glycoprotein species with affinity for concanavalin-A. Experiments with seawater trout CS provide evidence that an elevation of plasma calcium levels induced by exposure to high calcium water also stimulates the rate of hypocalcin synthesis of the CS.

The inverse relationship between the hypocalcin content of the CS and plasma calcium levels is in perfect agreement with the presumptive role of the CS in hypocalcemic control. The different macroscopical appearance of activated CS (blue and 'translucent' rather than white and opaque), is concordant with our observation of significantly decreased amounts of extractable protein in the CS of $CaCl_2$-treated fish. This latter observation also corroborates (electron) microscope studies on the glands by Lopez *et al.* (1984) and by Lafeber and Perry (1988), who showed significant degranulation of the glands of eel and trout after experimental hypercalcemia. Thus we may conclude that degranulation of the gland is reflected by the decreased content of extractable protein. In a recent report we have shown that the CS of freshwater fish normally store an abundance of RADH-I-immunoreactive hypocalcin (Wendelaar Bonga *et al.* 1988). Already in 1980, Aida and colleagues reported that coho salmon CS degranulate in response to high calcium levels *in vitro* and develop more extensive rough endoplasmic reticulum and Golgi systems; degranulation was a result of increased exocytosis. From these results it was tentatively concluded that plasma calcium levels may control CS synthetic and secretory activities. Our present observations corroborate the conclusion that the CS are under direct control by plasma calcium levels, as mild hypercalcemia such as induced by a $CaCl_2$-injection and by exposure of the fish to seawater diminished storage and enhanced hypocalcin synthesis rates of the CS. It is our experience that care should be taken with the dose of $CaCl_2$ used to induce hypercalcemia. Doubling the dose used in this study (*i.e.* injections of the same volume but 0.68 M instead of 0.34 M $CaCl_2$) inhibits CS synthetic activity (Flik, unpublished data).

A 2 kDa difference is found between the M_r of eel prohypocalcin and hypocalcin molecules and the corresponding molecules of the trout and goldfish. We tentatively conclude that this difference derives from differences in the protein core of the glycoproteins. Butkus *et al.* (1987) and Flik (unpublished data) have shown for eel and trout, respectively, that the glyco-moiety of the hypocalcins of these species comprises 4 kDa of the native monomeric molecules. Since the N-terminal amino acid sequences of eel and trout hypocalcin are highly conserved (see Lafeber *et al.* 1988), this extra 2 kDa protein moiety in the eel hypocalcin must be situated in the C-terminal region of the molecule. These observations seem to justify future research on total sequences of fish hypocalcins.

At first sight, one might conclude that the 70 kDa M_r species produced by the eel CS is a secretory protein. Secretory proteins (*e.g.* chromogranin A and SP-I), which are considered important markers for neuroectodermal cells, have been localized immunocytochemically in eel CS hypocalcin granules (Tisserand-Jochem *et al.* 1987). Then, in addition to the direct control of the CS by plasma calcium levels, a characteristic shared by the CS and calcitropic glands of the higher vertebrates, the CS might also have the same embryological origin. Secretory proteins of higher vertebrates have molecular radii of around 70 kDa (Wohlfarter *et al.* 1988), and are all glycoproteins with O-linked sugar residues (Majzoub *et al.* 1982). But such glycoproteins lack affinity for the lectin concanavalin-A. We conclude therefore, that the 70 kDa glycoprotein of the eel CS, which was collected by concanavalin-A adsorption, is not a secretory protein but we do not exclude the possible presence of a secretory protein in (eel) CS, as antisera, albeit polyclonals, to chromogranin-A and SP-I do indeed crossreact with eel and trout hypocalcin containing granules (unpublished observations).

We conclude that hypocalcin produced by teleost CS is a unique hypocalcaemic hormone. Newly-

synthesized hypocalcin is a dimeric glycoprotein, that may show interspecies variation in the size of the protein core of the molecule. During processing of the native dimeric prohormone, an 8 kDa protein-moiety is cleaved off to form a 56 kDa (trout and goldfish) or 60 kDa (eel) hypocalcin.

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