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Isolation and Characterization of Somatolactin, a New Protein Related to Growth Hormone and Prolactin from Atlantic Cod (*Gadus morhua*) Pituitary Glands†

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ABSTRACT: The characterization of cod somatolactin (SL), a new pituitary protein belonging to the growth hormone/prolactin family, is described. Cod SL has a molecular weight of 26 kDa and consists of 209 amino acids, of which eight are Cys. The protein has three disulfide bonds between residues Cys^{5}–Cys^{15}, Cys^{65}–Cys^{181}, and Cys^{198}–Cys^{206}. The Cys residues at positions 42 and 180 are not involved in disulfide bonding. The positions of these disulfide bonds are homologous to those found in prolactin and growth hormone. Cod SL has two possible N-glycosylation sites, but only one appears to have carbohydrate units attached. Chemical analysis showed the following sugars to be present: galactose, mannose, *N*-acetylneuramic acid, and glucosamine. A smaller variant (23 kDa) of SL has been isolated, which is believed to be deglycosylated. Sequence comparison revealed cod SL to be similarly related to both GH and PRL, but slightly higher identity was observed to the tetrapod hormones (27–33%) than to the teleost hormones (21–27%).

The pituitary hormones growth hormone (GH)[1] and prolactin (PRL) and placental lactogen (PL) of placental origin are structurally related and grouped together in the GH/PRL family. This family has recently been extended due to the discovery of placental proteins such as mouse proliferin (Linzer & Nathans, 1984), bovine PRL related cDNA I (Schuler & Hurley, 1987), and rat prolactin like protein A (Deb et al., 1989). These placental proteins, with the exception of human PL, have been found to be structurally more similar to PRL (30–40%) than to GH (15–21%), indicating that they may have evolved from a PRL gene (Duckworth et al., 1988). Human PL on the other hand, shows 85% identity with human GH (Miller & Eberhardt, 1983). A recent study has also isolated GH-related (rather than PRL-related) proteins from rat placenta (Ogilvie et al., 1990).

Recently, we discovered somatolactin (SL), a novel protein in pituitaries from Atlantic cod and flounder (Ono et al., 1990; Rand-Weaver et al., 1991). Analysis of the flounder cDNA sequence revealed it to be distant and similarly related to GH and PRL and thus the first new hypophyseal member of the GH/PRL family (Ono et al., 1990). By use of an antiserum raised against cod SL, the protein was shown to be produced in the pars intermedia of all teleosts tested (Rand-Weaver et al., 1991). Specifically, SL immunostaining was observed in the PAS-positive cells of cod, flounder, molly, killifish, and catfish and the chromophobic cells of rainbow trout. The PAS-positive cells have been considered to produce a factor involved in such diverse functions as calcium regulation (Ball et al., 1982; Olivereau et al., 1985), acid–base metabolism (Wendelaar-Bonga et al., 1986), adaptation to background (Ball & Batten, 1981), and reproduction [quoted in Ball and Baker (1969)].

We report here a simplified isolation procedure of cod SL and its characterization and primary structure determination. The relationship of cod SL with other members of the GH/PRL superfamily is discussed.

Materials and Methods

Isolation. Pituitary glands were taken from cod (*Gadus morhua*) caught in the Norwegian Sea (Lofoten), Norway, during February. They were frozen immediately on dry ice and stored at −80 °C until used. The glands were extracted with 0.05 M ammonium acetate, pH 9.0, for 1 h at 4 °C and chromatographed on Sephadex G-75 (1.9 × 100 cm) equilibrated and eluted in extraction buffer. Final purification of SL was achieved on a TSK gel ODS-120T reverse-phase (rp) HPLC column (0.46 × 25 cm, particle size 5 μm). Elution was performed by using a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 mL/min, and absorbance was monitored at 220 nm.

Electrophoresis. The molecular weight was estimated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) performed before and after reduction with

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[1] Abbreviations: GH, growth hormone; PRL, prolactin; PL, placental lactogen; SL, somatolactin; PAS-positive cells, periodic acid–Schiff positive cells; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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2-mercaptoethanol. The isoelectric points of the 23- and 26-kDa SLs were determined by two-dimensional electrophoresis (Pollard, 1986) using Ampholines of pH 4–6 and 5–8 (mixed in a 1:1 ratio) in the first dimension, followed by SDS–PAGE in the second dimension. Proteins were visualized following staining with 0.2% Coomassie Blue R-250 and removal of excess stain with methanol–acetic acid–water (1:1:17.5).

Deglycosylation. Cod SL (23- and 26-kDa variants) (0.03 mg) was deglycosylated by using Endoglycosidase F (Boehringer-Mannheim) (0.06 unit) for 18 h at 37 °C in 0.25 M sodium phosphate buffer, pH 8.6, containing 20 mM EDTA. The proteins were subjected to SDS–PAGE as described above.

Carbohydrate Analysis. The 26-kDa SL was analyzed for the presence of various carbohydrates. Neutral sugars were analyzed by the DABS–hydrazide method as described by Muramoto et al. (1987a). The presence of neuraminic acid was analyzed by the method of Hara et al. (1987) using a TSK gel ODS-120T column (0.46 × 25 cm, particle size 5 μm). The content of amino sugars was determined following labeling with dansylamino-PITC (Hirano & Wittmann-Liebold, 1986). Protein (0.038 mg) was hydrolyzed in 4 M HCl (0.08 mL) for 4 h at 100 °C under vacuum. The hydrolysate was dried and dissolved in 0.01 mL of distilled water and pyridine (0.01 mL) was added. This was then reacted with 0.01 mL of dansylamino-PITC solution (5 μmol/mL) for 40 min at 50 °C. Derivatized sugars were identified following separation by rpHPLC on a TSK gel ODS-120T column (0.46 × 25 cm, particle size 5 μm) eluted with 25% acetonitrile in 0.08 M acetic acid at a flow rate of 1 mL/min. Elution was monitored by fluorescence with excitation at 340 nm and emission at 540 nm.

Reduction and S-Carboxymethylation. Intact protein was reduced and S-carboxymethylated or directly S-carboxymethylated as described earlier for cod GH (Rand-Weaver et al., 1990).

Enzymatic Cleavage. Intact protein was digested with lysyl endopeptidase (E/S = 1/60 by weight) for 6 h at 37 °C in 0.05 M ammonium bicarbonate pH 8. Fragment LET15 was further reduced and S-carboxymethylated. Reduced and S-carboxymethylated SL was digested with *Staphylococcus* V8 protease (Sigma) (E/S = 1/30) and Endoproteinase Asp-N (Boehringer-Mannheim) (E/S = 1/200) for 18 h at 37 °C in 0.05 M ammonium bicarbonate, pH 8, and with Clostripain (E/S = 1/50) in 0.1 M sodium hydrogen phosphate buffer, pH 7.8, containing 2 mM EDTA for 2 h at 25 °C. S-Carboxymethylated SL was digested with trypsin (Sigma) (E/S = 1/60) for 6 h at 37 °C in 0.2 M ammonium acetate, pH 8.2.

Fractionation of Peptide Fragments. Following cleavage of cod SL with lysyl endopeptidase, V8 protease, or Clostripain, peptide fragments were fractionated by rpHPLC on a TSK gel TMS-250 column (0.46 × 25 cm, particle size 10 μm). Elution was carried out by using a linear gradient of 20–60% 2-propanol in 0.1% TFA at a flow rate of 0.5 mL/min. The unadsorbed fraction was further fractionated on a TSK gel ODS-120T column (0.46 × 25 cm, particle size 5 μm) by using a linear gradient of 5–40% 2-propanol in 0.1% TFA. The Asp-N and trypsin fragments were separated on a Wakosil 5C18-200 column by using a gradient of 5–60% 2-propanol. Elution was monitored at 210 nm. Amino acid analysis of fragments was performed as described previously (Rand-Weaver et al., 1990).

Sequence Analysis. N-terminal residues were determined by the Dansyl method (Gray, 1967). Sequence analysis was performed by automatic sequencing on a Shimadzu PSQ1 gas-phase protein sequencer. Phenylthiohydantoin amino acids were identified by Shimadzu LC System PTH-1 on a Wakopak WS-PTH column (0.46 × 25 cm, particle size 5 μm) at 40 °C with PTH amino acid mobile phase solvent (Wako Pure Chemicals). The data were analyzed by a Chromatopak C-R4A data processor (Shimadzu).

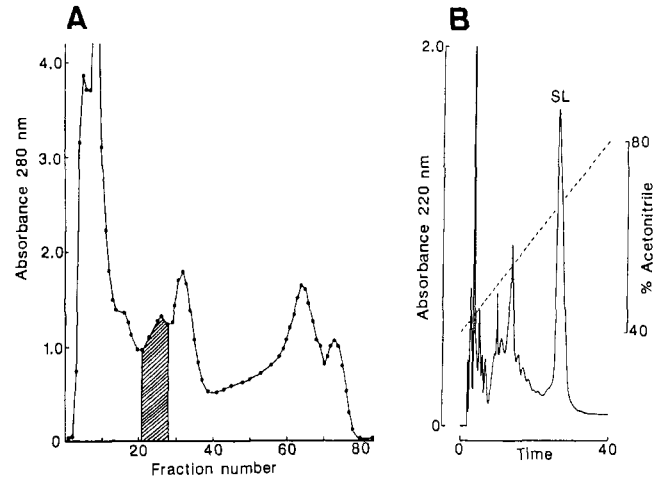

FIGURE 1: Purification of cod somatolactin (SL). (A) Pituitary gland alkaline extract was chromatographed on a Sephadex G-75 column (1.9 × 100 cm). The column was eluted with 0.05 M ammonium acetate, pH 9.0, at a flow rate of 15 mL/h and 3-mL fractions were collected. Absorbance was measured at 280 nm. (B) The hatched area of the elution profile in (A) was further applied to a reversed-phase HPLC column. A linear gradient of 40–80% acetonitrile in 0.1% TFA over 40 min was used to purify SL. The flow rate was 1 mL/min and absorbance was monitored at 220 nm.

Hydrophobicity Analysis. The hydrophobicity index of cod SL was analyzed by the method of Kyte and Doolittle (1982) using a window of six amino acids.

RESULTS

Isolation. Cod SL was extracted from pituitary glands under alkaline conditions as described previously for cod GH (Rand-Weaver et al., 1989). Initially, the extract was fractionated on Sephadex G-100 and the 20–30-kDa pool was separated on rpHPLC (Rand-Weaver et al., 1991). However, this necessitated many rpHPLC runs in order to obtain a large amount of SL, and it was therefore advantageous to apply the extract to a Sephadex G-75 column (Figure 1A). This effectively separated GH and SL so that a greater yield of SL was obtained per rpHPLC run (Figure 1B). By use of this modified procedure a yield of 1 mg of SL/g of pituitary wet weight was obtained.

Characterization. The HPLC-purified SL fraction was found to contain two proteins by SDS–gel electrophoresis. The major protein had a molecular weight of 26 kDa, whereas the minor component migrated at 23 kDa (Figure 2A). Using a shallow acetonitrile gradient for HPLC separation, we were able to obtain a small amount of the 23-kDa protein so that its relationship to the 26-kDa protein could be determined (Figure 2B). The amino acid compositions of the two SL variants were virtually identical (Table I), and both proteins had identical 32 N-terminal residues. The relative isoelectric points of the 26- and 23-kDa SLs were determined by two-dimensional electrophoresis. The 26-kDa protein separated into four major spots with pIs between 5.7 and 5.9, whereas only one major spot at pI 6.0 could be seen for the 23-kDa variant (Figure 2C).

Enzymatic deglycosylation of the SLs resulted in reduction of the molecular weight of the 26-kDa protein to 23 kDa,

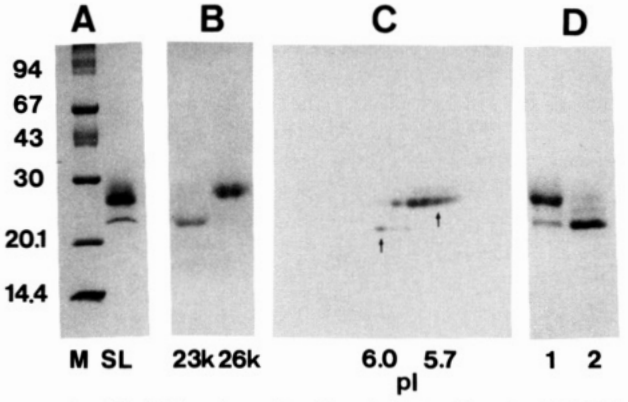

FIGURE 2: (A) SDS–polyacrylamide gel electrophoresis of HPLC-purified cod somatolactin (SL). Two bands were observed, the major and minor forms having apparent molecular weights of 26 and 23 kDa, respectively. Sample amount was 5 μg. M, standard molecular weight markers (kDa). (B) SDS–polyacrylamide gel electrophoresis of purified 23- and 26-kDa SLs. The two variants could be separated by HPLC using a shallow gradient of 60–65% acetonitrile in 0.1% TFA over 20 min. Sample amount was 3 μg. (C) Two-dimensional electrophoresis of cod SL. Cod SL (10 μg) was separated by isoelectric focusing in the first dimension, followed by SDS–polyacrylamide gel electrophoresis. Four spots with isoelectric points (pI) between 5.7 and 5.9 were observed for the 26-kDa SL, whereas only one main spot at pI 6.0 was detected for the 23-kDa variant. (D) Effect of enzymatic deglycosylation on the apparent molecular weight of cod SL. Cod SL (30 μg) was incubated with Endoglycosidase F (0.06 unit) for 18 h at 37 °C in 0.25 M sodium phosphate buffer, pH 8.6, containing 20 mM EDTA. A control sample was incubated under the same conditions but without enzyme. Samples (5 μg) were subjected to SDS–polyacrylamide gel electrophoresis. Lane 1, control; lane 2, deglycosylated SL.

Table I: Amino Acid Compositions of 23- and 26-kDa Somatolactin

amino acid	23 kDa	26 kDa
1/2 Cys[a]	3.0[b]	3.0 (8)[c]
Asp/Asn	11.6	11.5 (21)
Glu/Gln	15.9	15.6 (29)
Ser	7.2	6.4 (15)
His	1.4	1.4 (3)
Gly	4.2	3.0 (4)
Arg	4.3	4.2 (9)
Thr	4.4	4.3 (10)
Ala	3.4	3.3 (7)
Pro	4.4	4.8 (9)
Tyr	4.0	4.1 (8)
Val	8.0	8.6 (18)
Met	2.1	2.1 (5)
Ile	6.5	6.9 (14)
Leu	12.7	13.5 (27)
Phe	2.5	2.5 (5)
Trp[d]	1.3	1.1 (3)
Lys	6.1	6.5 (14)
total	100.1	99.8 (209)

[a] Determined as cystic acid. [b] Values indicate the number of residues/100. [c] Residues determined by sequence analysis. [d] Determined by the method of Muramoto et al. (1987b).

whereas there was no shift in the apparent molecular weight of the 23-kDa protein (Figure 2D). It is therefore likely that the 23-kDa protein represents a deglycosylated variant of SL. The carbohydrate composition of the 26-kDa SL was determined, and the protein was found to contain *N*-acetylneuraminic acid (1.5%), galactose (1.6%), mannose (1.8%), and glucosamine (0.7%), a total of 5.6% carbohydrate by weight.

Sequence Analysis. As characterization of the 23- and 26-kDa proteins indicated that they might differ only in carbohydrate content and the 23-kDa species was only a minor contaminant in the SL preparation, we used the SL preparation (23 and 26 kDa) without further purification for amino acid

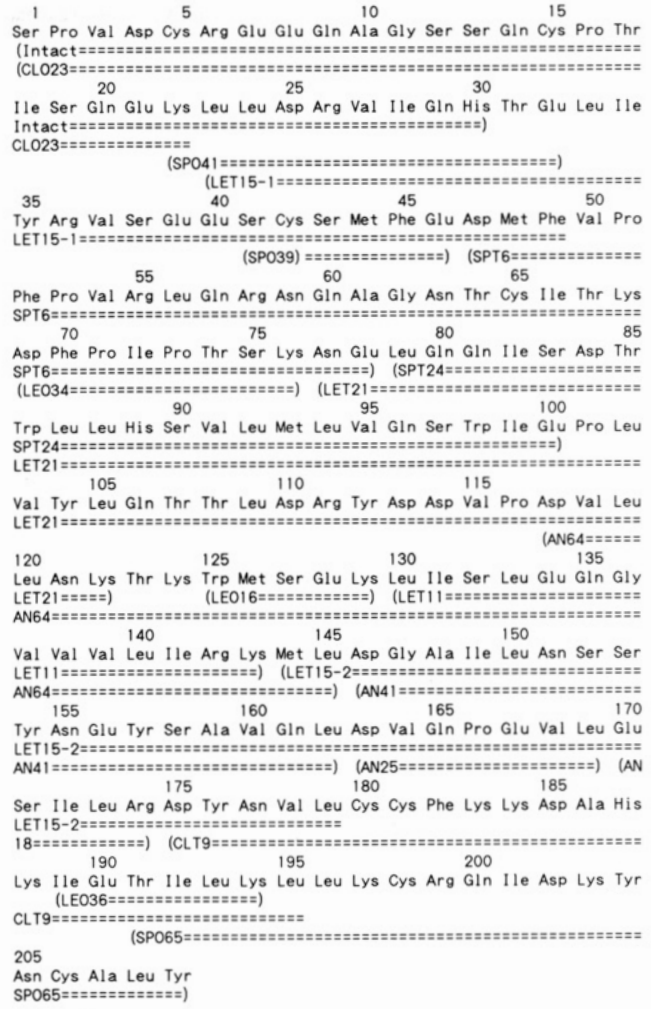

FIGURE 3: Proposed amino acid sequence of cod somatolactin. The enzymatic fragments used for sequence analysis are indicated.

sequencing. The complete primary structure of cod SL is shown in Figure 3, which also indicates the peptides used for sequence determination. Analysis of intact SL allowed the assignment of 30 N-terminal residues with the exception of cycles 5 and 15 where no amino acid was detected. The remaining sequence was obtained from enzymatically cleaved fragments, and it was found necessary to use several enzymes in order to provide complete overlap.

Separation of lysyl endopeptidase peptides of intact SL was achieved by rpHPLC, and six fragments were sequenced. LET15 was found to consist of two fragments joined by disulfide bonds and it was therefore further reduced and carboxymethylated. Complete separation of the two fragments could, however, not be achieved; LET15-2 eluted as a shoulder of the main LET15-1 peak. Consequently, the sequence of LET15-2 could only be obtained by deduction, knowing the LET15-1 sequence. However, the portion of the sequence covered by LET15-2 was confirmed by other enzymatic fragments (see below). The LE fragments sequenced determined a total of 141 residues.

Fragmentation of reduced and S-carboxymethylated SL with *Staphylococcus aureus* V8 protease gave additional five peptides. SPO41 confirmed part of the sequences obtained for the intact protein and the LET15-1 fragment, whereas SPT24 confirmed the sequence of LET21. SPO39 and SPT6 overlapped LET15-1 and allowed assignment of LE fragments in the order LET15-1, LEO34, and LET21. SPO65 overlapped four residues of LEO36 and determined an additional

15 residues; this fragment provided the C-terminal sequence since it did not contain Glu as the C-terminal residue but ended in Tyr. No further amino acid was detected in subsequent cycles.

Endoproteinase Asp-N fragment AN64 confirmed part of the sequence of SPT24 and allowed the ordering of LEO16, LET11, and LET15-2. No amino acid was detected in cycle 5 of fragment AN64, which was therefore believed to be Asn linked to carbohydrate moieties. Evidence for this was obtained by hydrolyzing the remaining sample of cycle 5 in 4 M HCl for 4 h at 110 °C. The hydrolyzate was subsequently analyzed by the HPLC system used for PTH amino acids. This resulted in the detection of PTH Asp, as hydrolysis of carbohydrate-linked Asn yielded Asp and constituted sugars. The fragments AN41, ANO25, and AN18 confirmed the sequence of LET15-2 between residues 146 and 174.

In order to provide overlap between LET15-2 and LEO36, modified SL was cleaved with Clostripain. Fragment CLT9 overlapped LET15-2, LEO36, and SPO65, thus providing the missing residues between these fragments. The sequence of fragment CLO23 was also obtained in order to confirm the N-terminal sequence of SL and identify the two missing residues as Cys.

Amino acid analysis of S-carboxymethylated nonreduced SL indicated that the protein contained two free SH groups. Assignment of the C-terminal disulfide could be made following sequencing of fragment LEO28 (residues 198–203 and 204–209), which was found to give two amino acids per cycle, consistent with a disulfide bond between Cys^{198} and Cys^{206}. The remaining two disulfide bonds were determined by analysis of trypsin-digested S-carboxymethylated nonreduced SL. Fragment T33 corresponded to residues 1–6 and 7–22, indicating a disulfide bond to exist between Cys^{5} and Cys^{15}. Fragment T64 was also found to give two amino acids per cycle (residues 59–68 and 175–183) and confirmed a linkage between Cys^{65} and Cys^{181}. The Cys^{42} (fragment T94) and Cys^{180} (fragment T64) are not involved in any disulfide bonding as these residues could be detected as CM Cys during sequencing.

The complete amino acid sequence of cod SL was thus established. The protein consists of 209 amino acid residues and contains eight Cys, six of which are involved in disulfide bonding. The protein has a calculated molecular weight of 24 190, which is lower than that estimated by SDS–polyacrylamide gel electrophoresis. The discrepancy can be accounted for by the carbohydrate moieties present on the molecule, and sequencing confirmed the presence of two possible N-glycosylation sites at positions 121–123 and 151–153. However, only the former site appeared to be glycosylated, as the nonglycosylated Asn at position 151 could be detected during sequencing.

Discussion

We recently isolated a new protein, somatolactin (SL), from pituitary glands of Atlantic cod (*G. morhua*), which was shown to be produced by the PAS-positive cells of the pars intermedia (Rand-Weaver et al., 1991). The cDNA sequence of flounder SL has been obtained and was shown to be structurally related to the pituitary hormones GH and PRL (Ono et al., 1990). We report here in detail on the isolation procedure, characterization, and amino acid sequencing of cod SL.

SL appears to be a major constituent of the cod pituitary as the two-step purification procedure of gel filtration and reversed-phase HPLC resulted in a yield of 1 mg/g wet wt of pituitary, which is approaching the amount of GH that can be obtained by using a similar method (Rand-Weaver et al., 1989). Two forms of SL were isolated, which differed in apparent molecular weights on SDS–PAGE, giving values of 26 and 23 kDa. The larger protein was always in abundance although different preparations varied in relative content of the two forms. The 26-kDa protein had an isoelectric point of between 5.7 and 5.9, which probably reflects microheterogeneity common to glycoprotein hormones (Keel & Grotjan, 1989). The glycoprotein nature of SL was confirmed by chemical sugar analysis, which showed the presence of neutral sugars (galactose and mannose), *N*-acetylneuraminic acid, and glucosamine.

The protein of molecular mass 23 kDa is believed to be a variant of the 26-kDa species on the basis of their similar amino acid composition and identical N-terminal amino acid sequence. As no reduction in molecular mass was observed of the 23-kDa protein following enzymatic deglycosylation and only one major spot at pI 6.0 was present after two-dimensional electrophoresis, the 23-kDa protein is believed to represent a deglycosylated variant of SL. It is not known at present if the lack of carbohydrates is due to sequence variation at the site of glycosylation on the 23-kDa protein. The multiplicity of pituitary hormones is well documented, and multiple forms of teleost GHs (Kawauchi et al., 1986; Yamaguchi et al., 1987; Kawazoe et al., 1988; Rand-Weaver et al., 1989) and PRLs (Yasuda et al., 1986; Yamaguchi et al., 1988) have been reported. The complete sequence analysis of the minor SL remains to be investigated.

The amino acid sequence was determined following enzymatic digestion of intact and reduced/S-carboxymethylated protein. Although about 67% of the sequence was obtained from lysyl endopeptidase fragments, the use of several other enzymes in order to provide overlapping sequences was required. The sequence showed SL to consist of 209 amino acid residues and contain eight Cys residues, two of which are located together at positions 180–181. Six Cys residues have been shown to be involved in disulfide bonding, whereas free SH groups were located at positions 42 and 180. It is interesting to notice that although cod SL has the potential for the formation of four disulfide bonds only three are formed, and these are located in homologous positions to those present in tetrapod PRL. Teleost PRL and all vertebrate GH lack the N-terminal loop but contain the large and C-terminal disulfide loops in homologous positions with SL.

Sequence analysis indicated two possible sites of N-glycosylation, but only one of these appeared to have N-linked sugars attached. This raised the possibility that the second site was located inside the molecule. Hydropathy analysis (Kyte & Doolittle, 1982) using a window a six amino acids indicated that whereas the Asn at position 121 was located in a hydrophilic region of the molecule, the Asn^{151} was predicted to be in a hydrophobic region. Although the functional significance of the carbohydrates in SL is not known, it is worth noting that flounder SL has only one predicted glycosylation site (Ono et al., 1990), which is in the same position as the one containing carbohydrates in cod (residues 121–123). Structural studies on other species should reveal if this glycosylation site has been conserved, thus suggesting a functional importance.

Alignment of the cod SL sequence with the deduced sequence for flounder SL (Ono et al., 1990) showed remarkable conservation of the SL sequence (78%) between these two species (Figure 4). In contrast, the sequence identity between GHs from cod and flounder is about 60% (Rand-Weaver et al., 1990). The most notable difference between the two SLs is that the flounder SL is two amino acids shorter at the C-terminal and contains seven Cys residues. The Cys^{180} in

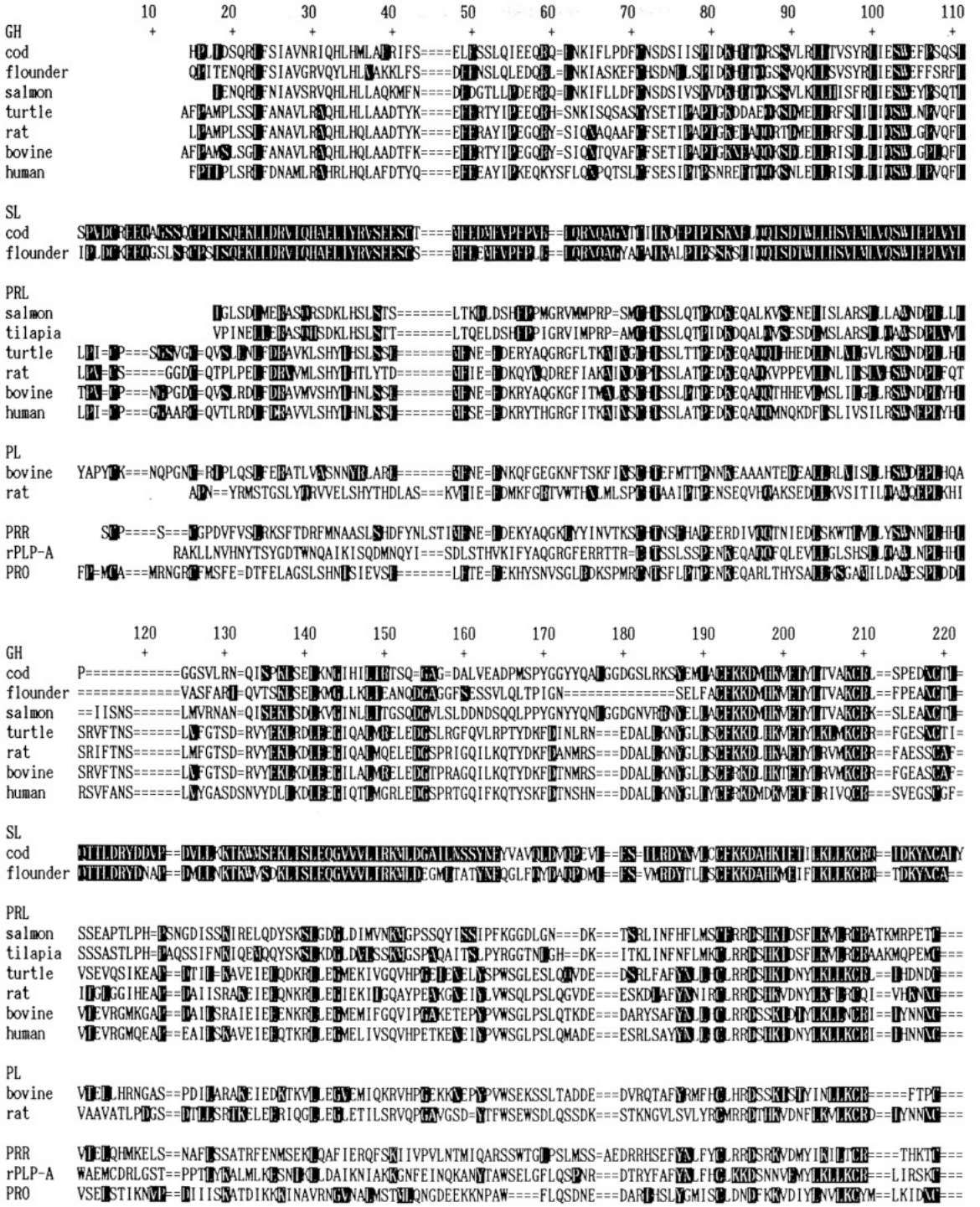

FIGURE 4: Alignment of the cod SL sequence with that of other members of the GH/PRL family. The sequences can be aligned at 222 positions and black boxes indicate identical residues compared to cod SL. Amino acid sequences used were those reported for cod (Rand-Weaver et al., 1990), flounder (Watahiki et al., 1989), salmon (Sekine et al., 1985), turtle (Yasuda et al., 1989), rat (Seeburg et al., 1977), bovine (Miller et al., 1980a), and human (Martial et al., 1979) growth hormone (GH); for flounder somatolactin (SL) (Ono et al., 1990); for salmon (Yasuda et al., 1986), tilapia (Yamaguchi et al., 1988), turtle (Yasuda et al., 1990), rat (Cooke et al., 1980), bovine (Miller et al., 1980b), and human (Cooke et al., 1981) prolactin (PRL); for bovine (Schuler et al., 1988) and rat (Duckworth et al., 1986b) placental lactogens (PL); for bovine placental prolactin-related protein I (PRR) (Schuler & Hurley, 1987); for rat prolactin-like protein A (rPLP-A) (Duckworth et al., 1986b); and for proliferin (PRO) (Linzer & Nathans, 1985).

cod is replaced by Ser^{180} in flounder, produced by a single base change mutation. The finding of this mutation supports our result that it is the Cys^{181} that is involved in disulfide bonding, assuming that structurally important Cys residues are conserved. The Cys–Cys feature found in cod has been shown to be present in a wide range of proteins including insulin and lutenizing hormone α-chain and has been thought to be of structural significance (Brown, 1976). It is unclear what significance the Cys–Cys feature has in SL since the double Cys is not conserved between cod and flounder despite the high overall sequence identity.

As already noted for flounder SL by Ono et al. (1990), the sequence of cod SL was found to share structural features with members of the GH/PRL family (Figure 4). The proteins can be aligned at 222 positions following introduction of gaps to maximize homology. Cys residues were aligned with those of PRL. SL appears to be similarly related to both GH and PRL, but with slightly higher identity to the tetrapod hormones

(27–33%) than to teleost hormones (21–27%). The higher identity of SL with the tetrapod hormones could indicate that SL has been more conserved than teleost GH and PRL during the evolution of these hormones. On the basis of sequence comparison of GH from representative species, a unit evolutionary period (UEP) of 6.3 million years (MY) for teleost GH and 9.6 MY for tetrapod GH has been calculated (Kawauchi & Yasuda, 1989; Yasuda et al., 1989), indicating a more rapid evolution of teleost GH. Additional sequence data from primitive vertebrates are required before UEP values can be calculated for PRL.

The extrahypophyseal members of the GH/PRL family, which are more similar to PRL than GH (with the exception of human PL) (Duckworth et al., 1988), show identities between 18–27%, the greatest being with bovine PL (Figure 4). The overall identity observed between SL and GH/PRL is within the range observed when comparing GH and PRL (Kawauchi et al., 1990). However, regions exhibiting in excess of 40% identity can be observed, and these coincide with the previously identified conserved regions Bg, Cg, and Dg in GH and Ap and Dp in PRL (Yamaguchi et al., 1987; Kawauchi & Yasuda, 1989). In addition to amino acid sequence homology, SL also appears to share the overall structural features of tetrapod PRLs with the presence of an N-terminal disulfide bond, lacking in teleost PRLs and all GHs.

No corresponding protein to cod SL has been reported from mammalian pituitary glands. However, in view of the fact that all pituitary hormones present in mammals have been isolated from lower vertebrates, it seems likely that SL will be shown to be contained in mammalian pituitaries. Indeed, there are indications that such a protein may exist. A 25-kDa protein similar to, but distinct from, PRL has been identified in ovine, bovine, mouse, rat (Sinha & Gilligan, 1985), and human (Sinha et al., 1987) pituitary extracts. Ono et al. (1990) were able to detect cross-reacting sequences with a flounder cDNA probe in rat, mouse, bullfrog, and human genomes, and we have partially purified a SL-immunoreactive protein from porcine and rat pituitaries (unpublished data).

In view of the structural similarity of cod SL to GH and PRL it may be speculated that SL shares biological activities with these hormones. However, we have not been able to demonstrate any growth-promoting activity of cod SL in rainbow trout (unpublished data). Although no clear function of SL is known at present, preliminary data obtained by homologous radioimmunoassay indicate that SL is present at higher levels in plasma obtained from mature cod as compared to immature fish. These results show some correlation with older observations indicating the pars intermedia PAS-positive cells, previously shown to be the site of SL production (Rand-Weaver et al., 1991), to become activated during sexual maturation [quoted in Ball and Baker (1969)]. More recent histological studies have shown the PAS-positive cells to become activated in fish exposed to hypocalcaemic conditions (Olivereau et al., 1981, 1985; Ball et al., 1982), acidification of water (Wendelaar-Bonga et al., 1986), and dark backgrounds (van Eys, 1980; Ball & Batten, 1981). However, our studies suggest that the data concerning the PAS-positive cells and sexual maturation should be reexamined and a possible role for SL during reproduction investigated in addition to ion- or coloration-controlling functions.

Acknowledgments

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Supplementary Material Available

Six figures showing the separation of enzyme-digested cod somatolactin by rpHPLC (Figures 1–5) and a hydrophobicity index of cod somatolactin (Figure 6) and one table detailing the amino acid sequence data for cod somatolactin, the yield (pmol) per cycle being indicated (10 pages). Ordering information is given on any current masthead page.

Registry No. SL, 131322-00-4; cod SL (reduced), 131322-02-6; cod SL, 131322-01-5; galactose, 59-23-4; mannose, 3458-28-4; *N*-acetylneuraminic acid, 131-48-6; glucosamine, 3416-24-8.

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Disulfide Assignments in Recombinant Mouse and Human Interleukin 4

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ABSTRACT: The disulfide pairings of mouse and human interleukin 4 (IL-4) proteins have been determined. The purified proteins, synthesized by recombinant DNA technology, are fully active as judged by their ability to stimulate an appropriate biological response in a variety of functional assays. Peptide maps were produced by digesting the proteins with pepsin and separating the resulting fragments by reverse-phase HPLC using linear acetonitrile–TFA gradients. Cystine-containing peptides were identified by determining which reverse-phase peaks showed an altered elution pattern after reduction. These peptides were purified further and defined by composition and sequence analysis. Three sets of disulfide-linked peptides were consistently identified for each protein. For mouse IL-4, the first and fifth, second and fourth, and third and sixth cysteines are joined. The disulfide bonds in human IL-4 are between the first and sixth, second and fourth, and third and fifth cysteines. A large double-loop region within the central three-fifths of each protein is stabilized by these bonds. Sequence analysis of the peptides containing the third and fifth cysteines of human IL-4 also demonstrated that only one of the potential N-glycosylation sites is used by C127 mammary tumor cells. Complete alkylation of mouse IL-4 under mild conditions completely destroyed its biological activity in a hematopoietic precursor cell proliferation assay.

Developmental maturation and functional activation of cells of the immune system are regulated by a group of extracellular growth and differentiation factors known collectively as interleukins and interferons. One of these interleukins, IL-4,[1] is a T and mast cell derived cytokine that stimulates a wide variety of biological effects in cells of hematopoietic origin. It was originally identified in studies of murine B cell dif-

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[1] Abbreviations: IL-4, interleukin 4; rmIL-4, recombinant mouse IL-4; rhIL-4, recombinant human IL-4; DTT, dithiothreitol; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin.

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