Synthesis and differentially regulated processing of proinsulin in developing chick pancreas, liver and neuroretina

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Abstract Regulated preproinsulin gene expression in non-pancreatic tissues during development has been demonstrated in rodents, *Xenopus* and chicken. Little is known, however, about the synthesis and processing of the primary protein product, proinsulin, in comparison with these events in pancreas. Using specific antisera and immunocytochemistry, immunoblot and HPLC criteria, we characterize the differential processing of proinsulin in developing neuroretina, liver and pancreas. The chick embryo pancreas expresses the convertase PC2, and largely processes proinsulin to insulin. In contrast, little or no mature PC2 is present in embryonic liver and neuroretina and the (pro)insulin immunoactivity identified is predominantly proinsulin.

Key words: Proinsulin; Insulin; Convertase; Neurogenesis; Chick embryo

1. Introduction

Two decades ago, a report by Havrankova et al. [1] initiated the long controversy of whether insulin is produced in the mammalian brain. Although the issue is not yet fully resolved for postnatal rodents, an insulin cDNA from adult rabbit brain has been isolated [2]. Many more reports reflect the expression of preproinsulin mRNA during development in tissues outside the pancreas in multiple species [3–10]. The ancestral rat insulin II gene has been unequivocally shown to be expressed and developmentally regulated in liver [3], yolk sac and brain [4,5]. In both mouse [6] and *Xenopus* [7] embryos, the insulin II gene is also expressed in the head region, prior to pancreas development. The chick embryo is an ideal model to study the expression of its single insulin gene in prepancreatic, avascular stages, as well as in the liver and the neuroretina, an avascular part of the central nervous system. Sensitive RT-PCR [8] and in situ hybridization [9] have demonstrated the wide distribution of preproinsulin mRNA during gastrulation and neurulation. The low levels of mRNA are translated into a biologically active secreted protein that attenuated apoptosis in the embryo in vivo and in culture [9,11]. In the chick embryonic liver, expression of preproinsulin mRNA was found to be differentially regulated compared to the pancreas. The main transcript in day 18 of development (E18) was similar in size in both tissues (~600 base pairs) although it was lower in abundance in liver [10]. No information exists on the translation of this liver transcript. During neurogenesis, the expression of preproinsulin mRNA in the neuroretina has been shown by RT-PCR [12] and in situ hybridization (Serna, J. et al., manuscript submitted). In addition, a (pro)insulin-related molecule could be immunoprecipitated from the culture medium of E5 neuroretina explants and the insulin immunoactivity found in vivo in the vitreous humor had the mobility of proinsulin in HPLC [13]. Others have also found insulin-related immunoactivity in chick embryo retinal extracts [14], but the identity of the protein produced, whether proinsulin or insulin, was not characterized.

In the pancreas, insulin is typically synthesized as a higher molecular size precursor, proinsulin, which undergoes limited proteolytic processing in the secretory granule of the β-cell to produce mature insulin and C-peptide [15]. This conversion is catalyzed by the action of two endopeptidases of the subtilisin/Kex2 family, the protein convertases PC2 and PC3. Both PC2 and PC3 are also synthesized as precursors that are processed to mature forms [16]. PC2 is initially synthesized as a 75-kDa precursor, which yields the active 64/66-kDa protein. Other members of this endopeptidase family include furin, highly expressed in the liver during development [17] but not capable of processing native proinsulin [18]. It is conceptually important to determine whether preproinsulin mRNA in non-pancreatic tissues yields as a final product proinsulin or insulin. The former is a poorly active metabolic analog of insulin [19] but has significant activity as a growth, differentiation [13] and survival [20] signal in neurogenesis. In this study, we have investigated the products originated by preproinsulin mRNA translation in the chick embryo neuroretina and liver, compared to the pancreas. In addition, the expression of the proinsulin-processing enzyme PC2 is analyzed throughout ontogeny in these three tissues.

2. Materials and methods

2.1. Chick embryos and tissue explant cultures

Fertilized White Leghorn eggs (Granja Rodríguez-Serrano, Salamanca, Spain) were incubated at 38.2°C and 60–90% relative humidity. At the designated day of development, the organs were dissected from the embryo, rinsed in cold phosphate buffer and used for RNA or protein extraction. Alternatively, pancreas, liver and neuroretina explants of embryonic day 15 (E15) were cultured at 37°C under 5% CO_2 for 4 h, prior to protein extraction, as previously described [8,13].

2.2. Antibodies

Insulin antibodies were bovine insulin antiserum (Sigma Chemical Co., St. Louis, MO, USA) and porcine insulin antiserum (Bio-Teck, Winooski, VT, USA), raised in guinea pig. Both recognize chicken insulin and, less efficiently, proinsulin, but not the IGFs, according to analysis by dot-blot. Rabbit anti-C-peptide antiserum (Rb40) was raised in our laboratory using a synthetic chicken proinsulin peptide

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Abbreviations: HPLC, high performance liquid chromatography; IGFs, insulin-like growth factors; IgG, immunoglobulin G; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TFA, trifluoracetic acid

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with the amino acid sequence Val^{39}-Glu^{58} [21]. The multiple antigen peptide method was used for synthesis (Universität Ulm, Germany). This antibody is specific for proinsulin and C-peptide; thus, it recognizes neither insulin nor the IGFs. PC2 antiserum ('Thumpa', [16]) was kindly provided by Dr. C.J. Rhodes (University of Texas, Dallas, TX, USA). IgGs were partially purified for immunocytochemistry by Affi-Gel Blue chromatography (Bio-Rad).

2.3. RT-PCR and Southern blotting

Extraction of total RNA from embryonic pancreas, liver and limb, and RT-PCR for preproinsulin expression analysis were performed as previously described [8]. The sequences of the chicken-specific oligonucleotides used for the RT-PCR were 5′-atggctctctggatccgatcactg-3′ (upstream) and 5′-gctagttgcagtagttctccagtt-3′ (downstream), and for the DNA hybridization 5′-acgctctccacacaccaggtagag-3′.

2.4. Protein extraction and HPLC

Pancreas, liver and neuroretina at the indicated embryonic age were homogenized with a ground glass homogenizer in 0.1–0.2 ml of 1 mM $NaHCO_3$ (pH 7.6) containing 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 50 μg/ml leupeptin, 10 μM E-64 and 10 μM tosyl-L-lysine chloromethyl ketone (all from Sigma). The homogenate was then incubated for 30 min on ice, centrifuged at 4°C for 5 min at $10000 \times g$, and the supernatant recovered for the PC2 ontogeny studies. Otherwise, proteins of 4-h cultured E15 pancreas, liver and neuroretina were acid ethanol extracted in 0.2 N HCl/75% ethanol (10 ml/g wet tissue weight) with an Ultra Turrax homogenizer as previously described [11]. Extracts were incubated at room temperature for 1 h in 0.1% (v/v) TFA and then partially purified in a C18 Sep-Pak cartridge (ODS, Waters Associates, Milford, MA, USA), as described elsewhere [22]. Eluates were dried by evaporation in a vacuum concentrator. Dried samples were reconstituted in 0.1% TFA and fractionated by HPLC (LKB system) on a C18 reverse-phase column (Vydac, Hesperia, CA, USA). Elution was at 1.0 ml/min with a 25–65% Buffer B gradient for 40 min (Buffer A = 0.1% (v/v) TFA; Buffer B = 80% (v/v) acetonitrile in 0.1% TFA). Fractions were evaporated to dryness and used for either radioimmunoassay [11] or dot-blot analysis to determine (pro)insulin immunoactivity. Alternatively, cultured pancreas extracts were incubated at room temperature for 1 h in 4% (v/v) acetic acid, loaded onto a C18 Sep-Pak cartridge previously equilibrated in 4% acetic acid, proteins eluted with methanol and dried.

2.5. Protein gel electrophoresis and immunoblotting

Pancreas, liver and neuroretina homogenized as described above were subjected to conventional SDS-10% polyacrylamide gel electrophoresis [23] for the PC2 ontogeny studies. For (pro)insulin analysis, E15 pancreas and whole retina were homogenized with an Ultra Turrax homogenizer and proteins extracted in acid ethanol (0.2 N HCl/75% ethanol, 10 ml/g wet tissue weight) as previously described [11]. The extracts were filtered in a Centricon-50 device (Amicon, Beverly, MA, USA) following the manufacturer's instructions. The filtrate was then concentrated with a Centricon-3 device (Amicon) and electrophoresed in a Tris-tricine SDS-14% Prosieve polyacrylamide (FMC, Rockland, ME, USA) non-reducing gel. Proteins were then transferred onto a nitrocellulose membrane (0.2 μm, Bio-Rad) using a semi-dry Trans-Blot unit (Bio-Rad). Membranes were subjected to immunoblot analysis for PC2 ('Thumpa' antiserum, 1:2000), proinsulin (Rb40 antiserum, 1:5000) or insulin/proinsulin (insulin antiserum from Bio-Teck, 1:2000), using the appropriate secondary antibody coupled to peroxidase. Proteins were finally detected by chemiluminescence (ECL, Amersham), as described [24].

For the dot-blot of extracts, dried HPLC fractions were reconstituted in 100 μl of phosphate buffer containing 10 μg of insulin-free bovine serum albumin and blotted onto a nitrocellulose membrane using a vacuum dot-blot apparatus. Insulin/proinsulin immunoactivity was then developed with an insulin antibody (Bio-Teck) as described above. For the dot-blot of the standard peptides, purified chicken insulin (Litron Lab Ltd., Rochester, NY, USA), our recombinant chicken proinsulin and chicken IGF-I and chicken IGF-II (both from GroPep, Adelaide, Australia) were spotted directly onto nitrocellulose and overlaid with the insulin antibody and the C-peptide antibody sequentially.

2.6. Immunocytochemistry

Sixteen micrometer cryosections from E15 eyes and pancreas with duodenum were prepared and immunostained as previously described [24] with either a guinea pig anti-insulin IgG (10 μg/ml) or a rabbit anti-C-peptide IgG (10 μg/ml) for 16 h at 4°C. As secondary antibodies, biotinylated goat anti-guinea pig IgG or goat anti-rabbit IgG (1:200, Amersham), and Cy2-streptavidin (1:200, Amersham) were used for 1 h, successively. Immunofluorescence was visualized by confocal microscopy (MRC 1024, Bio-Rad). Guinea pig and rabbit IgGs were used as negative controls.

3. Results

3.1. Preproinsulin gene expression in developing pancreas and liver

A highly sensitive and semi-quantitative RT-PCR analysis followed by Southern blot hybridization demonstrated the expression of preproinsulin mRNA in the chick embryo liver (Fig. 1), from E8 to E20 (hatching occurs at E21), extending our previous studies with Northern analysis [10]. The expression observed in liver at E8 and E10 was low; it increased markedly at E13 and E15, and later decreased until E20. On the contrary, the expression of the preproinsulin mRNA in the pancreas was much higher at every stage analyzed (considering the number of PCR cycles used) and showed little temporal regulation (Fig. 1). Preproinsulin gene expression in neuroretina has been previously reported to show an early and a late wave [12].

3.2. Ontogeny of the proinsulin-processing convertase PC2

To establish the possible extrapancreatic proinsulin processing, we searched for the proinsulin-processing enzyme PC2 in liver, neuroretina and pancreas of the chick embryo. In the developing pancreas, two specific PC2 immunoactive molecular forms of apparent molecular weight 75 kDa and 62/63 kDa were detected by immunoblot analysis (Fig. 2). The 75-kDa putative precursor molecular form was present in low abundance at all stages. Meanwhile, the 62/63-kDa putative mature form [16] was predominant and developmentally regulated, showing a progressive increase from E6 to E15. In the liver, the 75-kDa molecular form was observed, decreasing

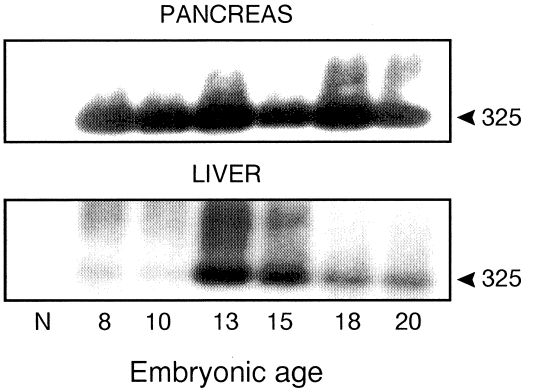

Fig. 1. Expression of the preproinsulin gene in embryonic pancreas and liver. Total RNA (1.23 μg) from tissues of the indicated age (day) were subjected to RT-PCR (20 cycles for pancreas and 30 cycles for liver) and Southern blot analysis. Autoradiograms show the amplified DNA fragment (325 base pairs). N, negative control without RNA template. Control hybridization of the blots with an IGF-I-specific oligonucleotide detected no signal. Limb RNAs of the same age embryos were positive for IGF-I expression but negative for preproinsulin expression (data not shown). For comparable data on neuroretina see [12].

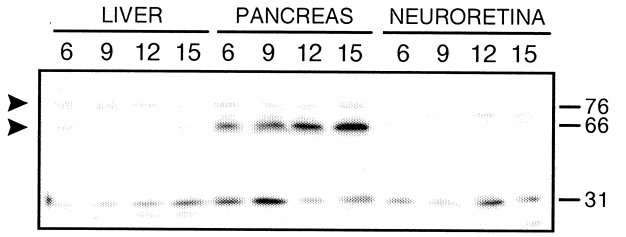

Fig. 2. Ontogeny of the proinsulin-processing enzyme PC2 in embryonic liver, pancreas and neuroretina. Fifty micrograms of total protein extracts of the indicated embryonic day were subjected to immunoblot analysis for PC2. The migration of molecular size standards of the indicated kDa is shown on the right. Arrowheads indicate the mobility of known specific PC2 molecular forms.

in abundance from E6 to E15, meanwhile the 62/63-kDa form was barely visible only in E6. In neuroretina, these two protein forms were absent and, although a 70-kDa band is weakly detected from E9 to E15, we cannot confirm that it is an alternative form of PC2.

3.3. *Proinsulin immunoactivity predominates in embryonic liver and neuroretina and insulin prevails in the pancreas*

After having demonstrated preproinsulin gene expression in developing liver and neuroretina as well as in the pancreas, and the near undetectable mature PC2 in both non-pancreatic tissues, we characterized the preproinsulin gene translated product at E15 by several methods.

Immunocytochemistry with two different antibodies allowed us to distinguish between insulin and proinsulin. The first is a commercial insulin polyclonal antiserum that recognizes better insulin, but also proinsulin, and the second is a C-peptide antiserum specific for chicken proinsulin, hence it does not recognize fully processed insulin (Fig. 5A). In the pancreas, both antibodies stained similar types of granular cells (Fig. 3B–D), some of which were in small clusters (arrowheads in Fig. 3B, and arrow in Fig. 3C) and, thus, identified as β-cells of not fully formed islets of Langerhans. Surprisingly, in the neuroretina, these two antibodies had different staining preferences. The anti-C-peptide IgG stained clusters of cells of the ganglion cell layer (arrowheads, Fig. 3E), in a non-granular, uniform manner, whereas the anti-insulin IgG only recognized isolated granular cells in the outer neural layers (Fig. 3F, G) and outside the pigmented epithelium (Fig. 3G). Pancreas sections treated with guinea pig non-immune IgG showed no specific staining (results not shown).

To further characterize the presence of either proinsulin or insulin in the chick embryo neuroretina, liver and pancreas, we performed a HPLC fractionation of both proteins prior to their immunodetection. A blank injection was run before sample injections in order to discard any previous (pro)insulin contamination of the column, and the pancreas extract was chromatographed after those of liver and neuroretina. Chicken insulin and human proinsulin were chromatographed last as standards, and detected by absorbance at 214 nm. Immunoactivity in the chromatographic fractions semi-quantified by dot-blot with an insulin antibody (Bio-Teck) that preferentially recognizes insulin, showed a major peak of immunoactivity in the pancreas (Fig. 4A). This peak eluted in the position of mature insulin and had a shoulder in the position of

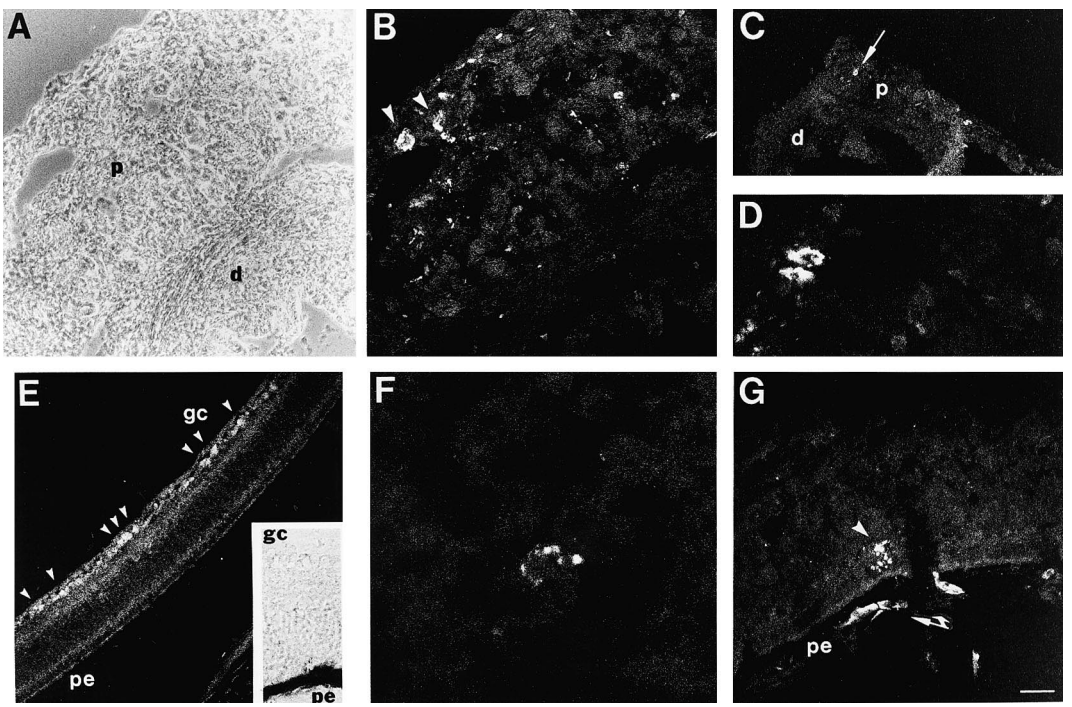

Fig. 3. Immunocytochemical distribution of insulin and proinsulin in E15 pancreas and retina. Pancreas/duodenum (B–D) and eye (E–G) cryosections were stained for immunofluorescence with an anti-C-peptide IgG (B, E) or an anti-insulin IgG (C, D, F, G), as described in Section 2. Phase-contrast images of pancreas (A) and retina (E, insert) sections are also shown. Arrowheads in B indicate clusters of immunostained cells. Arrow in C marks a group of at least two insulin-containing cells, which is shown in D at a higher magnification. Note that the duodenum is negative. Arrowheads in E indicate ganglion cells more intensively positive for proinsulin. Arrowhead and arrows in G point at three single (pro)insulin-containing cells within the retina (arrowhead) and outside the pigmented epithelium (arrows) as observed under phase contrast (not shown). Bar in G represents 80 μm for C, 40 μm for A, B, E, 20 μm for G, 10 μm for D and E, insert and 6.3 μm for F. p, pancreas; d, duodenum; gc, ganglion cell; pe, pigmented epithelium. The photographs are confocal microscopy images.

proinsulin. In both liver and neuroretina, however, only a small peak of immunoactivity corresponding to the elution position of standard proinsulin was observed (Fig. 4A). The HPLC blank run showed no immunoactivity. In a parallel HPLC fractionation of a larger liver extract, the fractions were quantified by insulin radioimmunoassay and the presence was confirmed of immunoactivity with the mobility of proinsulin (Fig. 4B). The E15 liver concentration of proinsulin was 3.4 ng/g wet tissue weight, probably a several-fold underestimation, since the standard used in the radioimmunoassay was chicken insulin, better recognized by the antibody. This corresponds to at least 1 ng per liver, whereas a specific IGF-I radioimmunoassay for this peptide measured only 0.38 ng per E15 liver. In addition, we confirmed the developmental regulation of proinsulin expression, since the E13 liver extract contained much less immunoactivity, 0.2 ng/g wet tissue weight.

Finally, we further demonstrated the synthesis of proinsulin in the E15 retina by immunoblot. We had previously demonstrated by metabolic labeling and immunoprecipitation, the synthesis and rapid secretion of a (pro)insulin-related immu-

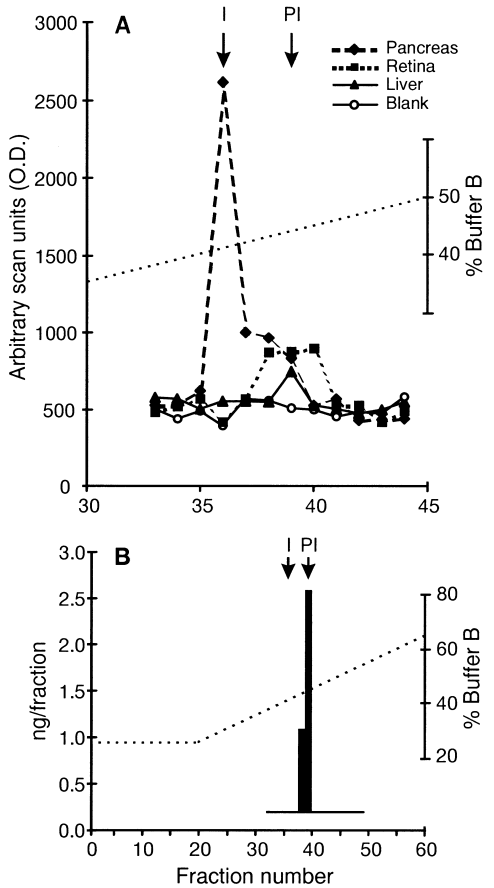

Fig. 4. HPLC analysis of insulin and proinsulin in E15 pancreas, liver and neuroretina. A: Pancreas (pool of 17 = 0.10 g of wet tissue weight), liver (pool of 2 = 0.40 g of wet tissue weight) and neuroretina (pool of 5 = 0.18 g of wet tissue weight) acid alcohol extracts were subjected to HPLC. Fractions (1 min) were analyzed by dot-blot with an insulin antibody (Bio-Teck) and ECL. B: Acidic liver extracts (pool of 19 = 5.7 g of wet tissue weight) were subjected to C18 reverse-phase HPLC and the collected 1.0-min fractions were tested in an insulin radioimmunoassay, that recognizes insulin and proinsulin, but not IGFs. Arrows indicate the elution position of chicken insulin (I) and human proinsulin (PI) standards.

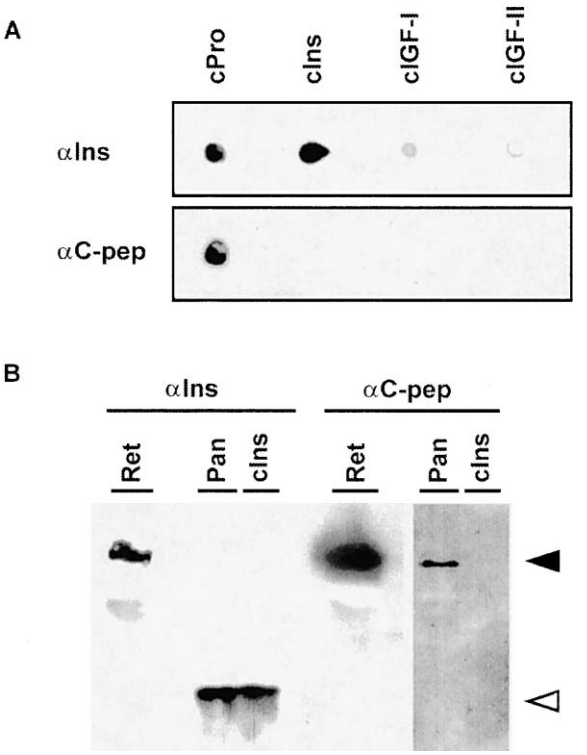

Fig. 5. Immunoblot analysis of insulin and proinsulin in E15 pancreas and retina. A: Specificity of the antibodies. Recombinant chicken proinsulin (cPro), IGF-I (cIGF-I) and IGF-II (cIGF-II) and purified chicken insulin (cIns) (100 ng of each) were immunoblotted first with the anti-C-peptide antibody (Rb40, αC-pep) and, after stripping of the blot, with the anti-insulin antibody (Bio-Teck, αIns). B: Pancreas (equivalent to 2.3 pancreas=0.07 g of wet tissue weight) and retina (pool of 12=0.36 g of wet tissue weight, containing traces of pigmented retina) extracts were subjected to Tris-tricine SDS-polyacrylamide gel electrophoresis under non-reducing conditions and then immunoblotted, using sequentially the anti-C-peptide antibody and the anti-insulin antibody as in panel A. Purified chicken insulin (500 ng) was electrophoresed as standard (open arrowhead). The mobility of proinsulin (solid arrowhead) in this type of gel coincided with a multimeric rather than monomeric form of standard proinsulin. The exposures were adjusted for best observation of the relevant band, therefore, the proinsulin band of the pancreas extract in the overlay with anti-insulin antibody was visible only in a longer exposure.

noactive protein in the cultured E5 neuroretina [13]. We now show that E15 retina also contains a protein with the electrophoretic mobility of proinsulin (Fig. 5B). This protein was detected by immunoblotting with both the insulin antibody and the C-peptide antibody, as would be expected for intact proinsulin. In contrast, in the pancreas extract, the insulin antibody identified a major band with the electrophoretic mobility of insulin and only after a long exposure a higher molecular weight band, putative proinsulin. Consistent with the specificity of the antibodies (Fig. 5A), the latter was better recognized by the C-peptide antibody (Fig. 5B).

4. Discussion

This study demonstrates that the insulin-related immunoactivity present in two non-pancreatic tissues of the chick embryo, neuroretina and liver, corresponds predominantly to unprocessed proinsulin, in contrast to the pancreas, in which insulin predominates. These findings strongly support the lo-

cal synthesis of proinsulin outside the pancreas. The presence of preproinsulin gene expression regulated during development in non-pancreatic tissues [3–14], although low in concentration, was already suggestive of local production of the factor. Studies at the protein level are scarce, however, because of the difficulty in characterizing a very low abundance small protein in embryonic tissues. Further, tissues such as liver, yolk sac or young embryos lack typical endocrine secretory granules, in which proteins can be concentrated about 200-fold [25]. In contrast, these tissues secrete proteins through the constitutive pathway that, if used by proinsulin, would result in continuous, rapid secretion [4,9,13]. The corresponding low cytoplasmic protein levels are thus much more difficult to detect [6,9]. We had previously postulated that the prepancreatic and extrapancreatic translation product of the preproinsulin gene could remain as proinsulin, and that it had a role in developmental events such as proliferation, differentiation and survival [9,13,20,26]. Recently, we have confirmed by immunocytochemistry that proinsulin is present in E1–E2 embryos (Serna, J., unpublished observation) where it modulates apoptosis [9]. Indirect evidence showed that the neuroretina produced proinsulin, since during proliferative stages (E6–E8), the immunoactivity accumulated in the vitreous humor, in contact with the neuroretina and ciliary processes, had the HPLC mobility of proinsulin [13]. In the present analysis, we chose to study the neuroretina at a later stage of development (E15), during neuronal maturation, to better compare it with a presumably more mature pancreas and with liver at its preproinsulin gene expression peak. For this purpose, we generated a specific chicken C-peptide antibody to discriminate immunologically the intact proinsulin from insulin, which lacks that region. The clusters of immunostained cells found in the pancreas were not yet completely arranged as typical islets, a feature similar to that found in other developing embryos [27]. Since they were similarly immunostained by an insulin antibody and the C-peptide antibody, these insulin-rich embryonic β-cells may contain some proinsulin stored in granules as well. In contrast, in the E15 neuroretina, the immunocytochemistry showed proinsulin-specific staining in patches of ganglion cells, reminiscent of the expression pattern of IGF-I mRNA [28] and compatible with the broad preproinsulin mRNA distribution detected by in situ hybridization (Serna, J. et al., manuscript submitted). In addition, a few isolated cells of stronger, insulin-specific granular staining appeared either in the outer layers of the retina or outside the pigmented epithelium, in the choroid. These cells are morphologically similar to macrophages/microglia, a type of granular cell reported to invade the chick embryo retina [29] and to contain NGF [30], among other growth factors, important in the balance of cell death/survival during neurogenesis. The definitive characterization of these cells, their production of preproinsulin mRNA and their capacity to process proinsulin into mature insulin will require future studies.

As for the liver, the insulin-related immunoactivity was also characterized as proinsulin by immunological and HPLC criteria. We did not perform immunocytochemistry, but preliminary in situ hybridization had shown diffuse preproinsulin mRNA in the embryonic liver [10]. The finding of proinsulin in both neuroretina and liver, in contrast with the predominantly processed insulin of the pancreas, concurs with the differential expression of PC2 in these tissues. While the pancreatic PC2 increased with embryonic age between E6 and E15, no similar 62/63-kDa band was detectable in the neuroretina and was only barely visible in the liver. This result is similar to that found in mammalian embryos, in which PC2 and PC3 have been detected in the developing pancreas, but not in the liver or the retina [17]. Both PC2 and PC3 function naturally in the regulated secretory pathway and require the granule environment to process proinsulin efficiently. In fact, no mature insulin has been detected in β-cell compartments other than the secretory granule [31]. Indeed, slow cleavage has even been described in transformed β-cells that lack another endoprotease, PC1/PC3 [32]. In these cells the ratio of proinsulin to insulin is higher than in normal β-cells and proinsulin uses the constitutive secretion pathway. Similarly, in a rat pancreatic tumor model, the absence of PC1 and PC2 coexisted with lack of conversion of proinsulin to insulin [33]. Other endopeptidases such as furin process precursors in the constitutive pathway. We cannot rule out that the ubiquitous furin, the PC2 precursor or other endopeptidase(s) may be able to convert chicken proinsulin into insulin more slowly, but it appears unlikely that the process is very efficient in neuroretina and liver during mid-development, in accordance with results shown here.

We can only speculate about the autocrine-paracrine role of this proinsulin in two tissues as different as neuroretina and liver at E15. It is possible that it has a cell survival function similar to that observed at earlier stages of embryogenesis [9,20] or it may have additional roles, for example, in neuronal maturation. The concentration of proinsulin found in the vitreous humor at E8 ($\sim$55 ng/ml) [13] is high enough to bind and activate the retina atypical insulin receptors [20]. It is also interesting that, in mid-embryogenesis, the production of IGF-I in the liver is negligible (see Section 3 and also [34]). As proposed in the fetal rat [3], liver (pro)insulin may also act in the chick embryo in an endocrine mode to accomplish both metabolic and growth effects. The situation of high levels of circulating proinsulin may be more physiological in lower vertebrates, such as fish, in which both insulin and proinsulin are considered growth factors [35]. In the chick, proinsulin appears also to deserve its own place as a developmental multifunctional tissue factor.

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