Cell Tissue Res (1996) 286:543–546

Cell & Tissue Research

Identification of a high molecular weight polypeptide in the subcommissural organ of the chick embryo

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Received: 14 March 1996 / Accepted: 23 May 1996

Abstract. The subcommissural organ is an ependymal brain gland that secretes, into the ventricular cerebrospinal fluid, high molecular weight glycoproteins that form Reissner's fiber. Precursor and processed forms of secretion have been demonstrated by immunoblotting in the subcommissural organ of mammals and fish. In the chicken only a processed form has as yet been identified. In the present report, we have studied the subcommissural organ of 13-day-old chick embryos using (1) an antiserum against bovine Reissner's fiber, and (2) the lectins, concanavalin A and *Limax flavus* agglutinin. Paraffin sections of the subcommissural organ and blots of subcommissural organ extracts have been analyzed. The ependymal cells of sectioned subcommissural organ are strongly stained with the antiserum. Concanavalin A binds to materials in all cytoplasmatic regions, whereas *Limax flavus* agglutinin identifies materials confined to the apex of the ependymal cells. In the blots, a band of 540 kDa is immunostained. This band is positive for concanavalin A positive but negative for *Limax flavus* agglutinin and is thereby regarded as representing a precursor form of the secretion.

Key words: Subcommissural organ – Secretory glycoproteins – Antibodies – Immunochemistry – Immunocytochemistry – Lectins – Chick embryo (White Leghorn)

Introduction

The subcommissural organ (SCO) is an ependymal brain gland located in the roof of the third ventricle (Oksche et al. 1993). Histochemical, immunocytochemical, autoradiographic and ultrastructural studies have demonstrated that SCO is highly specialized in the secretion of glycoproteins that condense into a thread-like structure, known as Reissner's fiber (RF), that continuously grows along the brain ventricles and the spinal central canal. The SCO is highly active during ontogenesis and is a persistent structure of the vertebrate brain. Mannose-rich glycoproteins are synthesized in the rough endoplasmic reticulum (precursor forms) and undergo maturation in the Golgi apparatus; this leads to the production of sialic-acid-rich glycoproteins (mature or processed forms) that are released into the cerebrospinal fluid of the third ventricle. Precursor and processed forms can be distinguished by their affinities to lectins (Rodríguez et al. 1986; Meiniel et al. 1988). Blotting studies of SCO extracts using specific antisera and lectins have confirmed the presence of mature and immature secretory materials in bovine (Nualart et al. 1991) and dogfish (Grondona et al. 1994; López-Avalos et al. 1996) SCO. Recently, Didier et al. (1995) have reported the presence, in the chick embryo, of a secretory compound that is considered to be a processed form on the basis of its lectin-binding properties. In the present study, we characterize a high molecular-weight glycoprotein in the subcommissural organ of chick embryos that may, according to its affinity to lectins, correspond to a precursor form.

This work was supported by grants PB 93–0979 from DGICYT, Madrid, and 95/1591 from FIS, Madrid.

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Materials and methods

Immunocytochemistry and lectin histochemistry

Ten chick embryos (*Gallus domesticus*, White Leghorn) at ontogenetic stage 39 (13 days old; Hamburger and Hamilton 1951) were dissected and the brains immersed in Bouin's fluid for 3 days. They were dehydrated, embedded in paraffin and cut into sagittal 10-μm-thick sections. Rehydrated sections were stained according to the peroxidase antiperoxidase (PAP) method of Sternberger et al. (1970). They were sequentially incubated in: (1) an antiserum against bovine RF (AFRU), developed in our laboratory according to Rodríguez et al. (1984), diluted 1:1000 in TCT solution, composed of 0.1 M TRIS buffer, pH 7.8, containing 0.7% non-gelling seaweed lambda carrageenan (Sigma, Madrid, Spain) as the saturating agent and 0.5% Triton X-100 (Sigma), for 18 h at 25°C in a moist chamber; (2) anti-rabbit IgG developed in goat (from our laboratory) diluted 1:50 in TCT, for 30 min at

25°C; and (3) rabbit PAP (Sigma) diluted 1:100 in TCT, for 30 min at 25°C. To reveal peroxidase, 3,3′diaminobenzidine tetrahydrochloride (DAB; Sigma) was used as the electron donor.

The lectins, concanavalin-A agglutinin (Con A), wheat germ agglutinin (WGA) and *Limax flavus* agglutinin (LFA), were used. For Con A and WGA binding, the sections were incubated in peroxidase-labeled lectin (Con A 5 μg/ml, WGA 3 μg/ml; Sigma) dissolved in TCT buffer for 2 h at 25°C in a moist chamber; DAB (Sigma) was used as the electron donor. For LFA binding, the sections were incubated in 3 μg/ml non-labeled LFA (Calbiochem, San Diego, Calif., USA) in phosphate-buffered saline (PBS) for 1 h and revealed by immunocytochemistry using an anti-LFA antiserum (from E.M. Rodríguez, Valdivia, Chile) diluted 1:5000 in TCT buffer for 18 h, as described above.

SCO extracts

We used 50 bovine SCOs obtained from a local slaughterhouse and 100 chick embryo SCOs obtained in our laboratory. Dissection, homogenization, sonication, and centrifugation were performed according to a procedure described previously (Pérez et al. 1993; Grondona et al. 1994). The extraction medium was composed of 50 mM ammonium bicarbonate, pH 8, containing the following protease inhibitors: 1 mM ethylene diamine tetraacetate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany), 1 μM pepstatin (Sigma), and 1 μM leupeptin (Sigma). The protein content of extracts was determined according to Bradford (1976).

Immunoblotting of chick embryo SCO extracts

SDS polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) by use of 100×100×0.75-mm slab gels, containing a 5–15% polyacrylamide linear gradient; bisacrylamide was used at a concentration of 2.6%. Molecular weight standards and samples (30-μg proteins) of the SCO and other brain areas were dissolved in 125 mM TRIS-HCl, pH 6.8, (Sigma), containing 2% sodium dodecyl sulphate (Serva, Heidelberg, Germany), 5% β-mercaptoethanol (Sigma) and 10% glycerol (Sigma), heated at 95°C for 2 min, and loaded on the stacking gel. Electrophoresis was performed at 15 mA for 1 h. Gels were transferred onto PVDF membranes (Millipore, Bedford, Mass., USA) according to the procedure of Towbin et al. (1979; for further details, see Grondona et al. 1994). The molecular weight standards used were: glycoproteins of the bovine subcommissural organ (540, 450, and 320 kDa; Nualart et al. 1991), myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa; Sigma). Blots of SCO and brain tissue were incubated with: (1) 5% non-fat milk in PBS buffer, pH 7.3; (2) AFRU, 1:1000 dilution for 18 h; (3) anti-rabbit IgG (raised in our laboratory) developed in sheep, 1:50 dilution for 2 h; (4) rabbit PAP (Sigma), 1:200 dilution for 1 h; and (5) 0.06% 4-chloro-1-naphthol (Sigma), 0.075% perhydrol (Merck), 20% methanol, in PBS, pH 7.3. Control blots were incubated with rabbit pre-immune serum.

Lectin binding

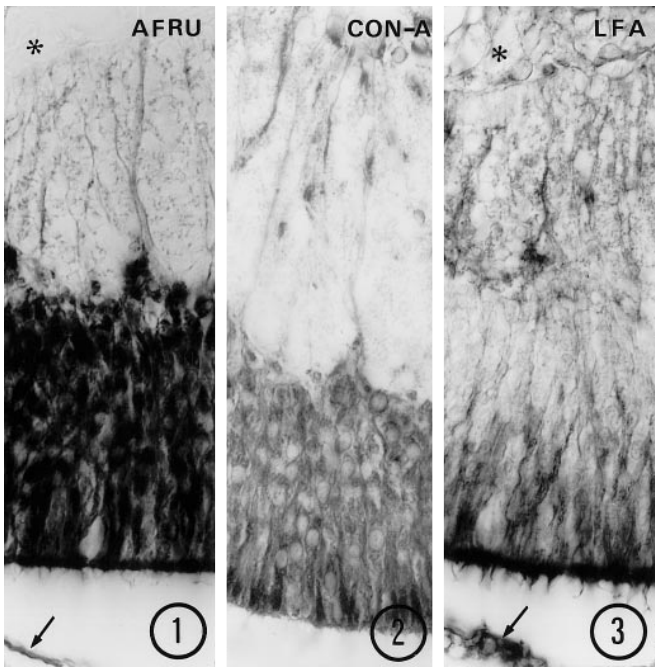
Blots parallel to those used for immunostaining were used for lectin binding. The blots were sequentially incubated with oxidized bovine serum albumin (Sigma) and with peroxidase-labeled lectins for 1 h. The lectins used were (1) 4 μg/ml Con A (affinity=mannose, glucose; Sigma), control transfers being incubated with Con A in the presence of 1 M D-mannose; and (2) 1 μg/ml LFA (affinity=sialic acid; Sigma), control transfers being incubated with LFA in the presence of 1 M sialic acid. Peroxidase was detected by the use of perhydrol and 4-chloro-1-naphthol.

Results and discussion

Schoebitz et al. (1986) have studied the SCO of the developing chick embryo with an AFRU similar to that used in this study. According to these authors, at day 13, the SCO of the chick embryo was fully developed and showed conspicuous immunoreactivity. In our material, immunostained products are present at the apical, perinuclear and basal cytoplasmic regions. A strongly immunoreactive band is evident at the apex of the ependymal cells. Immunoreactive fibrillary material of the pre-RF can be identified in the ventricle (Fig. 1). The secretory activity of the chick embryos has also been studied using an antiserum against SCO extracts from chick embryo with similar results (Karoumi et al. 1990a).

Con A lectin (mannose affinity) binds to materials in the perinuclear and apical cytoplasm (Fig. 2). LFA lectin (sialic acid affinity) weakly stains the apical cytoplasm and strongly stains the pre-RF and the band at the apex of the ependymal SCO cells (Fig. 3). These results are similar to those reported by Karoumi et al. (1990a) in the chick embryo with Con A and WGA.

In the SCO of several vertebrate species, it has been reported that Con A lectin binds to immature secretory products, whereas LFA or WGA lectins bind to sialic-acid-containing mature secretory products (Rodríguez et



Figs. 1–3. Details of a sagittal section through the subcommissural organ of a 13-day-old chick embryo.

Fig. 1. Immunostaining with an antiserum against bovine Reissner's fiber (AFRU). **Fig. 2**. Concanavalin-A agglutinin (Con A) binding. **Fig. 3.** *Limax flavus* agglutinin (LFA) binding. *Arrow*, Positive pre-Reissner's fiber; *asterisk*, leptomeninx. ×135

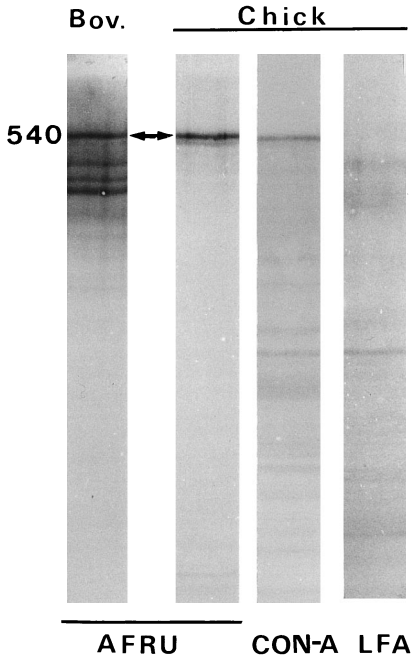


Fig. 4. Blots of SDS-PAGE of subcomissural organ extracts of the bovine and chick embryo; immunostaining with an AFRU and lectins Con A and LFA. In the bovine, four immunoreactive bands are evident, but only a band of molecular weight 540 kDa is AFRU-positive and Con-A-positive in the chick

al. 1986; Meiniel et al. 1988; Peruzzo et al. 1990; Grondona et al. 1994). Our results thus indicate that the SCO of the developing chick embryo must contain precursor (immature) and processed (mature) secretory products.

We have performed immunoblot and lectin-blot analysis of chick embryo SCO extracts. For a comparison, an extract of bovine SCO has been run in parallel lanes of the same electrophoretic gel. AFRU clearly reveals four bands in the bovine SCO extract. The largest polypeptide has an apparent molecular weight of 540 kDa. The other three range between 500 and 300 kDa (Fig. 4). Using a similar protocol, Nualart et al. (1991) have described four immunoreactive bands of molecular weights 540, 450, 320, and 190 kDa in blots of bovine SCO extract. All four bands are Con-A-positive but only the 450-kDa and 190-kDa bands are LFA-positive. Thus, the above authors regard the 540-kDa and 320-kDa bands as two precursor forms, and the 450-kDa and 190-kDa bands as two processed forms. In the dogfish, four AFRU-immunoreactive bands of molecular weights 600, 475, 400, and 145 kDa have been reported (López-Avalos et al. 1996); according to lectin affinities, the 600-kDa band can be considered a precursor polypeptide and the 475-kDa band a processed form.

In our study, we have used the same antisera and protocols as those of Nualart et al. (1991) and López-Avalos et al. (1996). The blot of chick embryo SCO extracts shows a conspicuous AFRU-immunoreactive band of a molecular weight of 540 kDa. This band is Con-A-positive and LFA-negative (Fig. 4). Thus, according to the above, it could be regarded as a precursor polypeptide, homologous to the 540-kDa precursor of the bovine (Nualart et al. 1991; present results) and the 600-kDa precursor of the dogfish (López-Avalos et al. 1996). The presence of high molecular weight precursor forms in the SCO of cow, dogfish, and chick embryo suggests that this may be universal among vertebrates.

LFA-positive bands of molecular weight under 500 kDa are evident in the chick blots (Fig. 4), but none of them is AFRU-positive. Thus, the processed form(s) stained by LFA in sections and in blots (Fig. 3) of our extracts may be in an amount too low to be detected by AFRU in immunoblot.

Karoumi et al. (1990b) have performed immunoaffinity chromatography of chick embryo SCO extracts using a specific antiserum. In blots of the eluted fractions, they found several bands of molecular weight lower than 250 kDa that were stained by lectins and thus considered to represent secretory glycoproteins. Using an antiserum against bovine RF and two-dimensional electrophoresis, Didier et al. (1995) have also studied the secretory material of the chick embryo SCO. They describe a compound of a molecular weight of 390 kDa that is immunoreactive to AFRU and that binds Con A and WGA lectins. Thus, they regard this compound as a mature (processed) form of SCO secretion. With their protocol, they have been unable to detect polypeptides of molecular weight higher than 500 kDa. The reason that Didier et al. (1995) have not found the precursor form in their study may therefore be that the precursor has a molecular weight over 500 kDa, as we report here. Taking together the results of Didier et al. (1995) and those of the present work, it can be concluded that the SCO of the chick embryo probably contains at least one precursor and one processed form of secretion, as appears to be the rule among vertebrates.

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Didier R, Dastugue B, Meiniel A (1995) The secretory material of the subcommissural organ of the chick embryo. Characterization of a specific polypeptide by two-dimensional electrophoresis. Int J Dev Biol 39: 493–499

Grondona JM, Pérez J, Cifuentes M, López-Avalos MD, Nualart FJ, Peruzzo B, Fernández-LLebrez P, Rodríguez EM (1994) Analysis of the secretory glycoproteins of the subcommissural organ of the dogfish (*Scyliorhinus canicula*). Mol Brain Res 26: 299–308

Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morphol 88: 49–92

Karoumi A, Meiniel R, Croisille Y, Belin MF, Meiniel A (1990a) Glycoprotein synthesis in the subcommissural organ of the chick embryo. I. An ontogenetical study using specific antibodies. J Neural Transm Gen Sect 79: 141–153

Karoumi A, Croisille Y, Croisille F, Meiniel R, Belin MF, Meiniel A (1990b) Glycoprotein synthesis in the subcommissural organ of the chick embryo. II. An immunochemical study. J Neural Transm Gen Sect 80: 203–212

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

López-Avalos MD, Pérez J, Pérez-Fígares JM, Peruzzo B, Grondona JM, Rodríguez EM (1996) Secretory glycoproteins of the subcommissural organ of the dogfish (*Scyliorhinus cani-*

cula): evidence for the existence of precursor and processed forms. Cell Tissue Res 283: 75–84

Meiniel A, Molat JL, Meiniel R (1988) Complex-type glycoproteins synthesized in the subcommissural organ of mammals. Light- and electron-microscopic investigations by use of lectins. Cell Tissue Res 253: 383–395

Nualart F, Hein S, Rodríguez EM, Oksche A (1991) Identification and partial characterization of the secretory glycoproteins of the bovine subcommissural organ-Reissner's fiber complex. Evidence for the existence of two precursor forms. Mol Brain Res 11: 227–238

Oksche A, Rodríguez EM, Fernández-LLebrez P (1993) The subcommissural organ. An ependymal brain gland. Springer, Berlin Heidelberg New York

Pérez J, Grondona JM, Cifuentes M, Nualart F, Fernández-LLebrez P, Rodríguez EM (1993) Immunochemical analysis of the dogfish subcommissural organ. In: Oksche A, Rodríguez EM, Fernández-LLebrez P (eds) The subcommissural organ. An ependymal brain gland. Springer, Berlin Heidelberg New York, pp 99–107

Peruzzo B, Pérez J, Fernández-LLebrez P, Pérez-Fígares JM, Rodríguez EM, Oksche A (1990) Ultrastructural immunocytochemistry and lectin histochemistry of the subcommissural organ in the snake *Natrix maura* with particular emphasis on its vascular and leptomeningeal projections. Histochemistry 93: 269–277

Rodríguez EM, Oksche A, Hein S, Rodríguez S, Yulis R (1984) Comparative immunocytochemical study of the subcommissural organ. Cell Tissue Res 237: 427–441

Rodríguez EM, Herrera H, Peruzzo B, Rodríguez S, Hein S, Oksche A (1986) Light- and electron-microscopic immunocytochemistry and lectin histochemistry of the subcommissural organ: evidence for processing of the secretory material. Cell Tissue Res 243: 545–559

Schoebitz K, Garrido O, Heinrichs M, Speer L, Rodríguez EM (1986) Ontogenetical development of the chick and duck subcommissural organ. An immunocytochemical study. Histochemistry 84: 31–40

Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish-peroxidase-antiperoxidase) and its use in identification of spirochetes. J Histochem Cytochem 18: 315–333

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354