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Original Article

The secretory material of the subcommissural organ of the chick embryo. Characterization of a specific polypeptide by two-dimensional electrophoresis

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ABSTRACT The subcommissural organ (SCO) is a cerebral gland that releases into the cerebrospinal fluid a carbohydrate-rich glycoprotein which condenses to form Reissner's fiber (RF). Western blots from two-dimensional gel electrophoresis were stained with lectins (Concanavalin-A, wheat germ agglutinin) and anti-bovine RF serum to identify the secretory products of the chick embryo SCO. Immunohistochemical investigations showed that the anti-bovine RF serum reacted exclusively with the secretion of the SCO. Comparative protein patterns of SCO, pineal organ and cerebral hemisphere extracts allowed us to characterize a specific polypeptide in the SCO electrophoretic profiles. The polypeptide was a highly acid compound (isoelectric point of 4.7) with a high molecular weight (390 kDa). On Western blots only this component was immunoreactive with the RF antiserum and it exhibited an affinity for the two lectins. On the basis of these results, this polypeptide may be considered as a specific component of the secretory material synthesized by the SCO cells of the chick embryo.

KEY WORDS: *chick embryo, subcommissural organ, glycoproteins, 2-D electrophoresis, immunochemistry*

Introduction

The subcommissural organ (SCO) is an ependymal derivative located in the dorso-caudal region of the third ventricle under the posterior commissure. This structure releases secretory compounds into the ventricular cerebrospinal fluid which condense to form Reissner's fiber (RF) running in the central canal of the spinal cord (Oksche, 1969; Rodriguez, 1970; Oksche *et al.*, 1993). Immunocytochemical studies using polyclonal (Sterba *et al.*, 1982; Rodriguez *et al.*, 1984; Schoebitz *et al.*, 1986; Karoumi *et al.*, 1990b) and monoclonal (R. Meiniel *et al.*, 1988, 1990; Didier *et al.*, 1992) antibodies against SCO extracts or bovine RF have established the early appearance of the SCO/RF complex during ontogenesis and the specificity of the material produced by the SCO cells. In addition, lectin histo- and cytochemistry have provided evidence that the secretory products of the SCO/RF complex correspond to complex-type glycoproteins (Rodriguez *et al.*, 1986; A. Meiniel *et al.*, 1988; Herrera and Rodriguez, 1990).

Although the SCO has a remarkable phylogenetic and structural constancy in all chordate classes (Oksche, 1961; Leonhardt, 1980), the exact protein composition of its secretory material has not been clearly established. In recent years, several studies have attempted to identify and characterize the glycoproteins secreted by this organ in various species by electrophoretical analysis (Meiniel *et al.*, 1986; Karoumi *et al.*, 1990a, 1991; Nualart *et al.*, 1991; Perez *et al.*, 1993). However, these studies show discrepancies in the number and electrophoretic characteristics of the secretory glycoproteins.

Using two-dimensional gel electrophoresis to increase resolution of all the components and electroblotting we re-investigated this biochemical aspect of the secretory material in the SCO of the chick embryo. Cellular proteins of the SCO were studied after fractionation into soluble and membrane proteins. As anti-bovine RF serum strongly reacts with the secretory material of the SCO in many vertebrates (Sterba *et al.*, 1982; Rodriguez *et al.*, 1984), we raised polyclonal antibodies against the bovine RF that served as probes for the characterization of the SCO specific secretion. Thanks to the combined use of immunoblotting and lectin binding on protein blots, we obtained useful information on the biochemical features of the material secreted by the SCO in the chick embryo.

Results

Immunohistochemistry

After incubation with the anti-RF serum, a strong immunoreactivity was visualized in the entire ependymal epithelium of the

Abbrevations used in this paper: SCO, subcommissural organ; RF, Reissner's fiber; NEPHGE, nonequilibrium pH gradient electrophoresis; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; NP40, nonidet P-40.

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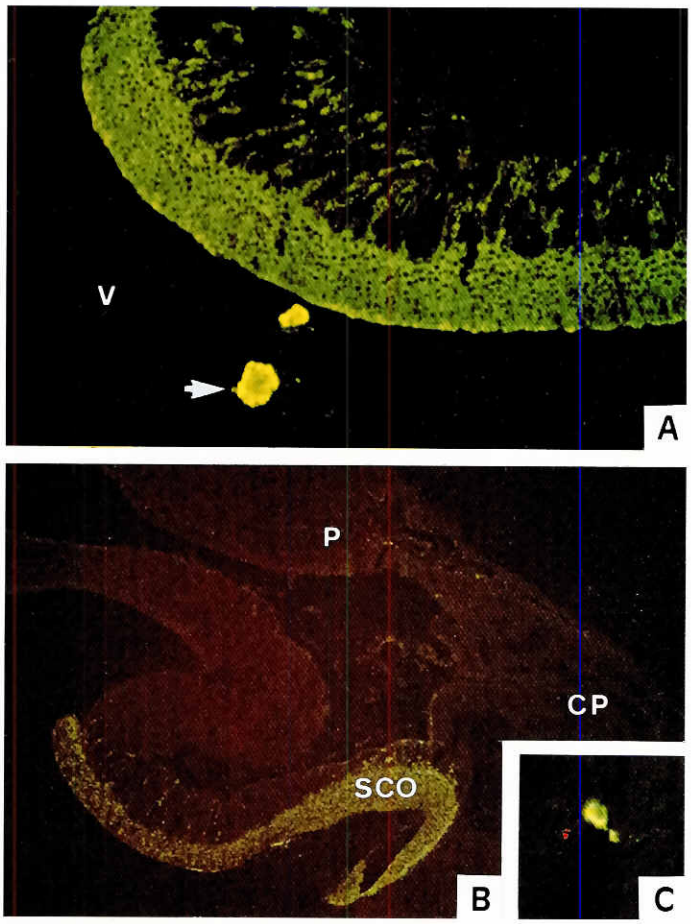

Fig. 1. Immunostaining of the subcommissural organ (SCO) and Reissner's fiber (RF) in a 19-day-old chick embryo using anti-bovine RF serum. (A and B) *Sagittal sections of the SCO.* **(A)** *Strong immunoreaction of the SCO and material (arrow) extruded into the ventricular cavity (V). x126.* **(B)** *The SCO is the only positive structure, no immunostaining in the pineal organ (P) or choroid plexus (CP). x51.* **(C)** *Transverse section of the spinal cord in the thoracal region. RF material is strongly immunoreactive. x315.*

SCO (Fig. 1A). The secretory material released into the third ventricle by the SCO cells also displayed an intense immunofluorescence. No labeling was detected in the pineal gland, the choroid plexus or the adjacent nervous tissue (Fig. 1B).

Reissner's fiber also displayed a positive reaction in the central canal of the thoracal spinal cord (Fig. 1C). No immunoreactive structures other than RF were observed.

First dimension gel analysis

Figure 2 shows the pH gradient established in the gels by carrier ampholytes after 12 h of electrophoresis towards the cathode. The pH gradient was linear along the first 10 cm and ranged from about 4.3 to about 8.9. As previously reported by O'Farrell *et al.* (1977), it was not stable in the most basic part of the gels, and there was a collapse of the pH gradient in the last 2 cm.

After electroblotting and treatment with Concanavalin-A (Con-A), which binds to glycopeptides rich in mannosyl and glucosyl residues, numerous Con-A positive bands were detected in the various extracts. Comparison of the SCO with the other brain tissue extracts showed a Con-A binding glycopeptide specific to the SCO electrophoretic profiles. This strongly Con-A labeled glycopeptide was present in both the soluble (Fig. 3A) and membrane (Fig. 3B) fractions of this organ with however a higher concentration in the soluble fraction. The latter was found near the top of the NEPHGE gel in the acid region at an isoelectric point of about 4.7.

Using wheat germ agglutinin (WGA), which binds to glycopeptides rich in N-acetyl-D-glucosamine and/or neuraminic acid residues, a large number of WGA binding proteins were found in each fraction. Examination of the various WGA positive patterns allowed us to detect a specific band in the soluble (Fig. 3B) and membrane (Fig. 3E) fractions of the SCO at the same pH as that previously described with Con-A.

On immunoblotting, only the band revealed by both lectins in the two fractions of the SCO extracts immunoreacted with the polyclonal antibody raised against bovine RF (Fig. 3C-F). In control experiments, in which the primary antibody was omitted, no staining was detected. With this antiserum no cross-immunoreactive polypeptides were visible on the Western-blots of pineal gland or cerebral hemisphere extracts (Fig. 3C,F).

Second-dimension gel analysis

After silver nitrate staining of two-dimensional gels, a complex polypeptide pattern was observed in soluble and membrane fractions of each tissue studied. More than 400 spots could readily be seen. The majority of components exhibited a molecular weight ranging from 30,000 to 200,000 daltons.

Analysis of the protein subunit patterns showed that the specific band previously identified after NEPHGE at an isoelectric point of 4.7 in the SCO extracts was resolved on second dimension gels into a single spot. This spot, present in both the soluble (Fig. 4A) and membrane (Fig. 4B) fractions, was characterized by a high molecular weight of about 390,000 daltons. It was absent from all other brain samples examined (Fig. 4C,D,E,F). No other qualitative differences were visualized in the SCO samples. All spots visible in the SCO fractions were tracked down either in pineal organ or in cerebral hemisphere extracts, although some quantitative differences were observed regarding several spots.

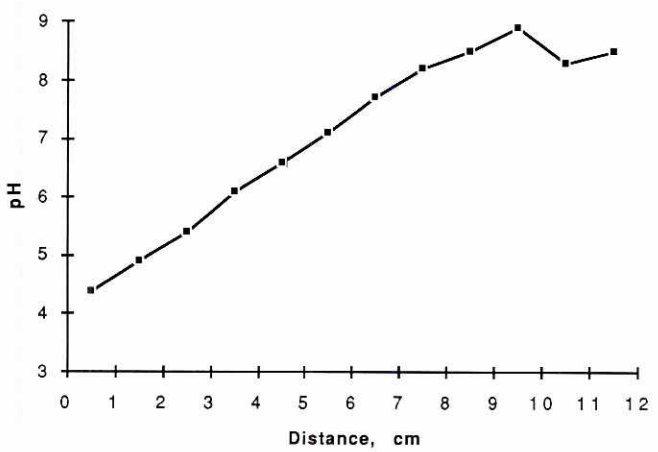

Fig. 2. pH gradient for the first dimension gel (NEPHGE). *The top of the gel (acidic end) is on the left.*

After electrotransfer, staining of Western blots by lectins Con-A and WGA revealed numerous spots in each fraction. The specific polypeptide of the SCO extracts displayed affinity for the two lectins used, as previously demonstrated in first dimension gels (Fig. 5A,B). Except for this polypeptide, these lectins revealed no additional specific glycopeptides in the SCO extracts.

Immunoblot analysis of the SCO extracts demonstrated that only this polypeptide was immunoreactive with the RF antiserum (Fig. 5C). No immunoreactive components were found in the other brain extracts.

Discussion

A comparative analysis of polypeptides synthesized in the chick embryo by the SCO and other brain structures (cerebral hemispheres, pineal organ) was performed using two-dimensional gel electrophoresis and Western blotting. The combined use of immunoblotting and blot lectin binding appears to be a suitable method to attempt to characterize the components of the secretory material of the SCO.

A comparison of protein patterns revealed that a specific polypeptide was present in the SCO extracts. This component, identified in both the soluble and membrane fractions, was absent in all the other cerebral structures studied. From the present study, a few characteristics of this polypeptide may be defined. It is a soluble protein most of which can be extracted from the SCO by homogenization in a low-ionic-strength buffer, although a certain amount remains trapped in the membrane fraction. Moreover, this compound is a highly acid molecule (isoelectric point of 4. 7) with a high molecular weight of about 390 kDa.

Immunofluorescent material was revealed by immunohistochemistry in the SCO ependymal cells and RF of the chick embryo after exposure to the bovine RF antiserum. The intense immunoreaction in both the apical lining and the entire parenchyma suggests that this antiserum recognizes the SCO secretory material throughout the secretory pathway. Using an anti-bovine RF serum, Sterba *et al.* (1982) and Schoebitz *et al.* (1986) failed to observe an immunoreactivity of RF in the chick embryo. According to Rodriguez *et al.* (1987), a "coat" formed by sialic acid residues could decrease or prevent the accessibility of the antibodies to the epitopes (Rodriguez *et al.*, 1987). In the present study, RF in the chick embryo immunoreacted strongly with our antiserum, suggesting that antigenic determinants are unmasked. One possible explanation for this discrepancy may be ascribed to variations in the number, nature and location of the antigenic determinants recognized by the various antibodies.

This specificity of the anti-bovine RF serum for compounds of the chick embryo SCO was further confirmed by the results of immunoblot analysis. In the SCO extracts, only the 390 kDa polypeptide was immunoreactive and no cross-immunoreactivity was found in the other brain structures studied. This polypeptide

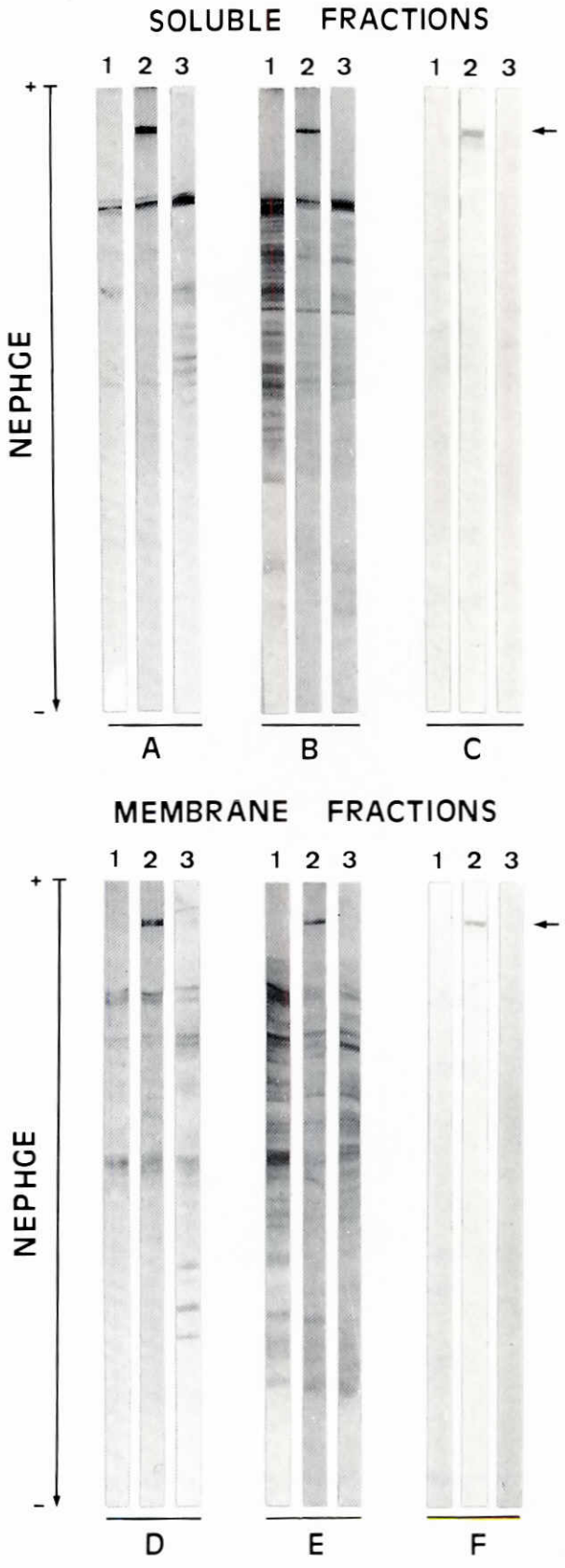

Fig. 3. Western-blots from NEPHGE. *Analysis of soluble and membrane fractions from cerebral hemispheres* **(lane 1)**, *SCO* **(lane 2)** *and pineal organ* **(lane 3)**. *A,D: Con-A binding. B,E: WGA binding. C,F: Immunoblotting using RF antiserum. Details on sample preparation are given in Materials and Methods. Position of specific band in the SCO fractions is marked by arrows.*

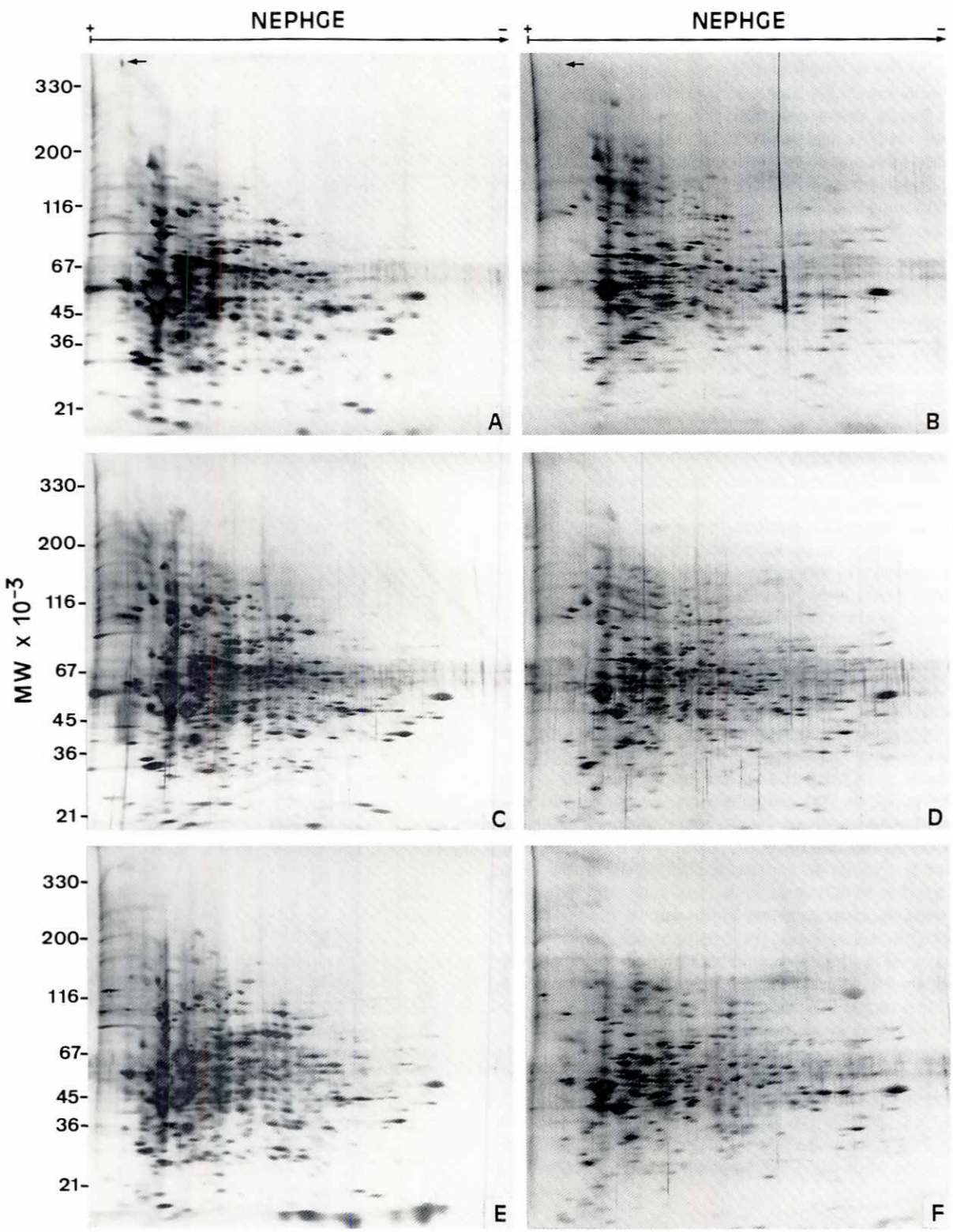

Fig. 4. Two-dimensional gel electrophoresis of SCO, pineal organ and cerebral hemispheres extracts from 19-day-old chick embryos. *Silver nitrate staining. Protein patterns of soluble* **(A)** *and membrane* **(B)** *fractions of SCO, soluble* **(C)** *and membrane* **(D)** *fractions of pineal organ, soluble* **(E)** *and membrane* **(F)** *fractions of cerebral hemispheres. Specific spot detected in the SCO extracts is indicated by arrows. MW, molecular weight standards in kDa.*

can therefore be regarded as a specific component synthesized by the secretory cells of the SCO that probably contributes to the formation of RF.

In mammalian SCO, it has been shown that both components of the secretory glycoproteins, i.e., protein and carbohydrate moieties, undergo an intracellular maturation process (Rodriguez *et al.*, 1986; A. Meiniel *et al.*, 1988; Rodriguez *et al.*, 1992). Thus, precursor material located in the rough endoplasmic reticulum is Con-A positive whereas apical granules and material released into the ventricular cavity are WGA-positive. The lectin staining of protein blots is a means of assessing the surface carbohydrate moieties on glycopeptides (Goldstein and Hayes, 1978) and has shown that the 390 kDa polypeptide visualized by RF antiserum in the SCO has affinity for both Con-A and WGA. Its affinity for WGA indicates that this component has passed through the Golgi apparatus and thus could represent the processed form. In our immunoblot analysis only this polypeptide was identified and we found no other specific glycoproteins binding Con-A but not WGA that could correspond to the precursor form as previously observed in the bovine SCO (Nualart *et al.*, 1991). The present finding in the chick embryo raises the following possibilities: 1) the precursor form could be a polypeptide of very high molecular weight that could not be revealed under our experimental conditions, especially by the acrylamide concentrations used; second dimensional gels can only reveal polypeptides with apparent molecular weights lower than 500 kDa. 2) In the SCO of the chick embryo, the peptide chain and glycosylation processing of the precursor form could give rise to a mature form with minor changes in both charge and size. Consequently, in our electrophoretic conditions the partition between these two forms would not be clearly detectable and they would co-migrate.

Most experiments attempting to identify the glycoproteins secreted by the SCO were performed using one dimensional gel electrophoresis (Meiniel *et al.*, 1986; Karoumi *et al.*, 1990a, 1991; Nualart *et al.*, 1991; Rodriguez *et al.*, 1992; Perez *et al.*, 1993). However, despite the information provided by these works, this aspect of the secretory material of the SCO is not entirely understood and is still a matter of discussion.

Using an antiserum against a crude extract of chick embryo SCO and after immunoaffinity chromatography, Karoumi et al. (1990a) detected numerous glycopeptides in the SCO eluted fractions of the chick embryo but none of these compounds was visualized in the present investigation. However, these authors speculated that the epitopes recognized by SCO antiserum do not correspond to the secretory product only but also to proteins specific of the particular phenotype of the secretory ependymocytes. Moreover, the fact that the antiserum against chick SCO did not cross-immunoreact with bovine SCO (Karoumi, 1990) implies that the anti-SCO serum does not recognize the same epitopes as anti-RF serum; RF antiserum would recognize epitopes conserved and synthesized by the SCO of most vertebrate species whereas antiserum against SCO extract would recognize epitopes which are class-specific. In addition, divergence in the number and biochemical characteristics of polypeptides revealed by the two types of antisera suggests that these various antigenic determinants are not shared by the same protein subunits. These possibilities are strongly supported by the findings of Perez *et al.* (1993) in the dogfish SCO. On immunoblots, bovine RF antiserum revealed a band with an apparent

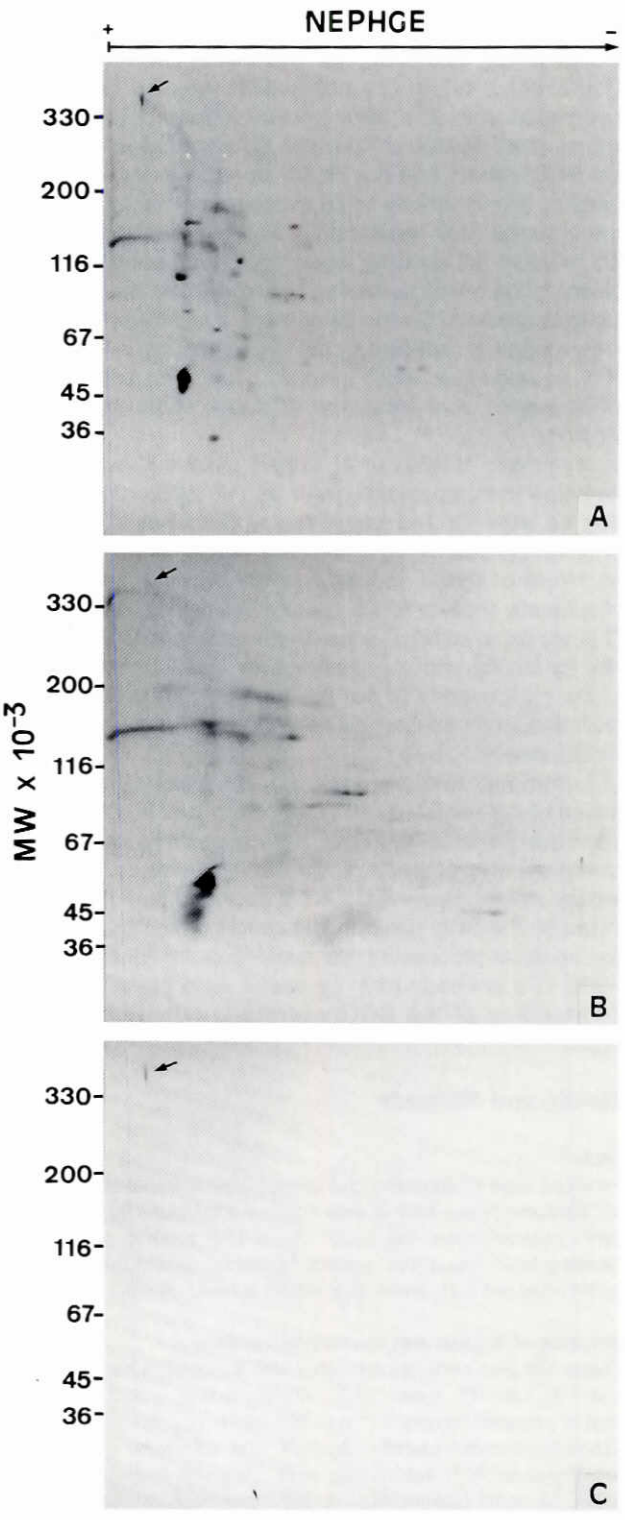

Fig. 5. Western blotting of two-dimensional gel electrophoresis. *Analysis of the SCO soluble fraction.* **(A)** *Con-A binding;* **(B)** *WGA binding;* **(C)** *immunoblotting with RF antiserum. Specific spot in SCO, marked by arrow, is reacted with the two lectins and recognized by the antibody.*

mass of 360 kDa while an antiserum against extract of dogfish SCO did not recognize this band but a polypeptide with a lower molecular weight (225 kDa). The discrepancy could also be due to the different procedures applied. It may be that specific glycoproteins revealed after immunoaffinity chromatography (Karoumi *et al.*, 1990a) are present in very small amounts in the whole SCO extract and not clearly detectable by our technical procedure. Immunoaffinity could increase their concentration and hence enhance their visualization. Furthermore, the high cross-linked gradient gel used by these authors probably restricts the resolution of components having high molecular weights, i.e., the 390 kDa compound. On the other hand, it is unlikely that the 390 kDa glycopeptide detected in the present study is a multimeric form or an aggregate which includes other components because reducing agents were present at all stages of the electrophoretic separation.

In mammals, Nualart *et al.* (1991) used a similar approach associating immunostaining with an RF antiserum and lectin binding on blots. On extracts of bovine SCO they detected three main glycopeptides of high molecular weight (540, 450 and 320 kDa). None of these compounds corresponds exactly in its electrophoretic mobility to the specific polypeptide detected in the SCO of the chick embryo. However this polypeptide has the same affinity for lectins and immunoreactivity to RF antiserum as the 450 kDa glycopeptide of the bovine SCO. Thus, we may also suspect that there are species differences in the molecular weight of similar proteins.

In summary, two dimensional gel electrophoresis and immunoblotting revealed the presence in the SCO cells of the chick embryo of a specific polypeptide with particular biochemical characteristics, i.e., acid molecule with a high molecular weight. However, it is not excluded that other proteins secreted by the SCO either in low concentration or submitted to an extracellular processing are involved in the RF formation. An anti-390 kDa antibody may be useful as a probe for a better understanding of the SCO secretory activity in the chick embryo.

Materials and Methods

Animals

Fertilized eggs of domestic fowl (*Gallus gallus domesticus*, Malvoisine strain) were purchased from a local supplier and incubated at 38°C. After 19 days of incubation, the SCO region with a small portion of the surrounding brain tissue, the cerebral hemispheres and the pineal organ were dissected out and immediately frozen in liquid nitrogen.

Preparation of soluble and membrane fractions

Tissue samples were homogenized with a glass-teflon homogenizer in cold 10 mM Tris-HCL buffer pH 7.5 (7 ml/g tissue) supplemented with a cocktail of protease inhibitors (1 mM phenylmethyl sulfonylfluoride, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis-[β-aminoethylether]-N,N'-tetraacetic acid, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml chymostatin, 10 μg/ml pepstatin A) and sonicated at low energy 4 times for 10 sec. The sonicated preparations were then centrifuged at 140,000xg for 90 min at 4°C in a Beckman ultracentrifuge. The supernatant constituting the soluble fraction was carefully collected and stored in aliquots in liquid nitrogen. The resulting pellet was resuspended in Tris-HCL buffer and centrifuged once again at 140,000xg for 90 min. The supernatant was discarded and the membrane pellet stored in liquid nitrogen until used.

Two-dimensional gel electrophoresis

The non-equilibrium pH gradient electrophoresis (NEPHGE) system of O'Farrell *et al.* (1977) was used for the separation in the first dimension to examine basic and acid proteins with the following modifications. NP40 was replaced by CHAPS, a zwitterionic derivative of cholic acid that improves resolution by inhibiting the formation of charge aggregates (Perdew *et al.*, 1983). Only pH 3.5-10 ampholytes were used. Isoelectric focusing was performed on vertical slab gels (14 cm wide, 12 cm long, 0.15 cm thick). The samples were prepared for electrophoresis as described elsewhere (Didier and Croisille, 1989). A protein sample of 1 mg proteins was applied across the whole width of the gel in a preparative manner to obtain about 70 μg proteins per cm. Focusing was performed at 400 v for 12 h. First dimensional gels were cut in longitudinal strips (1 cm wide). One strip was used for measurement of the pH, and the others were either equilibrated in equilibration buffer (3% SDS, 0.1 M DTT; 10% glycerol; 62 mM Tris-HCL pH 6.8) for 20 min and frozen at -80°C, or used for electroblotting.

Second dimension electrophoresis was performed on slab gels (5-15% linear gradient acrylamide, 16 cm wide, 12 cm long, 0.15 cm thick) with the discontinuous system of Laemmli (1970). The first dimension strips were laid over the stacking gel of the second dimension gel. To prevent intermolecular disulfide bound formation during electrophoresis, 10 mM 2-mercaptoethanol was added to the upper reservoir buffer (Fritz *et al.*, 1989). Gels were calibrated with a set of "low" and "high molecular weight" marker proteins (Pharmacia LKB, Uppsala, Sweden) ranging from 14,400 to 330,000 Daltons.

Staining

Two-dimensional gel staining was performed by the silver nitrate method as described by Gorg *et al.* (1985).

Protein electrotransfer

Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA) as described by Towbin *et al.* (1979).

For NEPHGE gel transfer, the buffer was 0.7% acetic acid and 10 mM 2-mercaptoethanol in distilled water. Blotting was conducted towards the cathode for 3 h at 70 V. For proteins separated by two-dimensional electrophoresis, electroblotting was done towards the anode with a transfer buffer of 25 mM Tris, 192 mM glycine and 15% methanol. This buffer also contained 0.005% SDS and 10 mM 2-mercaptoethanol to enhance electroblotting of the high molecular weight proteins. Blotting was performed at 70 V for 3 h.

Detection of glycoproteins

After electrotransfer, the blots were soaked for 1 h at 37°C with 0.1% Tween 20 in Tris-buffered saline (TBS) and glycopeptides were revealed with Con-A and wheat germ agglutinin (WGA). One blot was incubated with Con-A (30 μg/ml) for 1 h and then with horseradish peroxidase (50 μg/ml) for 1 h under constant stirring. Another blot was incubated in biotinylated WGA (2 μg/ml) for 4 h at 37°C and treated with the avidin-biotin-peroxydase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. The peroxydase activity was revealed by 4-chloro-1-naphtol (Con-A binding) or diaminobenzidine (WGA binding).

Polyclonal antibody

Bovine spinal cords were obtained at the slaughterhouse. Segments about 5 cm long were perfused through the central canal with Phosphate-buffered saline (PBS) and RF collected in the perfusate. Fibers were rinsed 3 times in water and then in 6 M urea for 10 min. They were incubated in 9 M urea overnight and sonicated. This preparation was emulsified with an equal volume of complete Freud's adjuvant and subcutaneously injected at multiple sites in a rabbit. The rabbit received a quantity of proteins equivalent to approximately 150 fiber segments. After 20 days it was again subcutaneously injected with the same quantity of RF

in incomplete Freud's adjuvant. The rabbit was bled 10 days after the last injection.

Immunocytochemistry

Cryostat sections (10 μm thick) of 19-day-old chick embryo SCO and thoracal spinal cord were prepared. After saturation with 3% bovine serum albumin (BSA) in PBS, the sections were incubated with a drop of the polyclonal antibody diluted 1/500 in PBS overnight. The slides were then washed with PBS and incubated with an anti-rabbit IgG (H+L) labeled with FITC (Bio-Yeda, Rehovot, Israel) diluted 1/400. After washing and mounting in glycerol water (1:1), they were examined with a Leitz fluorescence microscope.

Immunoblotting

Non-specific protein binding sites were blocked by incubating the blots in 10% non-fat milk in 50 mM TBS pH 7.5 for 1 h at 37°C. The blots were incubated for 3 h with polyclonal antibody diluted 1/250 in TBS containing 1% non-fat dry milk. After washing, they were incubated for 2 h in a 1/250 dilution of goat horseradish peroxidase-conjugated anti-rabbit IgG (H+L) (Byosis, Compiègne, France). The peroxydase reaction was developed with 4-chloro-1-naphtol. Control incubations were performed as described above except that the primary antibody was omitted.

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