Biochemical characterization of hamster oviductin as a sulphated zona pellucida-binding glycoprotein

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Oviductins are a family of glycoproteins, synthesized and released by oviductal secretory cells, which bind to the zona pellucida of the oocyte after ovulation. Hamster oviductin migrates as diffuse species of 160–350 kDa during SDS/PAGE under reducing as well as non-reducing conditions. In this report, we describe the one-step purification of hamster oviductin using either immuno- or lectin-affinity chromatography. Probing with specific lectins showed that the glycoprotein contains terminal α-D-GalNAc, and either terminal α-D-NeuAc or non-terminal β-D-$(GlcNAc)_2$ residues, but fails to react with concanavalin A and *Ulex Europeus* A-1 lectins which are specific for branched α-D-mannose and α-L-fucose moieties respectively. Intraovarian oocytes do not contain this glycoprotein and we demonstrate here that the immunoaffinity-purified oviductin readily binds to their zonae pellucidae *in vitro*, thus mimicking the *in vivo* phenomenon. Two major immunologically related forms of hamster oviductin (named α and β) were characterized using one- and two-dimensional gel electrophoresis. The α-form (160–210 kDa) has an acidic pI of 3.5–4.5 and the β-form (approx. 210–350 kDa) is localized at the cathodic site in the isoelectric focusing dimension; in between these two major forms lies a smear of minor-charge isomers. Peptide mapping of both major forms with papain and *Staphylococcus aureus* V8 protease yielded fragments of identical size. Moreover, the two forms share the same N-terminal sequence which display no significant homology with other reported proteins. Treatment with trifluoromethanesulphonic acid showed that a protein with the size and pI of the α-form can be generated from the β-form. Both the α- and β-forms are sulphated on O-linked oligosaccharide side chains but are not phosphorylated. Collectively, these results suggest that the hamster oviductin polymorphism observed in two-dimensional PAGE is a consequence of different glycosylation patterns and not the polypeptide chain itself. Hamster oviductin is mostly O-glycosylated and contains a few N-linked oligosaccharide side chains (approx. 10 kDa). We propose that hamster oviductin is a mucin-type glycoprotein which might act as a protective secretion influencing the first steps of the reproductive process necessary for the normal triggering of fertilization and early embryonic development.

INTRODUCTION

The oviduct provides the optimal milieu for fertilization and early development of the embryo. Numerous studies have demonstrated that this organ is involved in the final elaboration of the zona pellucida (zp) of the ovulated oocyte by secreting zp-binding glycoproteins. Such proteins have been described in the rabbit [1], pig [2,3], sheep [4], baboon [5], cow [6], mouse [7] and hamster [8–13]. The generic term 'oviductin' has been proposed [12] to collectively designate this new family of glycoproteins which are synthesized and released by the oviductal secretory cells and which later become associated with the ovulated egg and early embryo. It should be emphasized that the presence of oviductins has been reported in all mammalian species investigated thus far, and also in some amphibians [14,15].

Analyses by two-dimensional PAGE of bovine [16], sheep [4] and baboon [17] oviductins revealed a typical pattern of heterogeneous isoelectric forms ranging from acidic to basic pI. Recently, a partial cDNA sequence corresponding to the 85 C-terminal residues of a baboon oviductin was found to hybridize oviductal RNA from several species including the human [17], suggesting a certain degree of structural similarity for the polypeptide portion of oviductins. However, difficulties encountered to date in the isolation and purification of oviductins have delayed the elucidation of their structure and biological function(s). A purified oviductin would provide an invaluable tool for the direct study of the structural and functional properties of these glycoproteins. As they are added to the zp after ovulation, oviductins might play important roles in normal *in vivo* fertilization, in the process of implantation or could act as a protective barrier between the oocytes or preimplantation embryo and their environment.

This laboratory has previously described the *de novo* synthesis of a glycoprotein secreted by the hamster oviduct designated as hamster oviductin [12]. A monoclonal antibody (mAb 4,12) raised against hamster oviductin [13] reacts with the zp of ovulated oocytes and the secretory granules of oviductal secretory cells, but shows no affinity for the zp of intraovarian oocytes or the cumulus cells surrounding the ovulated eggs. The oviductal ciliated cells are not immunolabelled except for the external surface of the cilia expanding in the lumen of the tract [13,18,19]. Chemical deglycosylation of oviductin with trifluoromethanesulphonic acid (TFMSA) revealed the glucidic nature of the epitope recognized by mAb 4,12 [12]. The fact that human blood group A erythrocytes are agglutinated by mAb 4,12 indicates

Abbreviations used: Con A, concanavalin A; D-PBS, Dulbecco's phosphate-buffered saline; FITC, fluorescein isothiocyanate; hCG, human chorionic gonadotropin; HPA, *Helix pomatia* agglutinin; HRP, horseradish peroxidase; IEF, isoelectric focusing; i.p., intraperitoneal; mAb, monoclonal antibody; Me_2SO, dimethyl sulphoxide; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride); PVP, poly(vinylpyrrolidone); TBS, Tris-buffered saline; TFMSA, trifluoromethanesulphonic acid; UEA I, *Ulex europaeus* agglutinin I; WGA, wheat germ agglutinin; zp, zona pellucida; PMSG, pregnant mare serum gonadotropin.

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that the epitope comprises a terminal *N*-acetyl-α-D-galactosamine residue (G. Bleau, unpublished work).

In the present paper, we report on the purification of hamster oviductin to apparent electrophoretic homogeneity. We document the ability of the purified protein to bind to the zp of intraovarian oocytes *in vitro* and provide a description of certain biochemical properties including the sequence of the 18 N-terminal amino acids. Finally, we propose that hamster oviductin is a sulphated mucin-type glycoprotein.

MATERIALS AND METHODS

Materials

Mature female hamsters (*Mesocricetus auratus*) and BALB/c male mice were purchased from Charles River Inc., St-Constant, Québec, Canada. Pregnant mare serum gonadotropin (PMSG, Equinex) and human chorionic gonadotropin (hCG, APL) were from Ayerst. CDI-activated Reacti-gel 6X was purchased from Pierce. Horseradish peroxidase (HRP)-labelled wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin I (UEA I), *Helix pomatia* agglutinin (HPA) and concanavalin A (Con A) lectins as well as HPA–agarose (1.5 mg of lectin/ml) were obtained from Sigma. HRP and fluorescein isothiocyanate (FITC)-labelled rabbit anti-(mouse IgG) antibody were from ICN. Ampholytes (pH 3.5–10) were obtained from Pharmacia/LKB. Poly-(vinylidene difluoride) (PVDF) membranes (Immobilon-P, 0.45 μm) were purchased from Millipore Corporation. [^{35}S]-Methionine (specific radioactivity 1000 Ci/mmol), [^{3}H]mannose (specific radioactivity 23 Ci/mmol), [^{32}P]phosphate (specific radioactivity 900–1100 mCi/mmol) and [^{35}S]sulphate (specific radioactivity 395 mCi/mmol) were purchased from New England Nuclear. Na^{125}I was obtained from Amersham.

Preparation of mAb 4,12–agarose immunoaffinity matrix

Hybridomas (cell line 4,12 F6) producing an IgG monoclonal antibody (mAb 4,12) against hamster oviductin [13] were injected i.p. (10^6–10^7 cells) into BALB/c mice. mAb 4,12 was purified from ascitic fluid as previously described [13] and coupled to Reacti-gel 6X (1 mg/ml of gel) according to the manufacturer's specifications.

Affinity purification of hamster oviductin with mAb 4,12– or HPA–agarose

Female hamsters were superovulated by sequential i.p. injections of 25 i.u. of PMSG and 25 i.u. of hCG after 48–52 h. The animals were killed by cervical dislocation 17 h later and their oviducts were excised and homogenized (6 oviducts/ml which yielded approx. 3 mg/ml protein) at 4 °C in 50 mM Tris/HCl/150 mM NaCl, pH 8.0, containing 0.02% NaN_3, 35 μg/ml aprotinin, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 1 μM leupeptin, 100 μM EDTA and 0.2 mM benzamidine (homogenization buffer). The homogenate was centrifuged at 105000 *g* for 1 h at 4 °C to remove insoluble material. Portions of the supernatant (750 μg) were loaded on either mAb 4.12–agarose (2 ml; 1 mg of IgG/ml) or HPA–agarose (2 ml; 1.5 mg of lectin/ml) columns pre-equilibrated with the homogenization buffer. The samples were incubated with the gel for 30 min and the columns were then washed extensively with 10 bed vol. of buffer consisting of 50 mM Tris/HCl, 300 mM NaCl and 0.02% NaN_3, pH 8.0: this washing fraction is referred to as F1. Hamster oviductin was eluted from mAb 4,12–agarose columns with 3 bed vol. of buffer consisting of 200 mM glycine/HCl, 150 mM NaCl and 0.02% NaN_3, pH 2.5 (F2). The same conditions were used to elute hamster oviductin from the HPA–agarose columns except that the glycine buffer contained 200 mM α-D-GalNAc. The flow rate of the columns was controlled at 10–15 ml/h. The F1 and F2 fractions were neutralized, desalted and concentrated using ultrafiltration on Centriprep-30 and Centricon-30 devices (Amicon). The columns were routinely regenerated for multiple use by washing with a minimum of 10 bed. vol. of the starting buffer.

Incubation *in vitro* of intraovarian oocytes with immunoaffinity-purified hamster oviductin

For each experiment, eight female hamsters were injected i.p. with 25 i.u. of PMSG. The animals were killed 72 h later, their ovaries were excised and the large follicles were punctured using 30-gauge steel needles. The mature oocytes were recovered essentially as described [9] and washed three times for 5 min in Dulbecco's phosphate-buffered saline (Gibco) containing 1 mg/ml poly(vinylpyrrolidone) (D-PBS/PVP). In each of the five different experiments, three groups of 25 oocytes were used. The first group of oocytes was incubated for 1 h at 37 °C in the presence of portions of affinity-purified oviductin (pool from two hamsters) resuspended in 200 μl of D-PBS/PVP. The second group of oocytes was incubated for 1 h in 200 μl of oviductal fluid (200 μg of proteins), which was collected as described [12]. For the third group (control), the oocytes were incubated for 1 h in 200 μl of D-PBS/PVP containing 1% BSA (fraction V, Boehringer-Mannheim). The three groups of oocytes were then washed as described above and incubated for 30 min in 200 μl of hybridoma 4,12 F6 culture supernatant containing 1% PVP and 0.4% BSA. At this stage, five oocytes from groups 1 and 2 were separately incubated with the culture supernatant of the mouse myeloma cells (SP2/0-Ag14) as negative controls [13]. After another washing, all of the oocytes from each group were finally incubated for 30 min in FITC-labelled rabbit anti-(mouse IgG) (dilution 1:1000) in D-PBS/PVP containing 0.4% BSA. The oocytes were then washed in D-PBS, mounted on slides and examined for FITC fluorescence at 250× magnification using epifluorescence with a Leitz Orthomat.

Electrophoretic methods

SDS/PAGE under reducing conditions was performed as described by Laemmli [20]. Two-dimensional PAGE was carried out by the protocol of Dunbar et al. [21]. Unless indicated, electrophoretic transfer on PVDF membranes was performed as described by Towbin et al. [22].

Immunodetection and lectin staining of Western blots

Immunodetection was carried out essentially as described by Robitaille et al. [12]. Briefly, the PVDF membranes were blocked for 1 h in TBS buffer (25 mM Tris/HCl, 500 mM NaCl, pH 7.5) containing 3% (w/v) gelatin, allowed to react 1 h with the hybridoma 4,12 F6 culture supernatant, incubated for 1 h with HRP-labelled rabbit anti-(mouse IgG) (1:2000 dilution in 1% gelatin/TBS) and finally developed with HRP colour development reagent as described by the supplier (Bio-Rad). For lectin staining, the membranes were blocked as above, incubated for 1 h with HRP-labelled Con A, WGA, UEA I or HPA lectins [0.08 unit of purpurogallin/ml in 1% (w/v) gelatin in TBS] and developed as described above.

N-terminal sequence determination of hamster oviductin

Portions of affinity-purified hamster oviductin, using either mAb 4,12–agarose or HPA–agarose chromatography (pool from 30 animals in each case), were resolved by SDS/PAGE (5–10%

gradient gels) and electrotransferred on PVDF membranes essentially as described by Matsudeira [23]. The membranes were washed with deionized water and stained with Coomassie Blue as described [23]. The visualized bands of oviductin α (160–210 kDa) and β (approx. 260–350 kDa) were cut out and separately analysed for their N-terminal sequence. N-terminal sequencing was carried out on oviductin bound to the PVDF membranes using an Applied Biosystems 473A sequencer (Foster City, CA, U.S.A.) equipped with an on-line phenylthiohydantoin detection system as described by Matsudeira [23]. Five different analyses (three on immunopurified and two on HPA-lectin-purified material) were performed. The sequence of the 18 N-terminal residues of hamster oviductin was compared with the GenBank database using the TFASTA program in the GCG software package.

Peptide mapping of the α- and β-forms of hamster oviductin

Immunoaffinity-purified hamster oviductin (pool from ten animals) was radiolabelled by the chloramine-T procedure [24]. ^{125}I-Oviductin (100000 c.p.m. total per digestion assay) and BSA (20 μg) were subjected to SDS/PAGE under reducing conditions on 5–10% gradient gels. After electrophoresis, the gels were stained with 0.1% Coomassie Blue R-250 in 50% methanol/10% acetic acid, destained in 5% methanol/7% acetic acid and autoradiographed for 1 h at −20 °C. Based on the autoradiograms and the position of the high-molecular-mass standards, the regions of the gel corresponding to the α- (160–210 kDa) and β- (260–350 kDa) bands of hamster oviductin were localized and excised (see inset of Figure 4). The 67 kDa band in the control experiment with BSA was also excised. The isolated α- and β-forms of hamster oviductin and the control BSA were then separately digested *in situ* using *Staphylococcus aureus* V8 protease, papain, chymotrypsin or trypsin (Boehringer-Mannheim) essentially as described by Cleveland et al. [25].

TFMSA treatment

Partial chemical deglycosylation of the α- and β-forms of oviductin was achieved using TFMSA (Sigma) essentially as described by Edge et al. [26]. Briefly, immunoaffinity-purified ^{125}I-oviductin (100000 c.p.m. total per deglycosylation assay) was subjected to reducing SDS/PAGE on 5–10% gradient gel and the α- and β-forms were isolated as described above. Gel slices were electroeluted using a Bio-Rad electroelutor apparatus as described by the supplier. The eluate was then lyophilized in Teflon screw-cap reacti-vials. The α- and β-forms were assayed for deglycosylation after a mild treatment with 100 μl of TFMSA/anisole (2:1, v/v) at 4 °C for 0–180 min. After careful neutralization of TFMSA with ice-cold 50% aq. pyridine and extraction of anisole with diethyl ether (−40 °C), BSA (1 mg/ml) was added as a carrier. The reaction mixture was then dialysed at 4 °C against 25 mM ammonium acetate, pH 4.5, lyophilized and analysed by SDS/PAGE and two-dimensional PAGE. In control experiments, 20 μg of BSA per deglycosylation assay were subjected to SDS/PAGE on 12% (w/v) polyacrylamide gels, the gel was stained, the BSA bands were cut out, electroeluted, lyophilized and subjected to TFMSA treatment in an identical manner.

Metabolic labelling

Female hamsters were superovulated and their oviducts were isolated as described above. The oviducts were incubated in Tyrode's salt solution supplemented with 1 mg/ml sodium bicarbonate, 0.01 mg/ml sodium pyruvate and 100 units of penicillin/streptomycin solution (Gibco); 1 ml of medium was used for four oviducts. After an equilibration period of 15 min, [^{35}S]methionine (100 μCi/ml), $Na_2{}^{32}PO_4$ (250 μCi/ml), $Na_2{}^{35}SO_4$ (500 μCi/ml) or [^{3}H]mannose (100 μCi/ml) were added and the oviducts were incubated in a shaking water-bath for 6 h at 37 °C. For continuous labelling with [^{3}H]mannose, [^{35}S]sulphate and [^{32}P]phosphate, the culture media contained a reduced level of glucose (100 μM) or were sulphate- or phosphate-free respectively. In the experiments using tunicamycin (Boehringer-Mannheim), the oviducts were first cultured for 1 h in the presence of the inhibitor [7.5 μg/ml, prepared by diluting a 10 mg/ml stock solution in dimethyl sulphoxide (Me_2SO)], then transferred to fresh medium containing tunicamycin (7.5 μg/ml) and either [^{35}S]methionine or [^{35}S]sulphate (250 μCi/ml) and then cultured for an additional 5 h. Control cultures of oviducts, excluding tunicamycin, were treated in an identical manner with an equal amount of Me_2SO. In the experiments using [^{35}S]-methionine, labelled oviductal zp (see Figure 2c) were isolated as described previously [12].

Precipitation of hamster oviductin with HPA–agarose

Samples of the radiolabelled proteins released into the incubation media were incubated in the presence of packed HPA–agarose (75 μg of lectin/300 μg of protein) and protease inhibitors (100 μM EDTA, 1 μM leupeptin and 0.2 mM PMSF). After 1 h at 25 °C, the supernatant was discarded and the HPA–agarose beads were washed five times with 1 ml of buffer consisting of 50 mM Tris/HCl and 300 mM NaCl, pH 8.0. The bound glycoproteins were eluted by boiling the beads for 5 min in 50 μl of SDS/PAGE sample buffer. The beads were pelleted and the supernatants were analysed directly by SDS/PAGE.

Acid treatment of [^{35}S]sulphate-labelled hamster oviductin

Portions of the HPA precipitates were separated by SDS/PAGE (5–10% gels) and treated with hot (95 °C) 1 M HCl in the gel, followed by fluorography as described by Huttner [27].

Analytical methods

The molecular mass was determined using the high-molecular-mass calibration kit of Bio-Rad. Measurements of pI were achieved by cutting out and eluting the proteins from the gel as well as a comparison with the two-dimensional PAGE standards of Bio-Rad. Protein concentration was determined by the method of Macart and Gerbault [28].

RESULTS AND DISCUSSION

Single-step purification of hamster oviductin by immuno- or lectin-affinity chromatography

The protein content of oviductal fluid consists of a complex mixture originating from plasma transudation and the secretion of specific glycoproteins [29,30]. As oviductins are synthesized and released only in minute quantities by oviductal secretory cells, the characterization of these glycoproteins and the elucidation of their biological function(s) have been hampered. The high affinity and specificity of mAb 4,12 for hamster oviductin allowed the purification of this protein to homogeneity using affinity chromatography. As shown by silver staining of SDS/polyacrylamide gels (Figure 1a), oviductin is only a minor component of the total oviductal proteins (lane 1). Non-specifically bound oviductal proteins were eluted by washing the column with buffer containing 50 mM Tris/HCl and 300 mM

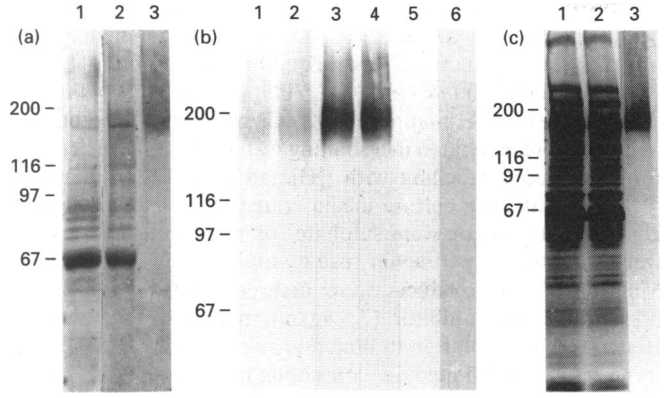

Figure 1 Single-step purification and lectin binding of hamster oviductin

(**a**) Hamster oviductin was immunoaffinity purified using mAb 4,12–agarose. Fractions were analysed using 5–10%-gradient SDS/PAGE under reducing conditions followed by silver staining by the procedure of Morrissey [31]. Lane 1, unfractionated oviductal homogenate (10 μg); lane 2, unretained fraction F1 (10 μg); lane 3, purified eluted hamster oviductin (pool of F2 fractions isolated from 3 mg of oviductal homogenate). (**b**) Portions of immunoaffinity-purified hamster oviductin (F2 fractions isolated from 750 μg of oviductal homogenate) were subjected to 5–15%-gradient SDS/PAGE followed by Western blotting. Lanes 1 and 2, immunodetection using mAb 4,12 with electrophoresis performed under reducing or non-reducing conditions respectively; lanes 3–6, probing of purified hamster oviductin (under reducing conditions) with HRP-labelled HPA, WGA, UEA I and Con A lectins respectively. (**c**) Hamster oviductin was affinity-purified on HPA–agarose, fractions were analysed by 5–15%-gradient SDS/PAGE under reducing conditions and the gel was silver-stained as in (**a**). Lane 1, unfractionated oviductal homogenate (30 μg); lane 2, unretained fraction F1 (30 μg); lane 3, purified eluted hamster oviductin (pool of F2 fractions isolated from 3 mg of oviductal homogenate). Molecular masses (kDa) indicated on the left.

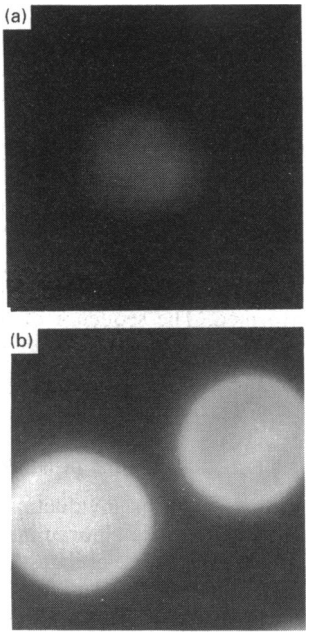

Figure 2 *In vitro* binding of purified hamster oviductin to the zp of ovarian oocytes

Intraovarian oocytes were incubated in the absence (control) or presence of immunoaffinity-purified hamster oviductin. The binding of the protein was detected using indirect immunofluorescence with mAb 4,12 and FITC-labelled rabbit anti-mouse IgG. The representative fluorescence micrographs show no specific reaction for a control oocyte (**a**), whereas the two oocytes incubated in the presence of purified hamster oviductin fluoresce strongly (**b**).

NaCl, pH 8.0 (Figure 1a, lane 2). Bound hamster oviductin (approx. 160–350 kDa) was eluted with buffer containing 200 mM glycine/HCl and 150 mM NaCl, pH 2.5 (Figure 1a, lane 3). The presence of hamster oviductin in the washing and elution fractions was also monitored by immunoblotting. The immunoreactivity was essentially limited to fraction F2 (Figure 1b, lane 1). No change in mobility was observed whether purified oviductin was subjected to SDS/PAGE under reducing or non-reducing conditions (Figure 1b, lanes 1 and 2 respectively). Hamster oviductin contains terminal α-D-GalNAc residues, as it reacted with HPA lectin (Figure 1b, lane 3); it was also recognized by WGA lectin (lane 4), indicating the presence of either terminal α-D-NeuAc or non-terminal β-D-$(GlcNAc)_2$; however, it failed to react with UEA-1 (lane 5) and Con A lectins (lane 6), which are specific for α-L-fucose residues and high-mannose/hybrid N-linked oligosaccharide chains respectively. As immunopurified oviductin reacts with peroxidase-labelled HPA lectin, this led us to use commercially available HPA–agarose as a second approach for the affinity purification of hamster oviductin. This method also yielded an efficient single-step purification, as the eluted material (F2) was found almost exclusively in the region of the gel where hamster oviductin migrates (Figure 1c, lane 3).

In vitro binding of purified oviductin to the zp of ovarian oocytes

Oocytes from mature ovarian follicles were isolated and incubated in the presence of immunopurified oviductin. Indirect immunofluorescence gave a strong signal for the oocytes incubated with purified oviductin (Figure 2b) as well as with total secreted oviductal proteins (results not shown). Control oocytes that were not exposed to purified oviductin (Figure 2a) and those incubated with SP2/0 myeloma culture supernatant (results not shown) before FITC-labelled anti-(mouse IgG) did not fluoresce. These results show that the purified oviductin can effectively bind to the zp of the ovarian oocyte *in vitro*, thus imitating the *in vivo* process of uptake by ovulated eggs.

Two-dimensional PAGE of hamster oviductin

The biosynthetically labelled proteins ([^{35}S]methionine incorporation) that are synthesized and released by the oviducts into the culture medium were analysed by two-dimensional-PAGE. Autoradiography (Figure 3) and immunostaining of the same Western blots with mAb 4,12 (not shown) revealed that a very prominent feature of hamster oviductin is its extensive charge heterogeneity. Hamster oviductin consists of two major isoelectric forms: one acidic form named α (pI 3.5–4.5) and another one found at the cathodic site, named β. In between these two major forms lies a smear of minor overlapping charge isomers. Both the α- and β-forms are secreted into the oviductal fluid (Figure 3a), become associated with the ovulated zp (Figure 3b) and are co-purified using mAb 4,12–agarose affinity chromatography (Figure 3c), thus indicating that they share a common antigenic determinant. The α-form has a lower-molecular-mass (160–210 kDa) and a more diffuse profile in the isoelectric focusing (IEF) dimension (pI 3.5–4.5) than the β-form which appears as a sharp band at the cathodic site in the IEF dimension but displays a more heterogeneous molecular mass in SDS/PAGE (approx. 210–350 kDa). The β-form is not really basic, as we observed that it does not migrate in either classical two-dimensional PAGE or in a non-equilibrium pH-gradient electrophoresis system (results not shown). The β-form always remains at the point of application whether the sample is solubilized in O'Farrell lysis buffer [32] containing 9.5 M urea, 2% Triton, 5%

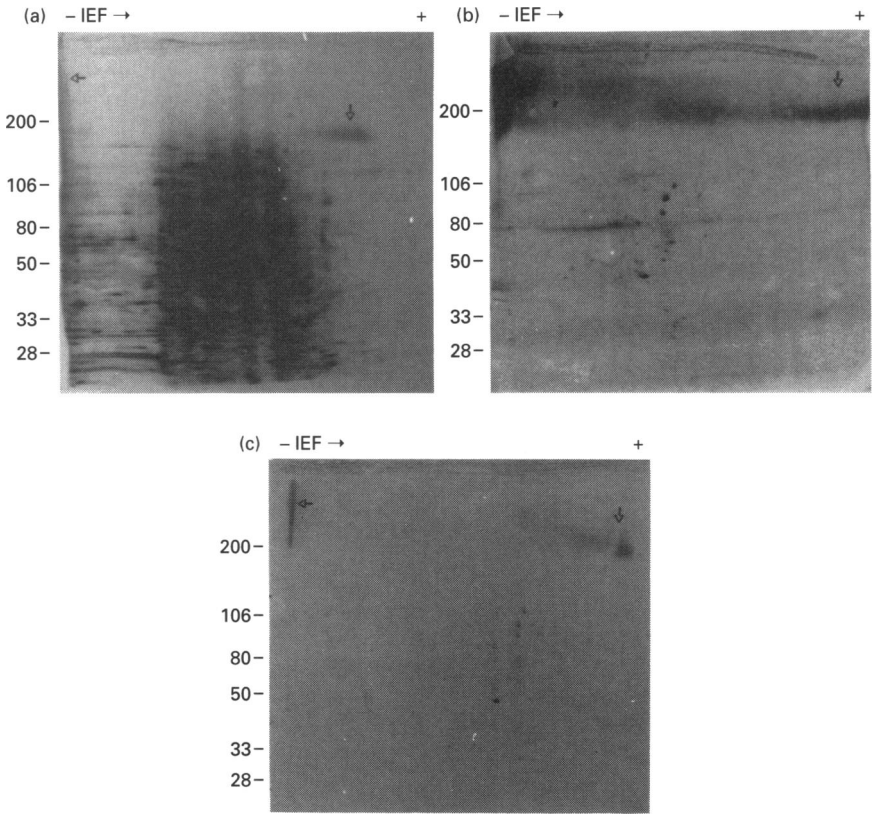

Figure 3 Two-dimensional PAGE of [^{35}S]methionine-labelled secreted oviductal proteins, zp proteins and immunoaffinity-purified hamster oviductin

Post-ovulatory oviducts were cultured in the presence of [^{35}S]methionine. The secreted products recovered in the incubation medium, the solubilized isolated zp proteins and immunoaffinity-purified hamster oviductin were analysed using IEF in the first dimension (horizontal, acidic end to the right) and reducing SDS/PAGE in the second dimension (5–10% acrylamide gradient). After Western blotting, the membranes were autoradiographed. (**a**) Secreted oviductal proteins (20000 c.p.m.); (**b**) zp proteins (60 zp, 5000 c.p.m.) and (**c**) purified hamster oviductin (5000 c.p.m.). The arrows indicate the positions of the α-form at pI 3.5–4.5 and the β-form localized at the cathodic site (as detected by immunostaining of the same Western blots, results not shown).

2-mercaptoethanol and 0.5% CHAPS or boiled in 0.05 M 3-[cyclohexylamino]-1-propanesulphonic acid buffer, pH 9.5, containing 2% SDS and 5% 2-mercaptoethanol [21], whereas the α-form migrates at pI 3.5–4.5 in both conditions. The poor mobility of the β-form in the IEF dimension may be due to extensive glycosylation, non-covalent aggregation or both. Carbohydrate-dependent self-association has been reported for ovine submaxillary and pig gastrointestinal mucins [33,34]. In the case of the ovine mucin, commonly used solvents for the dissociation of non-covalently bound protein subunits such as 0.1% SDS, 8 M urea or 1 mM 2-mercaptoethanol were ineffective; only high ionic strength or enzymic deglycosylation [35] could prevent aggregation. The glycosylated oviductal zp-binding proteins from cattle (97 kDa [16]), sheep (90–92 kDa [4]) and baboon (120 kDa [5]) closely resemble hamster oviductin in that they all resolve into two major isoelectric variants on two-dimensional PAGE: an acidic one and a so-called basic one. Polyclonal antibodies generated against each of the isoelectric forms of baboon 120 kDa oviductin cross-react with both the acidic and 'basic' components of the glycoprotein [5], showing that these two major isoelectric forms possess common antigenic sites, as found with hamster oviductin.

Peptide mapping of the α- and β-forms of hamster oviductin

Peptide mapping was performed in order to elucidate whether the α- and β-forms of hamster oviductin consist of two immunologically related but different glycoproteins or arise from different post-translational modifications. These experiments revealed a similar pattern of peptide fragments generated from both forms by papain and *Staphylococcus aureus* V8 protease (Figure 4, lanes 3–10). It is noteworthy, however, that most of the glycoprotein resisted digestion even in the presence of 10 μg of these enzymes. Moreover, chymotrypsin (lanes 11–14) and trypsin (results not shown) failed to digest the α- or the β-form of oviductin. In contrast, BSA treated in an identical manner was readily digested with 4 μg of trypsin, and the same amount of chymotrypsin resulted in the complete disappearance of the 67 kDa band (results not shown).

N-terminal sequence of hamster oviductin

Hamster oviductin, affinity-purified using either mAb 4,12– or HPA–agarose chromatography (pool from 30 animals in each case), was subjected to SDS/PAGE and electroblotted on to PVDF membranes. The Coomassie Blue-stained α- and β-bands were separately processed for sequencing and found to possess the same N-terminal sequence: YKLVAYFTNWAISRPVPA. A database search revealed no significant similarity to other reported protein sequences. These data, together with the peptide mapping results, strongly support the hypothesis that the α- and β-forms are indeed composed of a unique protein core that undergoes different post-translational maturation.

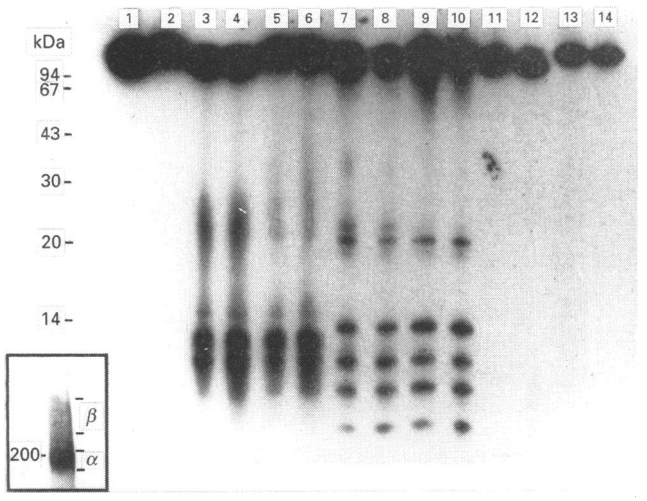

Figure 4 Peptide mapping of the α- and β-forms of purified hamster ^{125}I-oviductin

Autoradiogram of a reducing SDS/polyacrylamide gel (15%) showing the pattern of *in situ* protease digestion of the α- and β-bands of purified ^{125}I-labelled hamster oviductin. Lanes 1 and 2, undigested α- and β-bands (controls with no enzyme added); α- (lanes 3 and 4) and β- (lanes 5 and 6) bands treated with papain at 5 μg (lanes 3 and 5) or 10 μg (lanes 4 and 6) respectively; α- (lanes 7 and 8) and β- (lanes 9 and 10) bands treated with *Staphylococcus aureus* V8 protease at 5 μg (lanes 7 and 9) or 10 μg (lanes 8 and 10) respectively; α- (lanes 11 and 12) and β- (lanes 13 and 14) bands treated with chymotrypsin at 5 μg (lanes 11 and 13) or 10 μg (lanes 12 and 14) respectively. Inset: immunopurified ^{125}I-oviductin (100000 c.p.m. total) subjected to reducing SDS/PAGE (5–10% gels). The α- (160–210 kDa) and β- (approx. 260–350 kDa) regions were excised from the gel as indicated, based on the position of the high-molecular-mass standard of 200 kDa and the autoradiogram, and then subjected to *in situ* protease digestions.

TFMSA treatment of the α- and β-forms of hamster oviductin

Partial deglycosylation was used to qualitatively demonstrate whether the β-form could differ from the α-form by its higher degree of glycosylation. Both forms were treated with TFMSA under mild conditions, under which a rapid cleavage of peripheral sugars occurs along with a slow loss of serine- and threonine-linked GalNAc and retention of the first N-linked GlcNAc to asparagine [26]. As shown in Figure 5, the α-form does not seem to undergo any major changes even after 180 min of treatment: neither its molecular mass and heterogeneity during SDS/PAGE [which remains at 160–210 kDa (lane 9)] nor its IEF migration on two-dimensional PAGE (results not shown) are altered. In sharp contrast, the β-form, although still heterogeneous, undergoes a detectable shift in mobility as deglycosylation proceeds (Figure 5, lanes 4–6). The contribution of peripheral sugars to the charge heterogeneity of hamster oviductin was dramatically illustrated by the shift in electrophoretic behaviour exhibited by the TFMSA-treated β-form in the IEF dimension of two-dimensional PAGE compared with the untreated β-form. The isolated untreated β-form is mostly localized at the cathodic site (Figure 5c), whereas the migration pattern of the partially deglycosylated β-form drastically changes: the protein then migrates at an acidic pI of 3.5–5.0 (Figure 5c), resembling the situation encountered with the α-form. A similar treatment was applied to non-glycosylated BSA as a negative control; no proteolytic degradation occurred after such a treatment (Figure 5b, lane 4). These results led us to hypothesize that the β-form differs from the α-form by its higher degree of overall glycosylation, as, on partial deglycosylation, it behaves (molecular mass and pI) like the α-form. As alluded to earlier, the behaviour of the β-form in the IEF dimension of two-dimensional PAGE could result from non-covalent carbohydrate-dependent aggregation [33,34]. In this case, treatment with TFMSA would disrupt the existing polymeric structure and increase the mobility of the resulting product, thus making it resemble the less glycosylated non-aggregating α-form.

The α- and β-forms of hamster oviductin are sulphated on their carbohydrate moieties but are not phosphorylated

As the α-form migrates at a more acidic pH than the β-form, the possibility that phosphorylation or sulphation could account for this difference was investigated. After continuous *in vitro* labelling of oviducts, the culture media containing the secreted oviductal proteins were analysed by SDS/PAGE and two-dimensional PAGE and subjected to mAb 4,12–agarose affinity chromatography. No incorporation of [^{32}P]phosphate into secreted oviductin could be detected (Figure 6, lane 2) but several other secreted oviductal proteins were labelled (lane 1). On the other hand, [^{35}S]sulphate was incorporated into both the α- and β-forms as determined by SDS/PAGE after purification using mAb 4,12–agarose (Figure 6a, lane 4) and two-dimensional PAGE (Figure 6b, see arrows). Oviductin was found to be the major secreted protein labelled with [^{35}S]sulphate as shown in Figure 6(a), where the total secreted proteins (lane 3) are compared with the fraction purified by affinity chromatography through a mAb 4,12–agarose column (lane 4). As the tyrosine sulphate ester is acid-labile and hydrolysed faster than most carbohydrate sulphate esters [27], the HPA-adsorbed [^{35}S]sulphate-labelled oviductin was subjected to *in situ* acid hydrolysis essentially as described [27]. The comparison between the [^{35}S]sulphate labelling of the treated protein (Figure 7a, lane 3) and the untreated control sample (lane 2) indicates that sulphation occurs on the oligosaccharide residues. Moreover, when purified [^{35}S]sulphate-labelled oviductin was subjected to TFMSA treatment (Figure 7b), the intensity of the [^{35}S]sulphate-labelling drastically decreased as the deglycosylation proceeded (lanes 2–6). Taken together, these results confirm that sulphation takes place on the carbohydrate portion of the glycoprotein.

Effect of tunicamycin on the synthesis and post-translational modifications of hamster oviductin

For many glycoproteins, the carbohydrate groups confer important physical properties (for reviews see [38] and [39]) such as conformational stability [40,41], resistance to proteases [42] and mucosal protection [43,44]. Equally important are the roles of carbohydrate groups in biological recognition systems such as fertilization, where sequence diversity provides signals for gamete interaction [45,46]. Indeed, it was reported that mouse sperm bind to eggs via the terminal α-Gal of an O-linked oligosaccharide chain from a 3900 Da entity present on mZP3 [47]. As a clue for possible roles of hamster oviductin, we studied the type of linkage of the carbohydrate chains. Hamster oviductin is not a proteoglycan: treatments of the purified glycoprotein with heparinase III and/or chondroitinase ABC do not lead to any perceptible shift in molecular mass on SDS/PAGE (results not shown). Incorporation of [^{3}H]mannose into hamster oviductin (Figure 8, lane 10) indicates that it is *N*-glycosylated, and two-dimensional PAGE analyses of the labelled oviductin revealed that both the α- and β-forms possess mannose-containing oligosaccharides (results not shown). *In vitro* metabolic labelling in the presence of tunicamycin, an antibiotic that inhibits *N*-glycosylation of newly synthesized proteins [48], yielded labelled oviductin with a small shift in molecular mass of approx. 10 kDa

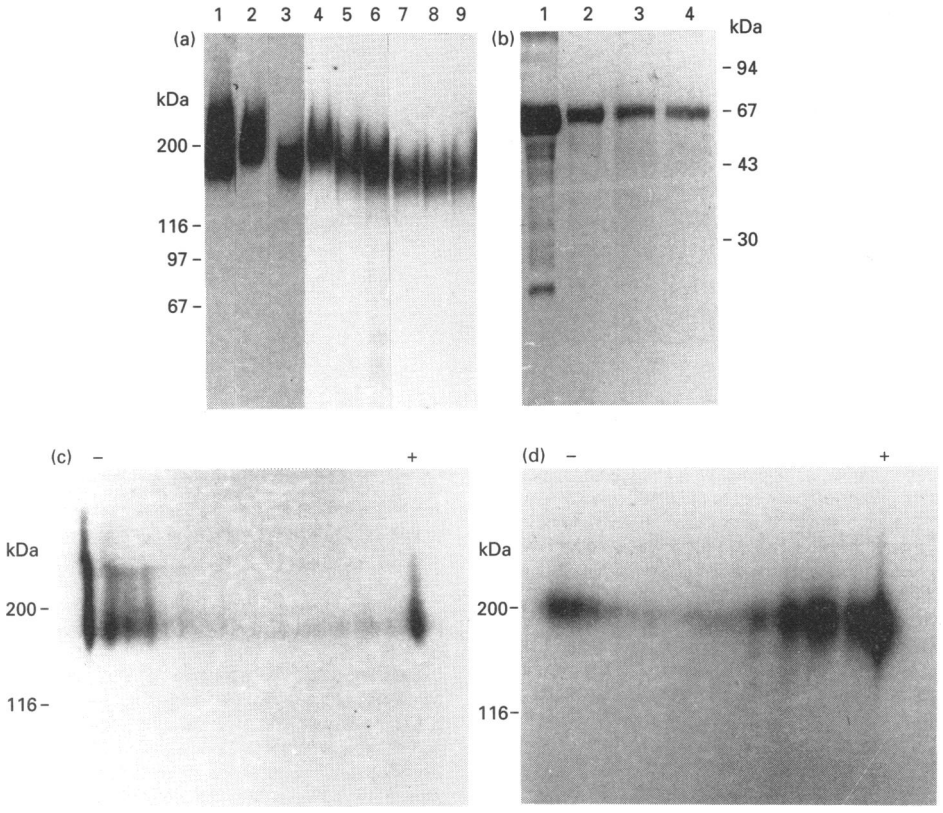

Figure 5 TFMSA treatment of the α- and β-forms of purified ^{125}I-labelled hamster oviductin

(**a**) Autoradiogram of a reducing 5–10%-gradient SDS/polyacrylamide gel monitoring the partial chemical deglycosylation of α- and β-forms with TFMSA. The α- and β-bands of immunoaffinity-purified ^{125}I-oviductin (100000 c.p.m. total per deglycosylation assay) were isolated from the reducing SDS/polyacrylamide gel, electroeluted and treated with TFMSA as described in the Experimental section. Lane 1, untreated total ^{125}I-oviductin (100000 c.p.m.); lane 2, untreated isolated β-band (100000 c.p.m.); lane 3, untreated isolated α-band (100000 c.p.m.); lanes 4–6, time course of TFMSA treatment of β-band (0, 90 and 180 min respectively); lanes 7–9, deglycosylation of α-band for 0, 90 and 180 min respectively. (**b**) Reducing SDS/polyacrylamide gel of BSA before and after chemical deglycosylation. Lane 1, 20 μg of commercial BSA (before electroelution); lane 2, untreated electroeluted BSA; lanes 3 and 4, BSA subjected to chemical deglycosylation with TFMSA for 0 and 180 min respectively. SDS/PAGE was performed on 12% gel with silver staining as described by Morrissey [31]. (**c**) and (**d**) Autoradiograms of two-dimensional PAGE of β-band treated for 0 (**c**) and 180 min (**d**) with TFMSA. The second dimension was performed on a reducing 5–10%-gradient gel as in (**a**): 1×10^6 c.p.m. were analysed in each gel.

(Figure 8, lanes 4 and 8), suggesting the presence of only a few *N*-glycan chains. Furthermore, treatment with tunicamycin did not modify the electrophoretic behaviour of oviductin during two-dimensional PAGE (results not shown). These results are consistent with our observation that probing of purified oviductin on Western blot with HRP-labelled Con A lectin did not yield any signal (Figure 1b, lane 6), suggesting that N-linked oligosaccharide side chains account for only a small proportion of the entire glycosylation of the glycoprotein and/or are probably hindered by the O-linked carbohydrate chains. Knowing that sulphation occurs on oligosaccharide side chains (Figure 7), the incorporation of [^{35}S]sulphate was evaluated in the presence or absence of tunicamycin so as to discriminate between sulphation on O- or N-linked glycans. The same 10 kDa shift in molecular mass was observed for [^{35}S]sulphate-labelled hamster oviductin in the presence of the drug (Figure 8, lane 8), suggesting that sulphation occurs on O-linked carbohydrates. The observations that hamster oviductin synthesized in the presence of the inhibitor of *N*-glycosylation remains very heterogeneous in one- and two-dimensional PAGE, conserves its immunoreactivity with mAb 4,12 and can bind to HPA–agarose (Figure 8) and WGA (result not shown) indicate that the protein is *O*-glycosylated, and is hence of mucin type.

Secretory mucin-type glycoproteins (for reviews see [49] and [50] constitute the viscous gel that covers, protects and lubricates most mucosal surfaces of the respiratory, gastrointestinal and reproductive tracts. Their protein backbone contains clustered regions rich in serine and threonine residues which are heavily glycosylated and negatively charged as a result of NeuAc and sulphate moieties. These glycosylated regions are highly resistant to proteolysis and are thought to confer the protective properties of mucins. Hamster oviductin displays several common properties with this family of proteins. It coats the oviductal epithelium, it is of high molecular mass, heterogeneous and *O*-glycosylated, negatively charged because of the presence of NeuAc and sulphate groups and possesses terminal GalNAc residues. Furthermore, it is resistant to digestion by trypsin and chymotrypsin, and hardly digested by other proteases. Like other characterized mucins, oviductin from hamster as well as other species could be a protective secretion acting as a selective physical barrier between the oocytes or early embryos and their environment.

The mechanism involved in the association of oviductins with the zp of the ovulated oocytes is still obscure. The possibility that hamster oviductin could bind to one or more of the recently characterized hamster zona pellucida proteins, hzp1, hzp2 or hzp3 [51], is at present being investigated. This report represents the first comprehensive biochemical analysis of a purified oviductin. The mechanisms determining the number, size and location as well as the composition of oligosaccharide chains have not been elucidated but polymorphism of hamster oviductin

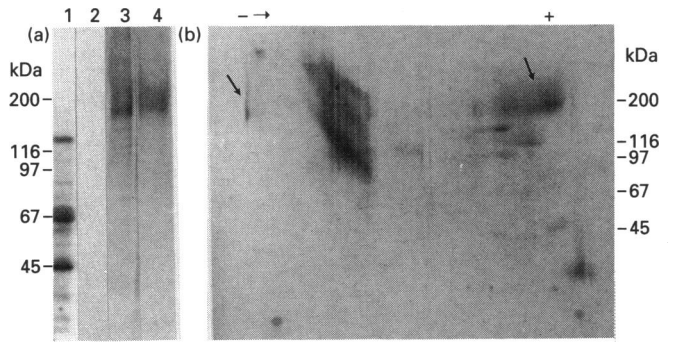

Figure 6 The α- and β-forms of hamster oviductin are sulphated but not phosphorylated

Oviducts were cultured for 6 h in the presence of [^{32}P]phosphate or [^{35}S]sulphate. The culture media containing secreted oviductal proteins were analysed by SDS/PAGE or two-dimensional PAGE or subjected to mAb 4,12–agarose affinity chromatography. (**a**) Autoradiogram of a 5–10%-gradient reducing SDS/polyacrylamide gel. Lane 1, [^{32}P]phosphate-labelled oviductal proteins released into culture medium (30 μg); lane 2, immunoaffinity-purified hamster oviductin isolated from 2 mg of [^{32}P]phosphate-labelled culture medium; lanes 3 and 4, the corresponding results obtained for [^{35}S]sulphate incorporation. (**b**) Autoradiogram of a Western blot after two-dimensional PAGE analysis of 150 μg of secreted [^{35}S]sulphate-labelled oviductal proteins. The arrows indicate the α- (at pI 3.5–4.5) and β- (at cathodic site) forms of hamster oviductin as confirmed by immunostaining of the same Western blot. The second dimension is a reducing 5–10%-gradient gel.

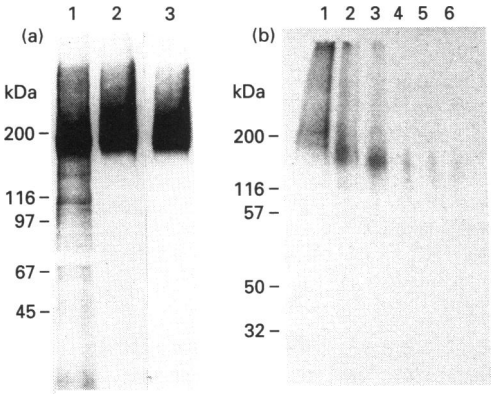

Figure 7 Hamster oviductin is sulphated on carbohydrate moieties

(**a**) [^{35}S]Sulphate-labelled hamster oviductin was subjected to *in situ* acid hydrolysis. Samples of [^{35}S]sulphate-labelled oviductal proteins secreted into the incubation medium were precipitated using HPA–agarose. Bound oviductin was eluted with electrophoresis buffer and subjected to reducing 5–15%-gradient SDS/PAGE. Sections of the gel were subjected to *in situ* acid hydrolysis with hot 1 M HCl or not (control) before autoradiography essentially as described by Huttner [27]. Lane 1, unfractionated oviductal proteins (30 μg); lane 2, HPA-precipitated proteins (isolated from 600 μg of culture medium); lane 3, HPA-precipitated proteins (isolated from 600 μg of culture medium) subjected to *in situ* hydrolysis with hot 1 M HCl in the gel before autoradiography. (**b**) [^{35}S]Sulphate-labelled hamster oviductin from culture medium (400 μg) was precipitated with HPA–agarose and then eluted with 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 200 mM GalNAc and 0.1% BSA as a carrier. After desalting using Centricon-30 devices and lyophilization in Teflon screw-cap reacti-vials, the material was treated with TFMSA. The samples were then analysed by 5–15%-gradient SDS/PAGE and autoradiography. Lane 1, untreated [^{35}S]sulphate-labelled HPA-precipitated hamster oviductin. Lanes 2–6, time course of TFMSA treatment of [^{35}S]sulphate-labelled HPA-precipitated hamster oviductin (15 min, 1 h, 2 h, 3 h and 4 h respectively).

may confer distinct roles, properties or localization to the different populations of glycosylated molecules. Structural analysis of the oligosaccharide moieties and the cloning of

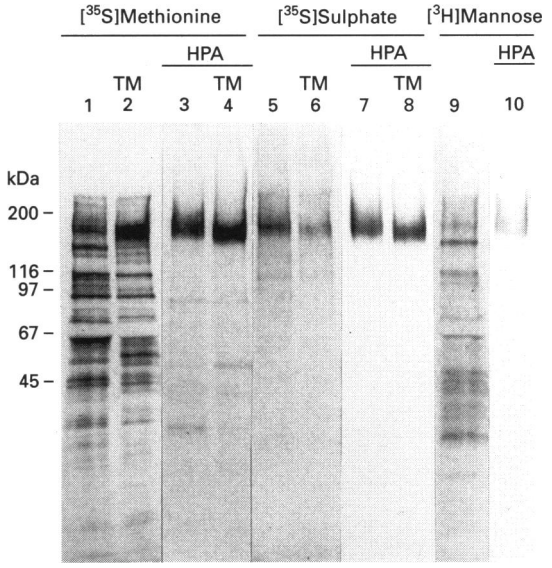

Figure 8 Effect of tunicamycin on glycosylation of hamster oviductin

Oviducts were cultured in the presence of [^{35}S]methionine, [^{35}S]sulphate or [^{3}H]mannose either with or without the *N*-glycosylation inhibitor, tunicamycin (TM). Portions of the radiolabelled culture media were precipitated with HPA–agarose. Unfractionated and HPA-precipitated proteins were subjected to 5–15%-gradient SDS/PAGE under reducing conditions and autoradiography. [^{35}S]Methionine (lanes 1–4), [^{35}S]sulphate (lanes 5–8) and [^{3}H]mannose (lanes 9–10) labelled proteins contained in culture medium of untreated (lanes 1, 3, 5, 7, 9 and 10) or tunicamycin-treated (lanes 2, 4, 6 and 8) oviducts. Lanes 1, 2, 5, 6 and 9 are unfractionated secreted oviductal proteins (30 μg); lanes 3, 4, 7, 8 and 10 are HPA precipitates from 300 μg (lanes 3 and 4), 600 μg (lanes 7 and 8) and 900 μg (lane 10) of culture media.

hamster oviductin cDNA should enable us to study both the molecular regulation and the biological role of this glycoprotein.

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