Evidence for shared epitopes within the ‘naked’ protein domains of human mucus glycoproteins

A study performed by using polyclonal antibodies and electron microscopy

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Polyclonal antibodies were raised in rabbits towards reduced subunits of human cervical mucus glycoproteins. The reduced subunits almost completely inhibited the antiserum, whereas the intact mucins and the heavily glycosylated fragments obtained after digestion of reduced subunits with trypsin (T-domains) caused only partial inhibition. Periodate oxidation of intact mucins, reduced subunits and T-domains caused no effect on the antibody response, and fragments obtained by more extensive proteolysis of the reduced subunits (P-domains) showed no inhibitory activity. By using electron microscopy, antibodies from T-domain-adsorbed antisera were revealed as bound to cervical mucin reduced subunits, either directly or with colloidal gold–Protein A. Binding sites (100–150 nm apart) were observed at the ends and at internal positions of the reduced subunits. We conclude that the antibodies do not recognize carbohydrate structures but are directed to two kinds of protein epitopes, one shared by whole mucins, reduced subunits and T-domains, and the other specific to the reduced subunit fragment. The latter epitopes are ‘cryptic’ and are probably shielded within folded protein domains stabilized by disulphide bonds. Human bronchial, cervical, gastric and salivary mucus glycoproteins share some of these cryptic epitopes.

INTRODUCTION

A layer of mucus coats the oral cavity and the cervical, gastrointestinal and respiratory tracts. The mucous secretions all contain large glycoproteins which are the constituents responsible for their rheological properties [for a review, see Carlstedt *et al.* (1985)]. The mucus glycoproteins (mucins) from these sources, although varying in compositional detail, are similar with respect to their basic ‘architecture’. In general, they are filamentous molecules polydisperse in size, with lengths ranging from 0.5 to 10 μ m (Sheehan *et al.*, 1986). The molecules are composed of subunits (M_r 2×10^6) obtained after reduction of disulphide bonds, and subsequent trypsin digestion yields high- M_r glycopeptides (T-domains) of M_r 300 000–400 000 (Carlstedt & Sheehan, 1984). An electron-microscopic study (Sheehan & Carlstedt, 1990) showed (i) that reduced subunits are composed of an array of heavily glycosylated domains with intervening naked peptide regions that become unfolded after reduction of disulphide bonds, and (ii) that T-domains may contain a residual naked peptide protruding from one end of the molecule. In the present study we raised polyclonal antibodies to reduced subunits from human cervical mucins and have used inhibition and direct-binding e.l.i.s.a. to characterize the nature of their epitopes. Furthermore, we have employed Protein A–colloidal gold to reveal, by electron microscopy, the distribution of antibody-binding sites on cervical mucin reduced subunits.

EXPERIMENTAL

Materials

Peroxidase-labelled goat anti-rabbit IgG and Freund’s complete and incomplete adjuvants were purchased from ICN

Biochemicals, High Wycombe, Bucks., U.K. Tween 20, 3,3',5,5'-tetramethylbenzidine dihydrochloride tablets, 3,3'-diaminobenzidine hydrochloride tablets and phosphate-buffered saline (PBS) tablets were from Sigma, Poole, Dorset, U.K.

Preparation of mucins and mucin fragments

Mucus glycoproteins were isolated from saliva and bronchial, cervical and gastric mucus essentially as described previously (Carlstedt *et al.*, 1983a). Cervical mucin reduced subunits and T-domains were prepared as described by Carlstedt *et al.* (1983b). Reduced mucins (bronchial, gastric and salivary) were obtained as described by Thornton *et al.* (1990). P-domains were obtained after papain digestion of reduced subunits in 1 M-NaCl/5 mM- Na_2EDTA /5 mM-cysteine hydrochloride/10 mM-sodium phosphate buffer, pH 7.0, for 15 h at 65 °C.

Preparation of antisera

Antiserum against cervical mucin reduced subunits was raised in Half-Lop male rabbits. The antigen, 0.5 mg in PBS, was mixed with an equal volume of Freund’s complete adjuvant and emulsified by repeated passage through a fine-bore needle. The emulsion was injected subcutaneously, and booster doses containing the antigen in PBS mixed with an equal volume of Freund’s incomplete adjuvant were given every 14 days. Blood samples were allowed to clot for 1 h at room temperature, followed by 16 h of clotting at 4 °C. The serum was separated from the clot by centrifugation (8000 g, 20 min), heated at 56 °C for 30 min, divided into aliquots and stored at –20 °C.

T-domain-adsorbed antibodies were produced by incubating antiserum (500 μ l) with T-domains (100 μ g/ml) and then performing rapid chromatography (25 min) on a column of Sepharose CL-2B (V_i = 15 ml) eluted with 50 mM-magnesium

acetate. Antibody-T-domain complexes were eluted in the void volume of the column and were discarded.

E.I.i.s.a. techniques

E.I.i.s.a. was performed by using nitrocellulose as the solid phase. Cervical mucin reduced subunits were deposited, by vacuum suction, on to a nitrocellulose membrane (pore size $0.2\ \mu\text{m}$) using a Schleicher and Schuell Minifold II 72-well slot-blot apparatus. The membrane was removed and incubated for 30 min in PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween 20 and then for 60 min with rabbit antiserum (diluted 1:250 in PBS containing 0.05% Tween 20). Unbound antibody was removed by washing three times (for 5 min each time) in PBS containing 0.05% Tween 20. The membrane was then incubated for 60 min with peroxidase-labelled goat anti-rabbit IgG (diluted 1:500 with PBS containing 0.05% Tween 20) followed by three washes (5 min each) with PBS containing 0.05% Tween 20 and one wash with PBS. The enzyme substrate [0.25 mg of 3,3'-diaminobenzidine hydrochloride/ml in PBS containing 0.03% (v/v) H_2O_2] was added, and the colour development allowed to continue for 10 min. The production of chromophore was stopped by the addition of 1% (w/v) NaN_3 , and the degree of colour produced was quantified as described by Thornton *et al.* (1989). The dilution of the first antiserum (1:250) was determined experimentally by a direct-binding e.I.i.s.a. and was chosen as the lowest antibody concentration producing optimal colour response for 40 mg of antigen deposited on the membrane (result not shown).

Inhibition mixtures containing a range of concentrations of whole mucins, reduced subunits of T-domains (0–2000 mg/ml) in PBS were incubated for 2 h at room temperature with antiserum (diluted 1:250). Cervical mucin reduced subunits were deposited, by vacuum suction, on to the nitrocellulose (40 mg/well), and the membrane was left inside the assembled slot-blot apparatus. The suction pump was turned off and the wells were treated with 3% BSA in PBS for 30 min. The wells were emptied and washed with PBS, after which the inhibition mixtures were added to the wells and incubated for 60 min at room temperature. The membrane was then treated as described above.

Electron microscopy

Electron microscopy was performed using the spreading technique described by Sheehan *et al.* (1986). In brief, cervical mucin reduced subunits (0.05–0.1 $\mu\text{g}/\text{ml}$) in 50 mM-magnesium acetate formed the hypophase on which a monolayer of benzyldimethylalkylammonium chloride was formed. After 10–15 min, carbon-coated grids were touched to the surface either washed with ethanol and rotary-shadowed with Pt/Tg at an angle of 7° or treated as described below.

T-domain-adsorbed antiserum was mixed with reduced subunits (200 μl at 0.1 $\mu\text{g}/\text{ml}$), incubated for 1 h, and antibody-molecule complexes were made as described by Sheehan *et al.* (1987). In brief, the reduced subunit-antibody complexes were collected for the void volume of Sepharose CL-2B ($V_t = 15\ \text{ml}$), spread for electron microscopy and shadowed as described above.

Alternatively, reduced subunit-antibody complexes, spread and picked up on to carbon-coated grids as described above, were stained with colloidal gold-Protein A (10 nm) by incubating the grids for 1 h on top of a 1 ml drop of the above reagent in PBS containing 0.1% (w/v) BSA and 0.1% (v/v) Tween 20. Subsequent washings ($2 \times 15\ \text{min}$) of the grids were similarly performed with PBS containing 0.1% (v/v) Tween 20 and finally with 50 mM-magnesium acetate (15 min). The grids were rinsed in ethanol and rotary-shadowed as described above.

RESULTS AND DISCUSSION

A polyclonal antiserum was raised in rabbits against cervical mucin reduced subunits. In an inhibition e.I.i.s.a. (Fig. 1) this antigen showed almost complete inhibition, whereas T-domains and whole mucins (results not shown) caused only partial

Fig. 1. Inhibition e.I.i.s.a. of cervical mucin reduced subunits, T-domains and P-domains

E.I.i.s.a. was performed using inhibition mixtures containing antiserum (1:250) preincubated with reduced subunits (●), T-domains (○) or P-domains (□) over the concentration range 0–2000 ng/ml as described in the text. I_e is the integrated intensity as defined by Thornton *et al.* (1989).

Fig. 3. Relative response of reduced human mucins in a direct binding e.I.i.s.a. using T-domain-adsorbed antiserum

Whole mucins (cervical) and reduced mucins (cervical, gastric, respiratory and salivary) over the range 2–128 ng were immobilized on a nitrocellulose membrane ($0.2\ \mu\text{m}$ pore size) and the e.I.i.s.a. was performed as described in the text. Cervical mucin reduced subunits (the original antigen) (○) showed the highest reactivity, whereas those from gastric (▲), respiratory (●) and salivary (△) mucins gave a diminished, but still significant, response in the assay. Whole cervical mucins (■) showed little reaction with the antibodies.

Fig. 2. Electron microscopy of cervical mucin reduced subunits

(a) Cervical mucin reduced subunits, spread on benzyldimethylalkylammonium chloride as described in the text, appear as flexible coiling filaments 200–600 nm in length. (b) When combined with T-domain-adsorbed antiserum in free solution and subsequently spread, the reduced subunits were revealed as having frequent thickened zones (arrows) approx. 100–200 nm apart. (c) Staining such preparations with colloidal gold-Protein A shows gold particles binding at similar intervals to that observed in (b). The bar represents 100 nm.

inhibition. The results could be explained by the presence of both carbohydrate and protein epitopes, since the reduced subunit is composed of alternating stretches of densely glycosylated and 'naked' protein. To investigate the presence of carbohydrate epitopes, direct-binding e.l.i.s.a. was performed on reduced subunits and T-domains before and after periodate oxidation. No decrease in antibody response was observed (results not shown), suggesting that carbohydrate epitopes are not present. Furthermore, fragments obtained by papain digestion of reduced subunits (P-domains) showed no evidence of inhibition (Fig. 1). These data suggest that the antiserum recognizes exclusively protein epitopes, some of which are present in whole mucins and T-domains. Previously obtained data (Sheehan & Carlstedt, 1990) suggest that the T-domains are not totally devoid of 'naked' protein, but have a residual stretch of peptide protruding at one end. The whole mucins are known to contain naked protein regions, most of which appear to be folded and thus not exposed on the surface of the molecule. The inhibition data (Fig. 1) therefore suggest that two different kinds of protein epitopes are recognized: one common to whole mucins, reduced subunits and T-domains, and the other specific to 'cryptic' sites only revealed after reduction of intramolecular disulphide bonds.

In a previous study on cervical mucins (Sheehan & Carlstedt, 1990), colloidal gold was found to bind selectively to regions of the protein core not coated by carbohydrate chains, provided that the gold and the molecules were allowed to react in free solution before spreading. When this procedure is used, reduced subunits bind gold at the end and at intervals along the molecules of approx. 100–180 nm. The appearance of cervical mucin reduced subunits in the absence of antibodies (Fig. 2a) contrasts with that for reduced subunits incubated with T-domain-adsorbed antisera (Fig. 2b). The antibodies are seen as thickened regions (approx. 100–200 nm apart) on many of the molecules. Staining such preparations directly on the grid with colloidal gold-Protein A shows a similar distribution of gold-binding sites (Fig. 2c). There was no interaction of the gold-Protein A or gold alone with the reduced subunit in the absence of antibodies, and the binding of these reagents to the reduced subunits is thus clearly mediated by the antibodies.

The T-domain-adsorbed antiserum was used to compare reduced subunits from human cervical, bronchial, gastric and salivary mucins (Fig 3). The bronchial, gastric and salivary mucin reduced subunit preparations showed a significant cross-reaction with the antibodies, and the data suggest that these mucins have epitopes in common which are exposed after reduction. This is corroborated by the observation that the whole cervical mucins react only weakly in this assay (Fig. 3).

Polyclonal antibodies to reduced rat gastric mucins interact almost exclusively with protein epitopes (Dekker *et al.*, 1989), and this antigen is prepared in a similar way to the cervical reduced subunits used here. Bara *et al.* (1988) have raised monoclonal antibodies to ovarian-cyst mucins directed to both

carbohydrate and protein epitopes. Antibodies against deglycosylated mucins have been used for cellular localization of mucin precursors (Marianne *et al.*, 1986; Perini *et al.*, 1989) and for screening cDNA libraries (Gum *et al.*, 1989). Antibodies raised to intestinal mucins showed a decreased reactivity against the reduced antigen (Mantle *et al.*, 1984) suggesting that the naked domains also in these mucins are stabilized by disulphide bonds. The antibodies described here are directed to two different peptide domains on the mucins, one of which is shared by intact mucins, reduced subunits and T-domains, and the other being exposed after reduction. The distribution of the latter epitope is consistent with that of naked peptide regions as previously revealed by electron microscopy (Sheehan & Carlstedt, 1990). This kind of epitope is shared by a variety of human mucins, indicating that the structure of the protein core within the naked regions is related.

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