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Interaction between secretory leucocyte proteinase inhibitor and bronchial mucins or glycopeptides

Physiopathological implications for the protection of mucins against proteolysis by human leucocyte elastase

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The interaction of secretory leucocyte proteinase inhibitor with bronchial mucins and glycopeptides was studied by means of c.d. spectroscopy. The interaction with mucins was characterized by an increase in organized structure of α-helical type, as evidenced by the appearance in the difference spectra of two positive bands at 208 and 218 nm. This phenomenon was correlated with the amount of inhibitor present in the mixtures, suggesting that the change was inherent to the inhibitor. Surprisingly, when the inhibitor was mixed with acid glycopeptides, difference c.d. spectra showed a decrease in organized structure, characterized by a negative minimum at 196 nm. Glycopeptides treated with neuraminidase gave similar profiles of difference spectra in three different mixtures, indicating that the interaction was smaller. The interaction between the inhibitor and mucins was also studied for its ability to modify *in vitro* the proteolytic activity of human leucocyte elastase. Mucins alone were degraded by that proteinase into glycopeptides of M_r 400000–500000, whereas mucins mixed with inhibitor before adding elastase were proteolysed to a lesser extent. These data demonstrate that the secretory leucocyte proteinase inhibitor interacts with mucins and consequently is capable of protecting the mucins against proteolysis by elastase.

INTRODUCTION

Secretory leucocyte proteinase inhibitor (SLPI), also called 'mucus proteinase inhibitor', is the major inhibitor of human leucocyte elastase (HLE; EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) [1] in the upper respiratory tract, where it is produced by the serous cells of the bronchial glands [2]. It is a strongly basic protein with M_r 11726 and pI > 10.5 (I. Van-Seuningen & M. Davril, unpublished work), organized in two separate domains of similar amino acid sequence, each of which contains four disulphide bridges [3].

Mucins are high-M_r polydisperse glycoproteins containing about 60–80% carbohydrate, mostly linked *O*-glycosidically to their peptide backbone via residues of threonine and serine. Sialic acid residues and sulphate are present on their carbohydrate side chains, conferring on them an acid character. Molecules of mucins appear to be linear and flexible threads with alternant highly glycosylated regions resistant to proteinases and 'naked' regions devoid of carbohydrate chain and sensitive to proteinases [4].

In diseases such as chronic bronchitis and cystic fibrosis, one can observe a hypersecretion and an increase of the mucus viscosity. Rheological properties of the mucus gel due primarily to the presence of mucins may be influenced by locally produced proteins, including lysozyme [5] and lactoferrin [6], this binding occurring via electrostatic forces. Recently, another locally produced protein, i.e. mucus proteinase inhibitor, has been shown to interact with mucins, this interaction having been demonstrated to be ionic [7,8]. In such diseases, the hypersecretion of mucus, due to the increase of the number of mucous cells which secrete mucins, can also be influenced by HLE, which was shown to be responsible for a bronchial-secretory-cell metaplasia when given intratracheally to hamsters [9,10]. Thus this enzyme could be considered as an agent of hypersecretion of mucus. HLE was also shown capable of proteolysing pig gastric mucins, thereby suggesting a role for it in the mechanisms of damage during chronic infection and inflammation of the respiratory tract [11].

C.d. spectroscopy is a very useful method to monitor structural transitions of proteins. C.d. bands of compounds occurring in the far-u.v. or amide region are dominated by contributions of the peptide bonds. Thus information about secondary structure are obtained from these c.d. spectra.

In the present work, mixtures of SLPI and bronchial mucins or glycopeptides were analysed by c.d. spectrocopy in order to reveal the existence of an interaction between these two components in solution. In order to study the influence, on the one hand, of entire molecules of mucins and, on the other hand, of their carbohydrate side chains on the interaction, SLPI was mixed with mucins, but also with two kinds of glycopeptides. The first one contained sialic acid and sulphate residues, whereas the second one had no sulphate and was partially desialylated. We also studied the influence of the interaction on the proteolytic activity of HLE towards mucins. The implication of sialic acid and/or sulphate residues in the binding, and the consequences of such an interaction on the activity of SLPI in pulmonary diseases where a hypersecretion is observed, are discussed.

MATERIALS AND METHODS

Materials

Pronase from *Streptomyces griseus* was purchased from Calbiochem. Neuraminidase (type VI) from *Clostridium perfringens* (EC 3.2.1.18) was from Sigma. Cellex E cellulose was

Abbreviations used: (r)SLPI, (recombinant) secretory leucocyte proteinase inhibitor; HLE, human leucocyte elastase; i.e.f., isoelectric focusing; PAS, periodic acid–Schiff.

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Table 1. Chemical compositions of mucins (column 1), acid glycopeptides (column 2) and desialylated glycopeptides (column 3)

Carbohydrate, sulphate and total amino acids are expressed as percentages of dry weight, and individual amino acid compositions are expressed as residues/100 residues.

	Composition		
Column ...	1	2	3
Fucose	16.0	18.7	15.1
Mannose	0.5	0.2	0.2
Galactose	21.5	23.8	22.4
N-Acetylglucosamine	17.9	16.0	19.3
N-Acetylgalactosamine	10.6	11.6	11.3
Sialic acid	1.4	2.1	1.5
Glucose	0.6	0.6	0.7
Total carbohydrate	68.5	73.0	70.5
Sulphate	3.2	5.5	0.0
Total amino acids	19.5	9.9	9.6
Aspartic acid	7.8	1.5	1.8
Threonine	14.4	33.1	31.4
Serine	10.3	15.6	15.2
Glutamic acid	7.7	2.8	3.4
Proline	8.2	11.1	10.3
Glycine	8.8	7.0	7.7
Alanine	7.9	9.8	10.5
Valine	5.8	4.3	4.4
$\frac{1}{2}$-Cystine	3.7	0.8	0.9
Methionine	0.7	0.3	0.0
Isoleucine	2.9	2.2	2.4
Leucine	6.8	4.0	4.6
Tyrosine	2.2	0.0	0.0
Phenylalanine	3.5	1.6	1.5
Lysine	3.0	1.1	1.2
Histidine	2.2	2.2	2.4
Arginine	4.0	2.4	2.1

provided from Bio-Rad. Sepharose 2B, 4B, 6B and Superose 6, M_r and pI markers were from Pharmacia. Servalyt Precote 3–6 was from Serva. All chemicals were of analytical grade.

Purification of recombinant SLPI (rSLPI), mucins and HLE

rSLPI, a gift from Synergen (Boulder, CO, U.S.A.) was obtained as previously described [12] and was identical with the native protein, which was prepared in our laboratory [7]. Mucins were purified from non-purulent sputum as in [8]. HLE was purified from purulent sputum by the method of Martodam *et al.* [13]. The purity of protein samples was monitored by SDS/PAGE and on acid polyacrylamide gels at pH 4.3 as described in [14].

Preparation of respiratory mucin glycopeptides

A 1 g portion of freeze-dried mucins was submitted to Pronase digestion as previously described [15]. A 650 mg portion of the freeze-dried supernatant of the Pronase digest was dissolved in 20 ml of 0.1 M-NaCl and applied to a Cellex E cellulose column (40 cm × 3 cm) equilibrated in 0.1 M-NaCl; elution was performed as is described in [16]. The so-called 'neutral' glycopeptides were purified by a first gel filtration on a Sepharose 2B column followed by a second gel filtration on a Sepharose 6B column (98.0 cm × 2.5 cm). The so-called 'sulphated' glycopeptides were purified on a Sepharose 4B column (98.0 cm × 2.5 cm). In the present paper they will be referred to as 'acid glycopeptides'. Fractions were checked for protein content by reading the A_{278} and for neutral hexose content by an automatic orcinol assay. Equilibration and elution of columns were performed as described in [16].

Neuraminidase treatment of glycopeptides

A 20.6 mg portion of the so-called 'neutral' glycopeptides, which contain a lower amount of sialic acid, was mixed with 0.6 mg (1 unit) of neuraminidase in 2 ml of 0.1 M-sodium acetate buffer, pH 5.0. Incubation was performed at 37 °C for 18 h with stirring and the solution was dialysed and freeze-dried. Further glycopeptide purification was carried out on the same Sepharose 6B column as that described above. The orcinol-positive peak was considered to be the glycopeptide fraction. In the present paper this fraction will be called 'desialylated glycopeptides'.

Electrophoretic studies

SDS/PAGE was carried out on 4–15 % -(w/v)-polyacrylamide gradient gels with the PhastSystem (Pharmacia) to check the purity of the glycopeptide fractions (40 μg of each were loaded). The gels were stained with Coomassie Blue for protein detection, with Sudan black for lipid and with the periodic acid–Schiff (PAS) reagent for carbohydrate. No additional bands were detected for protein and lipid, indicating the lack of contaminants in the mucin and glycopeptide preparations. Isoelectric focusing (i.e.f.) of glycopeptides of interest was performed with a Multiphor II LKB apparatus on a pH 3–6 gradient gel which was stained with PAS reagent [17].

Chemical analyses

Determinations of carbohydrate and sulphate contents, and amino acid analyses of mucins and glycopeptides, were performed as previously described [8].

C.d. spectroscopy

C.d. spectra were recorded with a Jobin Yvon R. J. Mark III dichrograph. Measurements were performed in the far-u.v. (250–190 nm) at room temperature in a cell of 0.01 cm path length. The protein concentrations were 0.8–1.0 mg/ml for single proteins and 1.0–2.0 mg/ml for mixtures in 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Ellipticities in degrees·cm^2·dmol^{-1} were expressed as $[\theta]$. The mean residue weight, determined from the composition of SLPI, was 109.3. For the mixtures with mucins and glycopeptides, the same weight was chosen. The theoretical spectrum calculated by the instrument corresponded to the sum of individual spectra of the components at their respective concentrations in the reaction mixture. The difference spectrum was calculated by subtracting the experimental spectrum from the theoretical spectrum.

Digestion of mucins by HLE

A 0.12 mg portion of HLE was added to 1.2 mg of mucins dissolved in 120 μl of 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, and the mixture was incubated at 37 °C for different times (1, 6 and 24 h). The reaction was stopped by removing samples and immediately freezing and freeze-drying them. Samples were dissolved in the same buffer as that described above at a concentration of 4.4 mg/ml. A 10 μl sample was loaded on a Superose 6 column equilibrated in the same buffer; the flow rate was 0.4 ml/min and the absorbance was monitored at 210 nm. Another 10 μl was also loaded on a 5–15 %-acrylamide gradient gel and SDS/PAGE was performed as described by Laemmli [18]. The gel was stained with the PAS reagent [17].

Elastase-inhibitory activity of the SLPI/mucin mixtures

SLPI was preincubated for 15 min at 20 °C with mucins

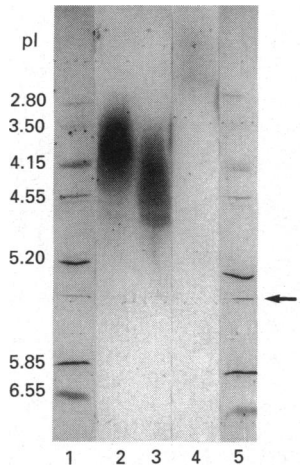

Fig. 1. I.e.f. of glycopeptides over the pH range 3–6

pI markers were stained with Coomassie Blue (slots 1 and 5), and glycopeptides (50 μg) were stained with PAS reagent. Slots 2 and 3, 'neutral' glycopeptides before (slot 2) and after (slot 3) neuraminidase treatment; slot 4, acid glycopeptides. Samples were applied at the position indicated by the arrow.

dissolved at the concentration and in the buffer described above. The amount of SLPI was calculated to have a molar excess of 2:1 over the HLE to be added. The same enzyme/mucin ratio (1:10, w/w) was chosen; the SLPI/mucin mixture was incubated with HLE and processed as described above for SDS/PAGE.

RESULTS

Chemical compositions

Total mucins (Table 1, column 1) are composed of 68.5% (w/w) carbohydrate and 19.5% (w/w) amino acids. Threonine, serine and proline, the most frequent residues in mucins, account for 32.9% of total amino acids. The two glycopeptide fractions obtained after Pronase digestion (columns 2 and 3) are quite similar. There is a noticeable enrichment in threonine (33.1% and 31.4%) and in serine (15.6% and 15.2%) when compared with mucins (14.4% and 10.3% respectively). The sum of these two amino acids accounts for approximately half of the peptide core (48.7% and 46.6% respectively). The content in sulphate is 3.2% for the mucins and 5.5% for the acid glycopeptides. The sialic acid content of glycopeptides is 1.5% after neuraminidase treatment (column 3), instead of 3.0% in the starting material (result not shown).

Purity and pI determinations

Mucins, acid and desialylated glycopeptides were shown to be free of protein and lipid contaminants by performing SDS/4–15%-PAGE (results not shown). pI values for glycopeptides were determined by i.e.f. in a pH 3–6 gradient (Fig. 1). 'Neutral' glycopeptides (slot 2) exhibit before neuraminidase treatment pI

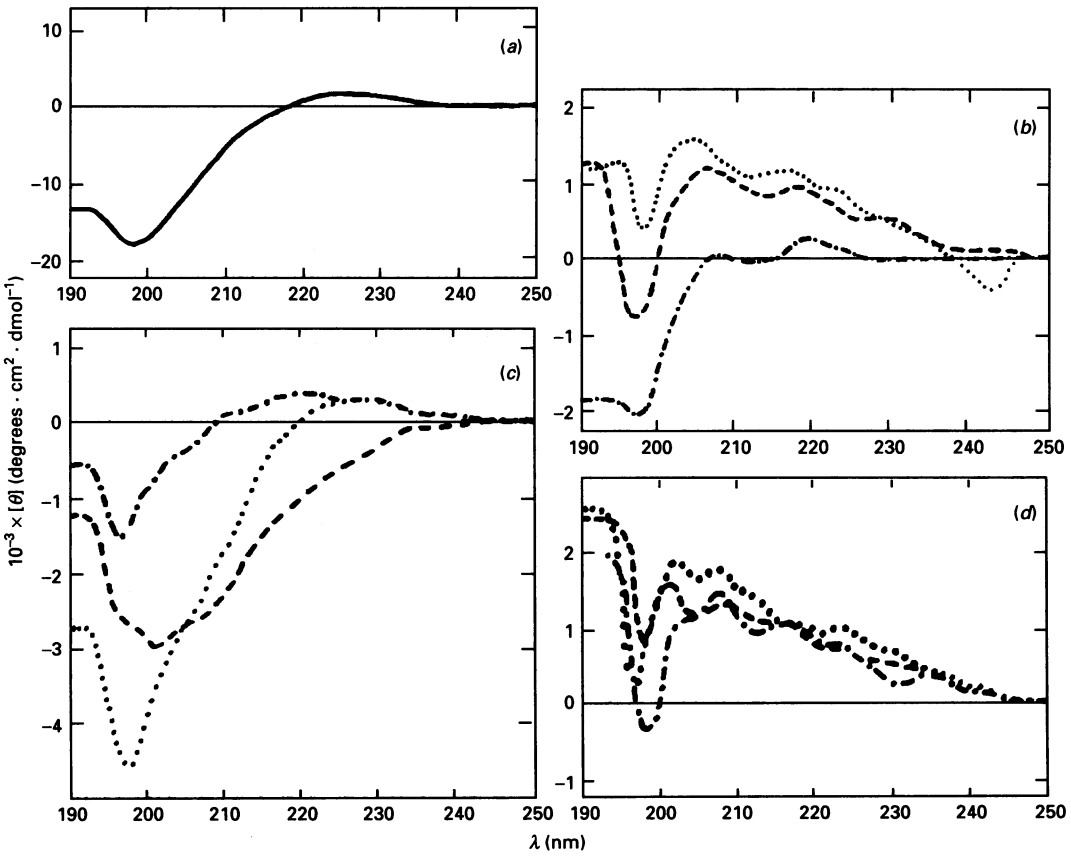

Fig. 2. C.d. spectra between 190 nm and 250 nm

Spectrum of SLPI alone (a); (*b*)–(*d*) difference spectra of SLPI/mucin mixtures (*b*), of SLPI/acid glycopeptide mixtures (*c*) and of SLPI/desialylated glycopeptide mixtures (*d*). Ratios (SLPI/mucins or SLPI/glycopeptides, w/w) were: ·—·—, 0.1:1; ——, 0.5:1; ·····, 1:1. Conditions are detailed in the Materials and methods section.

values ranging from 3.2 to 4.2, these values increasing to 4.0–5.0. after partial desialylation (slot 3). Acid glycopeptides have a pI of less than 2.80 (slot 4), this confirming their strongly acid character.

Far-u.v. c.d. spectra of SLPI, mucins and glycopeptides

The spectrum of SLPI is shown in Fig. 2(*a*). It is characterized by a positive band at 225 nm and a negative one at 197 nm. The same spectrum was obtained for the native and the recombinant proteins. Spectra of mucins, acid glycopeptides and desialylated glycopeptides were characterized by a negative shoulder between 225 and 230 nm, a minimum between 211 and 214 nm and a maximum between 198 and 201 nm (results not shown).

Far-u.v. c.d. difference spectra of SLPI/mucin mixtures

When calculating the difference between theoretical and experimental spectra, the acquisition of positive values demonstrates the formation of new structures after the interaction, whereas negative bands would indicate the loss of some organized structures.

Three mixtures were prepared in the ratios (SLPI/mucins) of 0.1:1, 0.5:1 and 1:1 (all w/w) in order to correlate the interaction with the amount of SLPI. C.d. spectra were recorded after a 15 min incubation of the mixtures at room temperature. The difference spectra are shown in Fig. 2(*b*). Three extrema are clearly obtained in all mixtures, at 218, 208 and 196 nm. It can be observed that the values of ellipticities at these three characteristic wavelengths increase with the amount of SLPI present in the mixtures, suggesting the formation of more organized structures in the molecule of inhibitor.

Far-u.v. c.d. difference spectra of SLPI/acid glycopeptide mixtures

Three mixtures were prepared with various ratios of SLPI/acid glycopeptide (0.1:1, 0.5:1 and 1:1, w/w) as described above. The negative band observed in Fig. 2(*c*) at 196 nm for the 0.1:1 and the 1:1 mixtures is shifted to 199 nm for the 0.5:1 mixture. The negative value of ellipticity increases with the SLPI/glycopeptides ratio. This result indicates a decrease of some organized structure correlated with the amount of SLPI in the mixtures.

Far-u.v. c.d. difference spectra of SLPI/desialylated glycopeptide mixtures

The three mixtures analysed were prepared in the same ratios as above. As shown in Fig. 2(*d*), the three difference spectra are very close to one another, indicating that maximum interaction had already been reached by the 0.1:1 ratio. Patterns similar to those obtained for SLPI/mucin mixtures were observed, and three contrast with those of the SLPI/sulphate-containing glycopeptide mixtures shown in Fig. 2(*c*).

Digestion of mucins by HLE

Degradation of bronchial mucins by HLE was studied by gel-filtration chromatography (Fig. 3) and SDS/PAGE (Fig. 4). Native mucins were eluted at the void volume of the column (17 min retention time), whereas mucins incubated with HLE were shown to be included (Fig. 3). After a 1 h incubation, one can see two unresolved included peaks at 18 and 20 min and a shoulder at 26 min, the last peak at 47 min being HLE. When mucins were incubated for 6 and 24 h with HLE, the same elution pattern was obtained: it showed one major peak at 21 min and a shoulder at 26 min, which was increased when compared with the 1 h-incubation pattern. The retention time of the shoulder corresponded to that of Pronase-digest

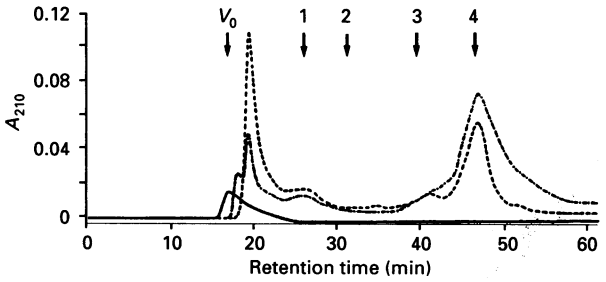

Fig. 3. Separation on Superose 6 of mucins digested by HLE

Mixtures of mucins/HLE in ratios 10:1 (w/w) (40 μg each of mucins) were loaded on the column (for conditions, see the Materials and methods section). ——, Mucins alone; –·–·, mucins/HLE after 1 h incubation time; – – – –, after 6 or 24 h incubation time. Markers: 1, Pronase-digest glycopeptides (M_r 1000000); 2, thyroglobulin (M_r 669000); 3, BSA (M_r 67000); 4, bronchial lysozyme (M_r 147000).

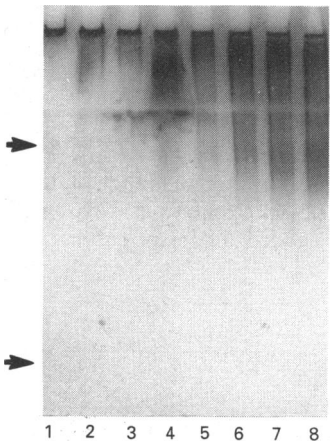

Fig. 4. SDS/5–15% -PAGE of mucins (40 μg) digested by HLE after, or without, preincubation with SLPI as described in the Materials and methods section

Slot 1, mucins alone; 2, SLPI/mucins; 3, SLPI/mucins after 1 h incubation with HLE; 4, after 6 h incubation with HLE; 5, after 24 h incubation with HLE; 6, mucins incubated with HLE for 1 h; 7, for 6 h; 8, for 24 h. The upper arrow indicates the position of Pronase-digest glycopeptides and the lower arrow that of the marker, α_1-acid glycoprotein (M_r 45000).

glycopeptides, which have M_r values of 400000–500000. The same fractions were loaded on a 5–15%-acrylamide gel, subjected to SDS/PAGE and stained with PAS reagent (Fig. 4). Native mucins (slot 1) were shown to be located at the top of the gel, in accordance with their high M_r. By constrast, mucins degraded by HLE migrated in the stacking gel, but also in the separating gel as bands more strongly revealed as the incubation time increased (slots 6–8). The staining pattern in this separating gel corresponded to that obtained with Pronase-digest glycopeptides (indicated by the upper arrow).

Effect of the SLPI–mucin interaction on the proteolysis of mucins by HLE

On SDS/PAGE (Fig. 4), the SLPI/mucin mixture (slot 2) was loaded as the reference. After 1 h of incubation with HLE (slot 3), the pattern was similar to the reference, whereas after 6 h of incubation (slot 4) a strong staining in the stacking gel and a faint staining in the separating gel could be observed, indicating that proteolysis of mucins had occurred. After 24 h of incubation, a similar result was obtained (slot 5). In comparison with mucins

incubated with HLE only (slots 6–8), where proteolysis had already occurred after 1 h (slot 6), one can conclude that SLPI efficiently prevents from this proteolysis during 1 h, but seems to lose part of its inhibitory activity at longer incubation times (slots 7 and 8).

DISCUSSION

Individual spectra of SLPI, mucins and glycopeptides show that these molecules adopt an inorganized secondary structure in solution. SLPI structure had already been studied by Grütter *et al.* [3] by the use of X-ray crystallography. Those authors observed, for both domains, an internal double-stranded β-sheet surrounded by a flat structure corresponding to the primary enzyme-binding segment. This small amount of secondary structure in the protein explains the c.d. spectrum that we obtained. The positive band at 225 nm is probably due to the presence of the eight disulphide bonds in the molecule. This phenomenon was already described for other small disulphide-rich proteins such as wheat-germ agglutinin or hevein [19]. Another explanation could be the contribution of β-turns, as described by Woody [20] for polypeptides including a preponderance of β-structures. SLPI, which contains 11 % of proline residues, has two proline pairs adopting a polyproline-II-like conformation [3]. C.d. spectra of mucins and glycopeptides are also typical of unorganized structures. They are in total accordance with those described by Feldhoff *et al.* [21]. Those authors also demonstrated a shoulder at 225 nm for intact mucins and a minimum band at 210 nm for their glycopeptides after Pronase digestion. They explained the difference to be dependent on the carbohydrate moiety of the glycopeptides.

Interaction between SLPI and bronchial mucins (or glycopeptides produced therefrom) has been demonstrated to occur in solution in a physiological buffer by the use of c.d. spectroscopy. The best-characterized interaction was obtained when SLPI was mixed with mucins. A change in the secondary structure, with the appearance of α-helical-type structure, could be observed. We can hypothesize that SLPI adopts a new conformation when it binds to mucins. An increase in the ellipticity with the amount of inhibitor present in the mixture supports this hypothesis. The interaction may be explained by the presence of positive charges on the surface of the strongly basic inhibitor with the acid residues of sialic acid and sulphate on the glycan chains of mucins. Such an ionic interaction between mucus proteinase inhibitor and mucins has already been demonstrated by us [7,8]. Moreover, two other basic proteins present in bronchial secretions, namely lysozyme [5] and lactoferrin [6], have been shown to interact with mucins by electrostatic forces.

In order to identify the type of linkage and to study the role of acid residues on the glycan chains of mucins in the establishment of their interaction with SLPI, two kinds of glycopeptides, different in charge, were prepared. Acid glycopeptides contained sialic acid residues (2.1 %) and sulphate (5.5 %). Characteristic difference spectra were obtained: a minimum between 196 and 199 nm was shown, which corresponded to a decrease of some organized structure. This interaction may be of clinical relevance, since mucin-type glycoproteins isolated from cultured epithelial cells from patients with cystic fibrosis have been shown to be oversulphated [22].

The second kind of glycopeptides investigated had no sulphate and contained only a few acid charges after incomplete removal of sialic acid groups. In this case only small differences were observed when varying the ratios in the mixtures with SLPI. From this experiment, it can be concluded that SLPI interacts weakly with desialylated glycopeptides, this interaction possibly occurring through the remaining sialic acid residues.

Finally, both glycopeptide fractions reacted with SLPI in a different manner when compared with macromolecular mucins, which would suggest that, besides the effect of acid charges discussed above, the conformation of mucins may have an influence on the interaction. Moreover, the major difference between mucins and glycopeptides is the presence on the peptide core of mucins of naked regions which are destroyed by Pronase treatment [4]. The loss of these naked regions did not significantly influence the c.d. spectra of the glycopeptides as compared with that of mucins, these spectra being largely dependent on the carbohydrate moieties (68–73 %; Table 1). Binding of SLPI with mucins, which is correlated with the presence of acid charges on the glycan chains, might be increased by peptide–peptide interaction.

SLPI is considered to be the major inhibitor of leucocyte elastase and cathepsin G in the upper respiratory tract where its physiological concentration is sufficient to counteract the action of these proteinases [23]. At this level, mucins are the predominant component of secretions. One role for these secretions in the defence of the respiratory tract is to act as a barrier against proteolytic attacks of enzymes released from neutrophils.

Assuming that the M_r of mucins ranges from 0.5×10^6 to 1.5×10^6 [4], one can calculate that 42 to 128 molecules of SLPI will bind to 1 molecule of mucin in the 1:1 mixture studied. This result is of clinical importance, since it assigns a role for SLPI as a protective agent in the mucus gel against proteolysis of mucins. Enzymes released from leucocytes or from bacteria were shown to be capable of hydrolysing pig gastric mucins [11]. In the human respiratory tract, the presence of the inhibitor bound in large quantity to the mucins may act as protective coat against HLE. Moreover, HLE has been shown to stimulate the release of mucins from primary cultures of hamster tracheal epithelial cells [24]. However, although HLE could degrade these mucins, the incomplete digestion observed by those authors [24] was suggested to be due in part to the presence of an SLPI counterpart, 'antileukoprotease', in the culture medium.

Our results confirm this hypothesis, since (1) HLE was shown to proteolyse human bronchial mucins rapidly into glycopeptides and (2) SLPI bound to mucins was capable of preventing their proteolysis for 1 h at 37 °C.

Concluding remarks

There is direct evidence for an interaction between SLPI and bronchial mucins. This binding leads to a modification of the secondary structure of the inhibitor. The role of sialic acid and sulphate residues has been demonstrated by the use of differently charged glycopeptides, the interaction being almost totally suppressed when desialylated glycopeptides were used. We also demonstrate that SLPI has a protective effect against proteolysis of mucins by HLE, thus allowing them to keep their rheological properties. This would be of clinical relevance in pathological states where qualitative and/or quantitative modifications of the secretions, and especially of mucins, are observed, such as in chronic bronchitis or in cystic fibrosis.

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