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Interaction of heparin with synthetic peptides corresponding to the C-terminal domain of intestinal mucins

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Unlike most other mucins described to date, two intestinal mucins, rat MLP (rat Muc2) and human MUC2 have a C-terminal tail that is enriched in cationic amino acids. The distribution of charge in each case resembles that of several well known heparin binding proteins. Peptides designated E20-14 and F13-15, corresponding to the C-terminal 14 amino acids of the two mucins, were synthesized and shown to bind ^{3}H-labelled heparin by a process that was saturable and mediated by strong electrostatic interactions, giving K_d values of 10^{-7} to 10^{-8} M. Using turbidometric analyses and native gel electrophoresis, we observed that peptide-heparin mixtures formed polydisperse aggregates that dissociated with a progressive increase in the concentration of heparin. Under certain conditions heparin protected the peptide from proteolysis by trypsin. Both heparin and dextran sulfate, the latter a highly sulfated synthetic polysaccharide, were potent inhibitors of ^{3}H-heparin binding to peptide E20-14, while less sulfated glycosaminoglycans were poorly- or non-inhibitory. Mucin in tissue dispersions and homogenates, or purified from rat intestine, did not bind to heparin, and failed to interact with an antibody specific for the peptide E20-14. Both mucin samples however, reacted with antibodies that recognize regions upstream of the C-terminal 14 amino acids. Immunofluorescent localization of E20-14 was confined to the basal perinuclear regions of goblet cells, whereas localization of an antibody to a flanking sequence on the N-terminal side of the C-tail, localized to mature mucin storage granules. These findings suggest that the heparin-binding C-tail of the mucin may be removed at an early stage of biosynthesis. Heparin-mucin complexes, if they form *in vivo*, are thus likely to be confined to the ER and/or Golgi compartments.

Keywords: heparin-peptide complex, MUC2, rat MLP (Muc-2), mucin, C-terminus

Introduction

We have earlier reported the isolation and sequence of a cDNA encoding the C-terminal 1121 amino acids of a large rat intestinal mucin designated MLP (mucin-like peptide) [1, 2]. The alignment and distribution of cysteine residues within the cysteine-rich 760 amino acid C-terminus of MLP were found to be very similar to several other reported secretory mucins, and to the human blood-clotting protein, von Willebrand factor (vWF). This common structural motif is assumed to be necessary for the proper folding of the polypeptide chain to allow intermolecular S–S bridges to mediate dimerization and polymerization. Overall amino acid sequence homology of the C-terminus of MLP with vWF or with other mucins was not a prominent feature however, with the exception of the human intestinal mucin MUC2, which shares approx 70% sequence identity with rat MLP over its C-terminal 730 amino acids [3]. Unlike vWF and most mucins, the extreme C-terminal end of both rat MLP and human MUC2 consists of a 14 amino acid segment that is enriched in positively charged residues. Since both mucins are expressed in normal intestinal goblet cells, it is possible that these positively charged domains could have a specialized function relevant to mucin biosynthesis or transport, or to one or more intestinal mucin functions such as the protective barrier role of secreted mucins, or epithelial growth and repair mechanisms. Presumably

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these functions would entail an interaction of the positively charged mucin domains with anionic molecules encountered within the cell, at cell membranes or in the local environment. Candidates for such an interaction include negatively charged phospholipids, nucleic acids and proteoglycans.

Binding to proteoglycans, particularly to heparin chains of heparan sulfate, is an attractive possibility, in part because heparan sulfate is particularly abundant in intestinal tissue [4]. In addition however, the clustering of the positively charged residues in the C-tail regions of MLP and MUC2 resembles the cationic clustering in peptides that have been shown to bind to heparin [5].

HS-proteoglycans are present in the extracellular matrix, basement membranes, intracellular and surface membranes of many cell types [4, 6, 7]. In one or more of these locations, HS-proteoglycans are involved in processes such as cell adhesion and migration, sequestration of growth factors, modulation of growth factor receptors, stabilization of the extracellular matrix, protection against proteolytic enzymes, modulation of the response to infection, inflammation and injury, and resistance to local metastatic spread of tumours [6, 8–13]. Mucus glycoproteins are also adhesive molecules, are present in almost all epithelial linings, and are involved in some of the same processes [14]. In the case of gastrointestinal epithelial injury and repair, luminal mucins together with fibrin, fibronectin and cell debris (which includes proteoglycans), form a thick mucoid 'cap' in the region of the basement membrane, over denuded injured sites. The 'cap' is required for epithelial regeneration beneath it [15, 16], a process that may be accelerated by heparin [17]. The potential exists therefore for mucins to interact with HS proteoglycans in several locations and in both physiological and pathological processes.

No investigations have been carried out to explore these possibilities. This is not surprising, given that both mucins and proteoglycans are adhesive, self-aggregating, polydisperse macromolecules. They are difficult to separate from each other, purify, quantitate and manipulate in the systematic way necessary for the demonstration of specific interactions. As a first approach to discovering a potential functional interaction of the C-terminal domains of intestinal mucins with anionic macromolecules, we have examined the heparin binding properties of synthetic peptides corresponding to the cationic C-terminal domain of each of the two intestinal mucins, rat MLP (also called RMuc-2 [18]) and human MUC2.

Materials and methods

Reagents

^{3}H-labelled heparin (0.57 mCi mg^{-1}, average molecular weight 13 000) was purchased from New England Nuclear (Wilmington, DE), and the following reagents from Sigma Chemical Co. (St Louis, MO): heparin from porcine intestinal mucosa, heparan sulfate from bovine intestinal mucosa, keratan sulfate, dermatan sulfate from porcine skin, chondroitin sulfate C from shark cartilage, dextran sulfate, heparinase and heparitinase. Heparin Affi-gel beads (containing 1 ± 0.2 mg heparin per ml gel) were purchased from Bio-Rad (Richmond, CA) and precast polyacrylamide gradient gels (10–20%) were purchased from Novex Experimental Technology (San Diego, CA). Trypsin-TPCK (236 U mg^{-1}) was obtained from Worthington Chemicals, New Jersey.

Synthetic peptides

The following peptides were synthesized by Multiple Peptide Systems (San Diego, CA) and purified by reverse phase-HPLC using a Vydac C-18 column with a 5 to 60% acetonitrile gradient in water containing 0.1% or 0.05% trifluoroacetic acid: E20-14 (molecular weight (MW) 1773), corresponding to the C-terminal sequence S R T R R S S P R L L G R K of rat MLP (1); G21-10 (MW 1507), corresponding to the sequence immediately adjacent to the C-terminus, R M V S L D C P D G S K L S; D139-19 (MW 1209), a further upstream sequence T G S T S S K P P T G S, that is based on residues 100–111 of rat MLP (2); and F13-15 (MW 1697), which corresponds to the C-terminal sequence S R R A R R S P R H L G S G of humun MUC2 [3]. Molecular weights and compositions of each peptide were confirmed by mass spectrometry and amino acid analyses, respectively, as performed by Multiple Peptide Systems and the HSC/Pharamacia Biotechnology Service Centre (Toronto, Ontario).

Peptide binding to heparin Affi-gel beads

Routine assays involved gentle agitation at 22 °C for 15 min, of mixtures of peptide (2 μg) and 25 μl of heparin Affi-gel (containing 25 μg heparin) in a total volume of 300 μl containing 0.01 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.5, in 0.15 M NaCl (PBS). The mixture was centrifuged briefly in an Eppendorf centrifuge and the pellet washed twice with 1 ml of PBS containing increasing concentrations of NaCl (0.15–0.55 M). The final pellet was resuspended in tricine SDS-PAGE sample buffer (Novex), boiled for 5 min to release the bound peptide, and the solution (20 μl) subjected to electrophoresis through polyacrylamide gradient gels (10–20%) in 0.1% SDS. Peptide bands were detected by Coomassie blue staining.

Electrophoresis on native gels

Triplicate solutions were prepared, each containing 2 μg of peptide and heparin (0.4–30 μg) in 4 μl of 10 mM Tris HCl buffer, pH 6.8, containing 0.15 M NaCl (TBS). After

the addition of native PAGE sample buffer (Novex Pharmaceuticals), samples were electrophoresed on 4–20% gradient polyacrylamide native gels (i.e. without SDS). One gel was stained with Coomassie blue (for protein), one with Alcian blue (to detect heparin), and one with sequentially added Alcian blue and silver (AB/S) [19]. For glycosaminoglycans and proteoglycans, the sequential stain is much more sensitive than either silver or Alcian blue alone (i.e. pg versus μg detection), and is thus capable of detecting the entire range of diffuse bands that result from the extensive size heterogeneity characteristic of heparin. In addition, bands containing glycosaminoglycans and stained with AB/S change colour from blue-brown to brown-black when protein is present, a feature that makes this stain combination particularly valuable for the detection of complexes between peptides and heparin. Control samples contained peptide alone or heparin alone. Additional experiments were carried out using dextran sulfate, keratan sulfate, dermatan sulfate, chondroitin sulfate C or heparan sulfate instead of heparin. Separate experiments were also conducted in which mixtures of heparin and peptide were incubated briefly (10 min at 22 °C) with 7.5 ng of trypsin-TPCK (enzyme:-peptide ratio 1:267 by weight) prior to electrophoresis through native gels.

Turbidity measurements

Peptide E20-14 (final concentration 0.1 mg ml^{-1}) was mixed at room temperature with heparin (final concentration 0.02–0.15 mg ml^{-1}) in TBS, pH 7.4, to give a final volume of 400 μl. At different time intervals (10–300 min) the absorption at 450 nm was measured spectrophotometrically. Control samples contained heparin alone or peptide E20-14 alone.

Solid phase assay of ^{3}H-labelled heparin binding to immobilized peptides

Peptide solutions (2 μg peptide in TBS, pH 7.5) were slot-blotted onto a PH 79 nitrocellulose membrane using the Minifold II Manifold apparatus of Schleicher and Schuell. Control samples consisted of buffer alone. Each slot blot was rinsed with TBS (50 μl), the filter dried overnight at room temperature, and cut into strips. Strips were incubated for 2 h in TBS in a final volume of 10 ml containing ^{3}H-labelled heparin (0.08–4 μCi ml^{-1}) to give a concentration range of 0.14–7 μg ml^{-1} (10–475 nM heparin). For inhibition experiments, non-radioactive heparin or dextran sulfate (5–30 μg ml^{-1}) or other GAGs (30 μg ml^{-1}) were premixed with labelled heparin (2 μg ml^{-1}) prior to incubations with immobilized peptide. The strips were subsequently washed four times (5 min per wash) with TBS, immersed overnight in Ready Protein Scintillation cocktail (Beckman Canada, Mississauga, Ont), and counted in a Beckman LS-6000/C scintillation counter. Heparin binding data were analysed with the microcomputer-based EBDA/LIGAND program [20] as modified for microcomputers [21].

Mucin preparations

Rat small intestinal mucin was purified by CsCl density gradient centrifugation as described previously [22]. In addition, homogenate supernatant solutions (crude mucin) were prepared by homogenizing intestinal scrapings (1:100 w/v) in 0.01 M phosphate buffer, pH 7.4, containing 10 mM EDTA, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 0.2 mM TPCK, 0.2 mM TLCK and 5 mM NEM (final concentrations), or by simply dispersing scrapings in a mixture containing the same protease inhibitors and 6 M guanidine hydrochloride. Mixtures were then centrifuged at 30 000 $\times$ **g** for 30 min and the filtered supernatant used immediately for immunoassays or applied to heparin Affi-gel columns as described above for peptides.

Antisera

Polyclonal antisera were developed in rabbits against each of the peptides E20-14 and G21-10 (the region immediately adjacent to the E20-14 sequence), and the specificity of each confirmed by ELISA procedures as described earlier for other synthetic peptides of rat intestinal MLP [1]. A third antibody used in the present study was one recognizing the entire 118 kDa C-terminal glycopeptide of MLP and described in earlier publications [1, 22].

Immunofluorescent localization of peptides E20-14 and G21-10

Antisera were adsorbed against goat liver powder prior to use in immunolocalization experiments. Frozen sections of rat small intestine were fixed in methanol, air dried and blocked with 4% normal goat serum in PBS containing 0.1% Triton X-100 and 0.1% Tween-20. Sections were washed, incubated overnight at 4 °C with immune serum or preimmune (control) rabbit serum (1:100 dilution v/v), washed thoroughly, and reincubated for 1 h at 22 °C with fluorescein (FITC)-tagged goat antirabbit IgG (Vector Laboratories, Inc. Burlington, CA). In some experiments sections were pretreated for 1 h at 37 °C with 1 mU 100 μl^{-1} of heparinase or heparitinase, or with Target Unmasking Fluid (Pharmingen, San Diego, CA), prior to blocking and exposure to the E20-14 antiserum. In other experiments, sections were treated at 90 °C with β-mercaptoethanol immediately before the E20-14 antiserum was added. In selected experiments the antibodies for peptide E20-14 or G21-10 were premixed with peptide E20-14 or G21-10 (0.05–1 μg ml^{-1}) prior to incubation

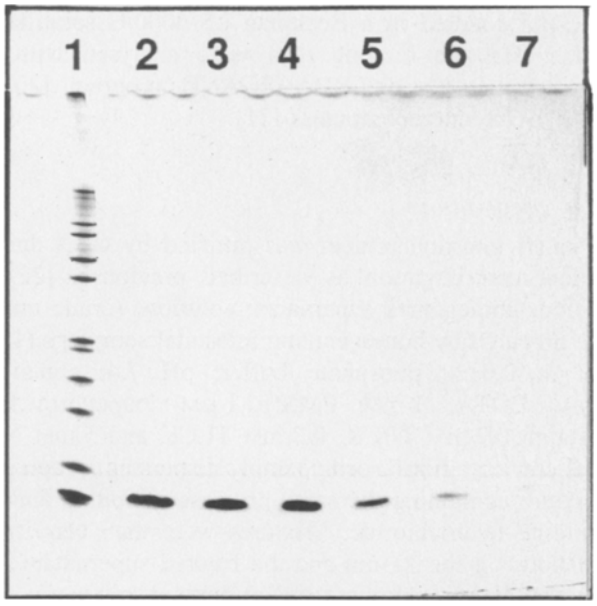

Figure 1. Influence of ionic strength on the binding of mucin peptide E20-14 to heparin-agarose affinity beads. Samples of peptide E20-14 (2 μg) were incubated with heparin Affi-gel beads for 15 min, centrifuged, and each pellet washed twice with PBS containing a different concentration of NaCl. The remaining bound peptides were released by SDS, subjected to SDS PAGE, and the peptide detected by coomassie blue staining. Lane 1, molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14, 6, 3.5 and 2.5 kDa). Lane 2, peptide (2 μg) alone (i.e. no previous exposure to Affi-gel beads). Lanes 3–7 represent peptide samples which were bound to heparin Affi-gel and not removed by solutions containing 0.15, 0.25, 0.35, 0.45, and 0.55 M NaCl, respectively.

with the sections. Sections were washed and examined under a Reichart-Jung Poyvar epifluorescence microscope.

Results

Binding to Affi-gel heparin

The synthetic peptide E20-14, corresponding to the C-terminal 14 amino acids of rat intestinal mucin MLP [1] contains six residues (five arginines and one lysine) bearing positively charged side chains. On 10–20% gradient polyacrylamide gels the peptide was detected at an M_r position of approximately 2 or 3 kDa (Fig. 1, lane 2). After incubation of the peptide with an excess of heparin conjugated to Affi-gel beads, none of the peptide was removed from the beads by washing with 0.15 or 0.25 M NaCl (lanes 3, 4). Partial removal occurred with 0.35 M NaCl (lane 5), but the majority was only removed by a higher ionic strength (0.45 M NaCl or more). Thus the E20-14 peptide binds to heparin and the interaction appears to be mediated by relatively strong electrostatic bonds. Peptide D139-1, which corresponds to a different region of MLP, is enriched in hydroxy amino acids, and contains only one lysine residue. This peptide failed to bind to heparin Affi-gel beads (not presented).

Electrophoresis on native gels

Electrophoresis of E20-14 peptide-heparin mixtures on 4–20% gradient native gels was used to determine if the peptide and heparin formed complexes. Separation of compounds on native gels depends upon both size and charge-to-mass ratios [23, 24], and therefore cannot be used to give precise estimates of molecular weights. Positively charged molecules do not enter native gels, and in the case of negatively charged molecules, increased mobility usually correlates with decreasing molecular size. As expected, the positively charged peptide E20-14 did not enter the gel. Coomassie blue also failed to detect heparin, even though heparin did enter the gels and was detected by Alcian blue as a broad band of high mobility (labelled I in Fig. 2a, lane 7). However the more sensitive sequential AB/S staining procedure (Fig. 2b, lane 7) not only showed band I but also revealed a diffuse band of intermediate mobility (band II). These findings are consistent with the polydisperse nature of commercial heparin (molecular weight range 4000–20 000). In mix-

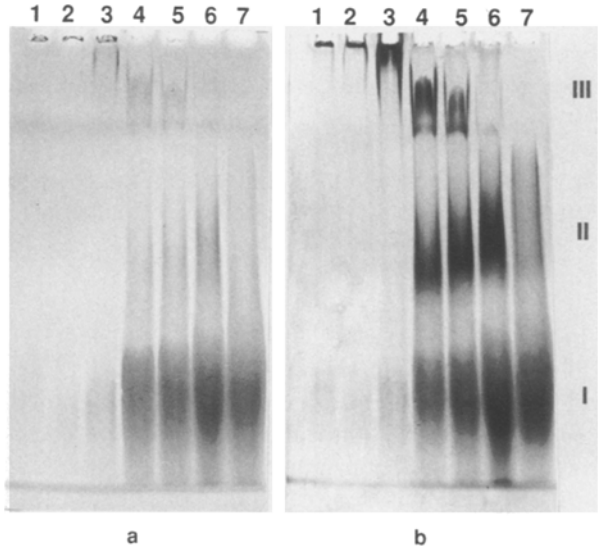

Figure 2. Detection of heparin-peptide E20-14 complexes in native gels. Peptide and heparin were mixed in TBS pH 6.8 and then electrophoresed through polyacrylamide native gels (4–20%). Lanes 1–6 each contained 2 μg of peptide plus increasing heparin (0.4, 0.5, 1, 5, 10, and 30 μg, respectively). The molar ratios of peptide:heparin were calculated as 36.4, 29.7, 14.6, 2.93, 1.47, 0.49, respectively, based upon the molecular weights of 1773 for E20-14 and 13 000 (average) for heparin. Lane 7 contained heparin alone (30 μg). Gels were stained with Alcian blue (a) or combined Alcian blue/silver (b).

tures containing a fixed amount of E20-14 peptide (2 μg) and increasing amounts of heparin (0.4–30 μg), the staining patterns indicated the formation of polydisperse complexes that were seen as brown-black bands after AB/S staining (Fig. 2b). As the molar ratio of peptide:heparin decreased from 36.4 to 0.488 (lanes 1–6 respectively), a low mobility complex (band III) appeared first at the top of the gel, gradually entered the gel, broadened, increased in mobility, and then disappeared. At the same time the faster mobility bands I and II became broader and more intense. Confirmation that the peptide and heparin were both present in all three bands is provided in Fig. 3. AB/S staining was performed on mixtures containing the same range of peptide and heparin concentrations as in Fig. 2, but was carried out before and after brief digestion (10 min) with trypsin (E:S 1:267 w/w). Trypsin abolished the low mobility band III and also markedly decreased the intensity of bands I and II, indicating that the peptide was a prominent constituent of each complex. Therefore under conditions of a high molar excess of peptide, large aggregates (band III) of heparin and peptide were formed. As more heparin was added, the aggregates dissociated into smaller more negatively charged complexes (I and II).

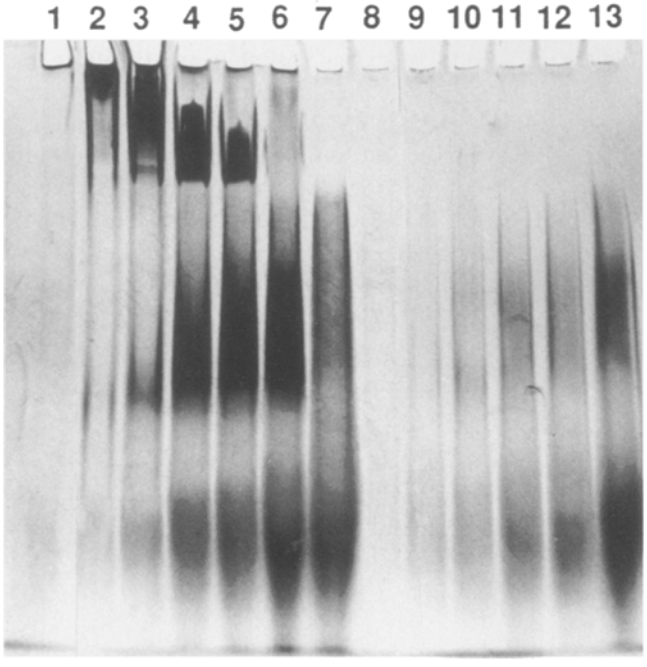

Figure 3. The effect of trypsin on heparin-peptide E20-14 complexes. Duplicate samples containing peptide E20-14 and increasing concentrations of heparin were mixed and electrophoresed as described in Fig. 2. One group (lanes 1–6) was incubated for 10 min without, and the other (lanes 8–13), with trypsin TCPK (E:S 1:267 by weight). The molar ratios of peptide:heparin in lanes 1–6 and 8–13 were 36.4, 14.6, 7.3, 2.9, 1.5, and 0.49, respectively. Lane 7 contained 30 μg heparin alone. Gels were stained with combined Alcian blue/silver.

An additional feature of the heparin:peptide association was noted in Fig. 3. Not only was the trypsin-sensitive peptide E20-14 present in all of the bands, some of the peptide was protected from trypsin degradation when it was mixed with 30 μg of heparin. This is shown by the lighter staining of bands in lane 7 (heparin alone, 30 μg) than in lane 13 (2 μg peptide plus 30 μg heparin). If trypsin had destroyed all or most of the peptide (which it did under conditions of low heparin, as in lane 10 for example), the staining intensities of the bands in lanes 7 and 13 would have been the same (i.e. equivalent to heparin alone). Since this was not the case, heparin partially protected the C-terminal peptide from proteolysis.

Qualitatively similar complex formation was detected by native gel electrophoresis of mixtures of E20-14 peptide (2 μg) and heparan sulfate or dextran sulfate (not presented). In both of these cases however, the complexes remained mainly at the top of the gels (position III), with only a very faint smear at a lower position (equivalent to II), suggesting that the complexes were quite large. In the same experiment, no evidence was obtained to suggest that the less sulfated dermatan sulfate, keratan sulfate, or chondroitin sulfate formed complexes with peptide E20-14.

Turbidity of heparin-peptide mixtures

Some peptides that bind to heparin have been reported to undergo conformational changes indicative of α-helix formation during the binding process, as revealed by circular dichrosim [25]. This technique could not be used in the present study because solutions containing heparin (0.5–1 $\mu g\,\mu l^{-1}$) and E20-14 (0.1 $\mu g\,\mu l^{-1}$) became turbid almost as soon as the two compounds were mixed. This suggested that insoluble aggregates were forming, a process that was examined in more detail by performing turbidometric studies. Neither E20-14 (100 $\mu g\,ml^{-1}$) nor heparin (30–150 $\mu g\,ml^{-1}$) alone caused an increase in turbidity (i.e. light absorption at 450 nm) over a period of 300 mins of observation (Fig. 4). However, mixtures of the two components caused a dramatic concentration-dependent increase in turbidity as a function of time. In general, higher molar peptide:heparin ratios correlated with higher turbidity. The highest ratios (24:1 and 36:1) appeared to cause large but unstable aggregates, which dissociated after 20–30 min of incubation. The highest increase in turbidity that remained stable with time was noted with a peptide:heparin molar ratio of 18:1. This is approximately the same ratio that earlier was noted to produce large aggregates (i.e. slow mobility band III) in native gels (Fig. 2).

Solid phase-binding assays

Peptide E20-14, and the comparable C-terminal peptide F13-15 of the human mucin MUC2, were separately

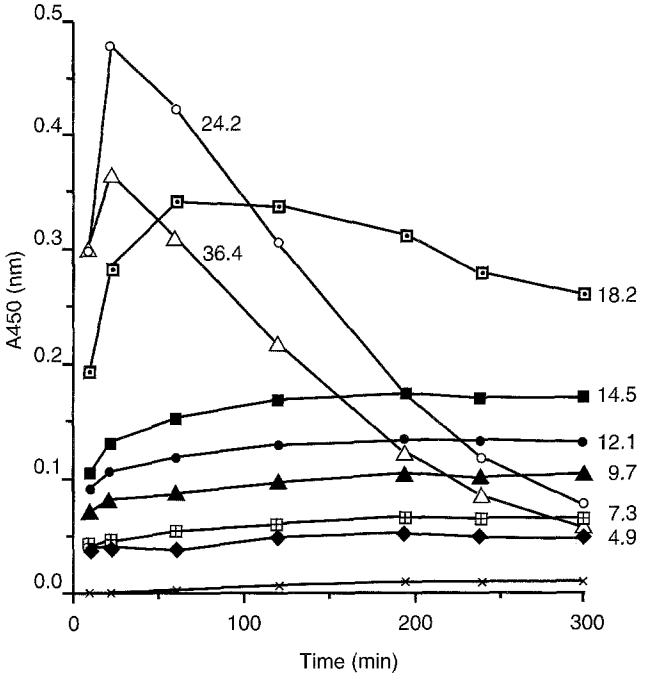

Figure 4. Turbidity of peptide E20-14-heparin mixtures. Mixtures containing a fixed concentration of the peptide (0.1 mg ml^{-1}) and varying concentrations of heparin (20–150 μg ml^{-1}) were incubated at room temperature and light absorbance at 450 nm measured at specified times. The molar ratios of peptide:heparin are indicated beside each curve. Neither peptide alone nor heparin alone gave A450 readings >0.02 (lowest curve).

immobilized on nitrocellulose membranes in a slot-blot manifold, and incubated with increasing concentrations of ^{3}H-heparin. Direct binding plots (Fig. 5a and 5b) show that each of the cationic peptides bound heparin in a concentration-dependent fashion that reached a plateau at approximately 200 nM heparin. Linear Scatchard plots suggested that a single class of receptor sites for heparin exists on each of the peptides. For E20-14, the K_d was 7.4×10^{-8} and the B_{max} 1.26×10^{-9} M. The comparable constants for F13-15 were 1.3×10^{-7} and 1.29×10^{-9}M respectively. The slightly lower affinity for F13-15 may be a reflection of its lower positive charge (one less lysine) than E20-14. There was negligible binding of heparin to the serine and threonine-rich peptide D139-19 (not presented). Based on the average molecular weight of heparin of 13 000 and the measured molecular weights for E20-14 and F13-15 of 1773 and 1697 respectively, it was calculated from B_{max} data [10] that the theoretical maximum binding to E20-14 corresponds to 90 mol, (and for F13-15, 91 mol), of peptide per mol of heparin.

Inhibition of heparin binding to mucin peptides by other polysaccharides

Solid phase binding assays were repeated with peptide E20-14 and ^{3}H-heparin, except that non-labelled heparin or the synthetic polysaccharide, dextran sulfate, was pre-mixed with ^{3}H-heparin before it was added to the incubation. Figure 6 shows that both unlabelled polyanions competed with ^{3}H-labelled heparin binding. The IC_{50} (the concentration required for 50% inhibition) for heparin was 1.54 μg ml^{-1} and for dextran sulfate it was 0.769 μg ml^{-1}. The lower value for dextran sulfate is consistent with its higher sulfate content [9, 26]. At a concentration of 30 μg ml^{-1}, heparin caused 96%, and dextran sulfate caused 98% inhibition. Similar experiments with heparan sulfate (30 μg ml^{-1}) caused 28% inhibition, but chondroitin sulfate, dermatan sulfate and keratan sulfate were without effect (not presented). Thus only the most richly

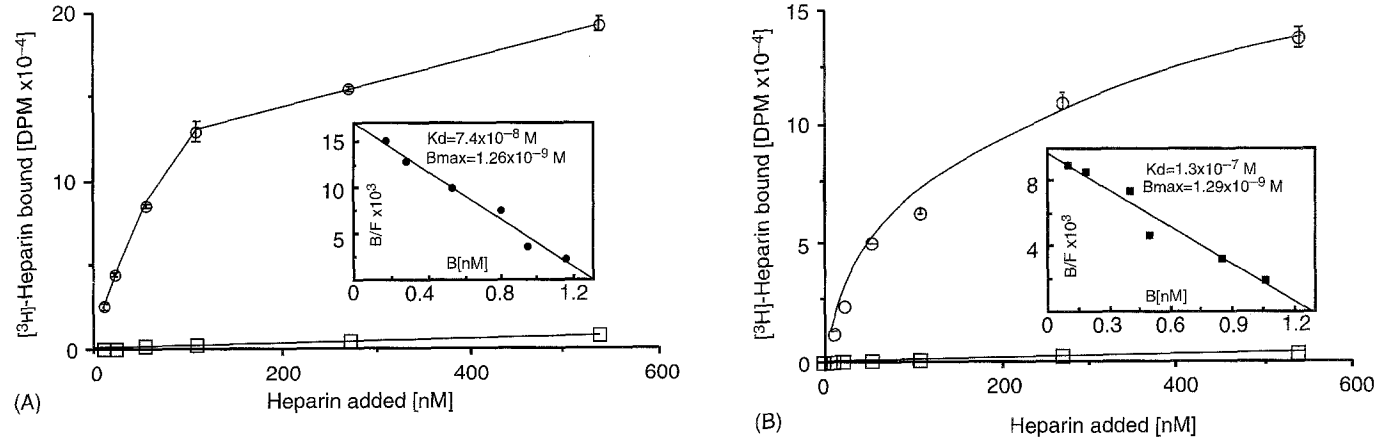

Figure 5. Solid phase assay of the binding of ^{3}H-heparin to peptides E20-14 (of MLP) and F13-15 (of MUC2). Peptide solutions (2 μg peptide in 50 μl of TBS, pH 7.5) were slot-blotted onto nitrocellulose membranes and incubated with ^{3}H-labelled heparin 10.8–538 nM). Control samples (lower curves) contained TBS instead of peptide. Binding data were analysed by Scatchard plots (inserts) using the EBDA/LIGAND computer program. (A) Peptide E20-14: K_d 7.4×10^{-8} M; B_{max} 1.26×10^{-9} M (equivalent to 6.3 pmol of heparin per μg peptide). (B) Peptide F13-15: K_d 1.3×10^{-7} M; B_{max} 1.29×10^{-9} M (6.4 pmol of heparin per μg peptide). Molar values are based on the average molecular weight of heparin of 13000.

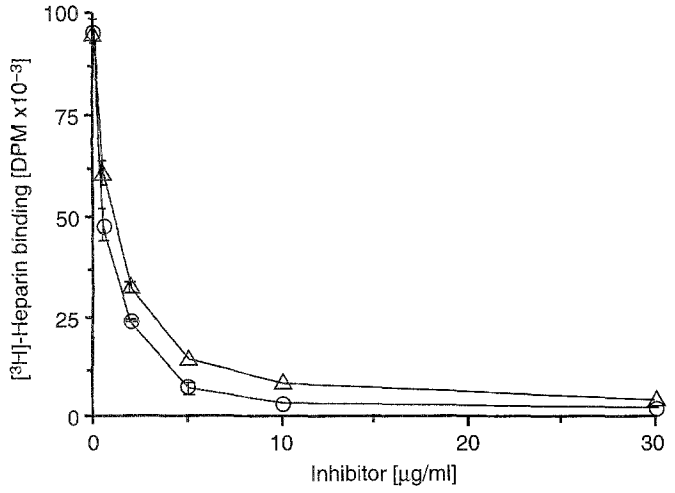

Figure 6. Inhibition of ^{3}H-labelled heparin binding to peptide E20-14 by non-labelled heparin and dextran sulfate. Binding assays were as described for Fig. 5 except that ^{3}H-heparin (2 μg ml^{-1}) was pre-mixed with increasing amounts of non-labelled components prior to adding to slot-blotted peptide (2 μg). The IC_{50} values for dextran sulfate (–○–) and heparin (–△–) were 0.769 and 1.538 μg ml^{-1} respectively.

sulfated polysaccharides were able to inhibit binding of heparin to the mucin peptide, and the inhibition varied in proportion to the degree of sulfation of the inhibitors [11, 26].

Intact mucin and heparin interaction

When purified rat intestinal mucin (instead of peptide E20-14) was used in ELISAs with the antiserum to the E20-14 peptide, no specific immunoreactivity could be detected (not presented). It was considered possible that the cationic C-terminal domain had been proteolytically cleaved from the mucin during its purification from homogenates. Thus an effort was made to determine whether mucin present within initial epithelial homogenates or dispersions (and protected by a battery of proteinase inhibitors and/or guanidine HCl) could react with the antibody and/or bind to heparin. Homogenate supernatant samples reacted with the antibody that recognizes the large C-terminal 118 kDa glycopeptide of rat intestinal mucin [1], but they did not react with the antiserum to the peptide E20-14. Homogenate samples were applied to heparin Affi gel columns and eluted with a NaCl step gradient as described earlier for the E20-14 peptide. Eluted fractions were then subjected to SDS-PAGE and Western blotting. Neither the wash fraction nor the NaCl gradient fractions reacted with the anti-E20-14 antiserum. As judged by a positive reaction with the anti-118 kDa antiserum however, the rest of the mucin was in the wash fraction (data not presented). Thus the mucin in homogenate supernatants did not bind to heparin and did not appear to contain the cationic C-terminal epitope.

Immunofluorescent localization

It was considered possible that the E20-14 domain of mucin in the homogenate or in purified mucin samples was present but was not accessible for reaction with its antibody. We therefore tested the reactivity of mucin *in situ*, by performing immunolocalization experiments on tissue sections. The E20-14 sequence was localized (Fig. 7A) to the basal region of goblet cells in the perinuclear area below and above the nucleus and along the lateral margins of the base of the theca, where RER, Golgi and condensing vacuoles are concentrated. There was no signal over the apical granule mass, where mature mucin is stored in goblet cells. Assuming that the C-terminal domain of the mature mucin in granules might still be 'buried' or covered by bound heparin (or other constituents), sections were pretreated with heparinase, heparitinase, mercaptoethanol or Target Unmasking Fluid in a boiling water bath for 10 min. However none of these treatments caused fluorescence to appear over the granule mass, and it was not possible to judge whether the bright signal over the RER and Golgi became any brighter after heparin-degrading enzymes were used. The experiment was therefore inconclusive with respect to demonstrating heparin binding to intracellular mucin, but did suggest that the extreme C-terminal 14 amino acids of the mucin might be present only in the basal region of goblet cells.

Specificity of the antibody for the E20-14 epitope was confirmed by demonstrating that basal immunofluorescence was abolished by preincubating the antibody with its peptide antigen (Fig. 7B). The peptide G21-10, which corresponds to the sequence immediately adjacent to the C-terminus of the mucin, gave no inhibition (not presented).

Peptide G21-10 was localized to the mature granule mass of goblet cells and secreted mucin, and in some cells, fluorescence was also detected in the supranuclear area below the theca (Fig. 7C). Therefore, with mucin in its natural configuration *in situ*, the peptide region immediately adjacent to the C-tail was accessible to its own antibody. Localization of this antibody was not inhibited by preincubation with the peptide E20-14 (not presented).

Discussion

Features of heparin-binding proteins

In addition to several blood clotting factors, many other proteins have been shown to exhibit strong heparin-binding properties, including a variety of growth factors [27–40], enzymes [41–46] serine protease inhibitors [10, 47–50], including mucus protease inhibitors [51], actin [52], antimicrobial peptides [53] and extracellular matrix components [25, 54–57]. It is through these inter-

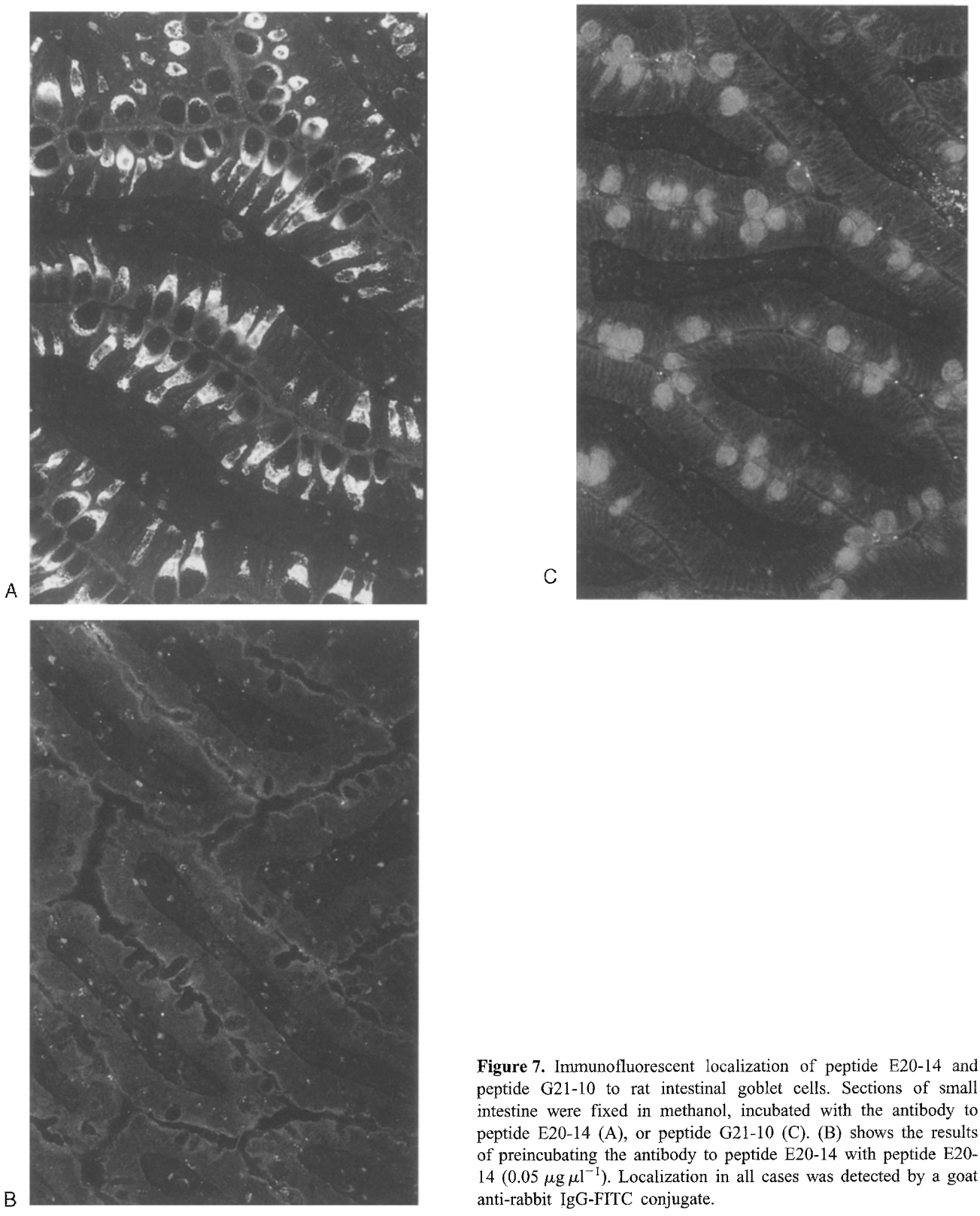

Figure 7. Immunofluorescent localization of peptide E20-14 and peptide G21-10 to rat intestinal goblet cells. Sections of small intestine were fixed in methanol, incubated with the antibody to peptide E20-14 (A), or peptide G21-10 (C). (B) shows the results of preincubating the antibody to peptide E20-14 with peptide E20-14 (0.05 $\mu g\,\mu l^{-1}$). Localization in all cases was detected by a goat anti-rabbit IgG-FITC conjugate.

actions that heparin and heparan sulfate proteoglycans influence a wide diversity of cellular processes.

Positively charged domains of about 14–20 amino acids in heparin binding proteins are responsible for the electrostatic interactions that mediate binding to the sulfate residues of long heparin chains. However the peptide domains possess more than simply an abundance of cationic residues [56, 57]. The important prerequisite is

a sequence in which alternating clusters of two or three adjacent positively charged residues (lysine or arginine) are separated by short stretches containing two to five uncharged residues [5, 38, 49, 58–61]. The present study suggests that the unique C-terminal domains of rat MLP and human MUC2 mucins share this requirement. A somewhat similar sequence may also be present near the C-terminal end of the human lung mucin MUC5. The MUC2 peptide F15-13 has a sequence of XBBXBBXXB XXXXX, the relevant MUC5 sequence is XBBBBXXX XXBBXB, and the MLP peptide, E20-14, contains a palindromic-like sequence of XBXBBXXXBXXXBB (where B refers to an arginine or lysine residue and X to a non-charged amino acid). Another palindromic pattern XBBXXBBBXXBBX defines the consensus heparin binding sequence in the A1 domain of von Willebrand factor [58].

As is true of other heparin binding peptides, the mucin peptides in the present study exhibited a preference for the most highly sulfated polysaccharides (i.e. heparin, dextran sulfate (a synthetic GAG analogue), and to a lesser extent, heparan sulfate), and were inhibited from binding to heparin by concentrated NaCl (0.4–0.5 M) or exogenously added heparin. As judged by the K_d values of heparin for the peptides (10^{-7}–10^{-8} M), the affinity is comparable to that of heparin binding by vWF [58, 62], growth factors [28, 34, 63, 64], fibrinogen [65], antithrombin III [66] and lipoprotein lipase [45].

Aggregate formation

At high molar concentrations relative to heparin (>14:1) the mucin peptide caused turbidity and decreased electrophoretic mobility of heparin, suggesting the formation of large heparin-peptide aggregates. A similar aggregation and partial charge neutralization of heparin occurs with mixtures of heparin and diamine salts [67]. In the case of E20-14 peptide, a molar ratio of approx. 18:1 (peptide:-heparin) was optimum for relatively stable aggregate formation, as judged from turbidity measurements and native gel electrophoresis. This suggests that the cationic peptide forms ionic bridges between sulfated polysaccharide chains of heparin. At higher ratios, the largest aggregates were unstable, presumably because an excess of positively charged peptide weakens the bridging function. At lower peptide:heparin ratios, the net charge of the mixture would become more negative, and sulfate repulsion between heparin chains probably accounts for dissociation of the large aggregates and a consequent lowering of solution turbidity.

Functional considerations

The physiological significance of the binding of mucin peptides to heparin is unknown at the present time. Purified mucin and mucin within intestinal homogenates did not bind to heparin, and immunolocalization studies suggested that the E20-14 sequence is not retained beyond the basal perinuclear region of goblet cells. Thus the cationic C-terminus of the mucin, which is a part of the expressed mucin peptide, may be cleaved during post-translational maturation before the mucin is packaged into mature granules. Mucin-heparin interactions, if they occur within goblet cells, would therefore be most likely in ER, Golgi or condensing vacuole compartments. Heparan sulfate proteoglycan is normally intercalated into membranes [7, 9] and heparin chains of the proteoglycan have been reported to have the capacity to anchor cationic proteins to membranes. This includes the tethering of positively charged histamine to heparan sulfate of mast cell granule membranes [68], carboxypeptidase to mast cell granule matrix [69], and lysozyme to leukocyte lysosomal granules [41]. Further research is needed to determine if the same anchorage mechanism could be operative for mucin molecules in ER vesicles or immature granules of goblet cells.

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