Characterization of quail intestinal mucin as a ligand for endogenous quail lectin

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The S-type lectins have been shown to be components of mucosal scrapings, and in avian systems these lectins have been localized immunohistochemically to the mucosal surface and goblet cells of the intestine. The interaction of lectin specifically with purified mucin has not, however, been established. Quail intestinal mucin was purified by two subsequent isopycnic density-gradient centrifugations in CsCl and chromatography on Sepharose Cl-2B. Purified mucin, obtained from the void volume of the Sepharose column, was characterized by SDS/PAGE, amino acid and carbohydrate analyses, sensitivity to thiol reduction, and cross-reactivity with antibody preparations to rat and human intestinal mucins on Western blots. Antibody raised against purified quail mucin partially cross-reacts with purified rat, rabbit and human intestinal mucins, and specifically labels the mucosal surface and goblet cells of quail intestine by the immunoperoxidase technique. Protein eluted by lactose from an affinity matrix composed of quail intestinal mucin possessed the same molecular mass on SDS/PAGE as intestinal lectin and reacted on Western blots with a lectin-specific antibody. The data clearly demonstrate the co-localization of lectin and mucin in the quail intestine and also the ability of the lectin to specifically interact with the purified mucin, raising the question of the role of endogenous lectins in secretions.

INTRODUCTION

A continuous layer of mucus protects the gastrointestinal tract by serving as a physical barrier between the underlying mucosa and the luminal contents of the intestine. Mucus glycoproteins (mucins) are the major structural components of mucus and are responsible for the visco-elastic properties characteristic of the mucus layer (Neutra and Forstner, 1987). The rich and varied carbohydrate content of the mucus gel has allowed it to become the target of many lectin associations. For example, many instances of bacterial binding to purified or partially purified mucin have been reported (Cohen et al., 1983; McSweegan and Walker, 1986; Mouricout and Julien, 1987; Yamamoto and Yokota, 1988; Mantle et al., 1989b; Wanke et al., 1990; Sajjan and Forstner, 1990a; Mack and Sherman, 1991). Bacterial binding to mucin may, however, involve association with the N-linked oligosaccharide of the link protein (Sajjan and Forstner, 1990b) or other protein components of the mucus (Sanford et al., 1989). Protozoan parasites such as *Entamoeba histolytica* (Petri et al., 1990; Chadee et al., 1991; Saffer and Petri, 1991) and *Giardia lamblia* (Lev et al., 1986) also possess lectin adhesions that are thought to mediate binding of the organism to mucus. In addition to such exogenous lectins, endogenous lectins have also been found in mucus secreted on the skin or mucosal surfaces. For example, mucus covering the skin of *Xenopus* and fish is rich in β-Gal-specific lectins (Bols et al., 1986; Shiomi et al., 1989). Families of β-Gal-specific endogenous lectins have been found at the mucosal surfaces of rat (Cerra et al., 1985; Clerch et al., 1988) and human (Sparrow et al., 1987) lung, and rat (Leffler et al., 1989) and rabbit (Harrison et al., 1984; R. Fang and H. Ceri, unpublished work) intestine. Endogenous lectins capable of binding heparin are secreted at the mucosal surfaces of airways, with increased secretion found in cystic fibrosis (Ceri et al., 1988, 1991). The role played by endogenous lectins in mucus secretions is not known. In the avian intestine, only a single β-Gal-specific lectin is secreted on mucosal surfaces. This lectin is localized to the mucosal surface and to goblet cells in chicken and quail intestines (Beyer et al., 1979; Beyer and Barondes, 1980; Fang and Ceri, 1989). The relative simplicity of the avian system offers an ideal opportunity to study the role of β-Gal-specific lectins in mucus secretion and to establish whether a direct association of lectin with avian mucin occurs. We now report the purification and characterization of quail intestinal mucin and its ability to serve directly as a ligand for the quail intestinal lectin.

MATERIALS AND METHODS

Isolation and purification of mucin

Quails (*Caturnix caturnix japonica*) were obtained from the Quail Genetic Stock Center, Department of Animal Science, University of British Columbia, Vancouver, BC, Canada. After an overnight fast, quails were decapitated and the small intestine removed. The lumen was flushed with PBS [75 mM NaH_2PO_4/K_2HPO_4 (pH 7.2), 75 mM NaCl] containing 5 mM Na_2EDTA, 1 mM phenylmethanesulphonyl fluoride and 10 mM *N*-ethylmaleimide as proteolytic inhibitors. Mucosal scrapings were collected and homogenized in the same buffer. Mucin was purified from homogenates by two consecutive equilibrium density-gradient centrifugations in CsCl followed by chromatography on Sepharose Cl-2B (Pharmacia) (Mantle et al., 1984; Mantle and Thakore, 1988). The void volume fractions were collected. Mucins from human, rabbit and rat small intestine were prepared as above.

Chemical analysis

The amino acid composition of purified mucin was determined by acid hydrolysis under vacuum followed by analysis on a Beckman 6300 apparatus using ninhydrin detection. Amino sugars were analysed on the same machine following standard acid hydrolysis. Monosaccharide analysis of quail mucin hydro-

Abbreviation used: HRP, horseradish peroxidase.

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lysates was carried out by the method of Chaplin (1982). Protein was assayed using the Bradford method (Bradford, 1976), total glycoprotein by the periodic acid/Schiff reagent assay (Mantle and Allen, 1978), and DNA by the method of Gendimenico et al. (1988). Sulphate was determined as previously described (Mantle et al., 1981).

Gel electrophoresis

PAGE of mucin was carried out by the method of Laemmli (1970) using a 5.7% stacking gel and a 7.5% running gel. Gels were silver-stained using the Bio-Rad kit following the manufacturer's directions, or sections of the gel were cut and stained for carbohydrate using the periodic acid/Schiff method.

Western blot analysis

Electrophoretic transfer from SDS/PAGE to Immobilon-P (Millipore) was conducted as previously described (Fang and Ceri, 1989). Immunoreactive bands were developed using first rabbit antibody to quail intestinal mucin (see below) followed by goat anti-rabbit serum conjugated to horseradish peroxidase (HRP) (Sigma). Finally, HRP-conjugated rabbit anti-HRP (Sigma) antibody was added. Bands were visualized using the chromogen 3,3′-diaminobenzodine tetrahydrochloride (Sigma) in PBS containing H_2O_2 (Fisher).

Thiol reduction and trypsin digestion

Thiol reduction of mucins was carried out at 100 °C in the presence of 0.2 M 2-mercaptoethanol over a series of time points. Mucin was alkylated by the addition of 0.5 M iodoacetamide (Mantle and Thakore, 1988). Trypsin digestions were carried out at an enzyme/substrate ratio of 1:1000 for 72 h at 37 °C using bovine pancreatic trypsin (type III; Sigma) (Mantle, 1991). Alternatively, digestions were carried out at high enzyme/substrate ratios that are known to digest the non-glycosylated protein components of mucin (Mantle et al., 1984). The effect of thiol reduction or trypsin digestion on mucin was assayed by both SDS/PAGE and chromatography on Sepharose Cl-2B (Pharmacia), as described above.

Preparation of antibody

Antibody against purified quail intestinal mucin was developed in rabbits by subcutaneous injection of a mixture of 100 μl of purified mucin (approx. 40 μg), 100 μl of Pertussus vaccine (Sigma) and 400 μl of Freund's incomplete adjuvant (Gibco) as previously described (Mantle and Thakore, 1988). Booster doses of mucin antigen in Freund's incomplete adjuvant with Pertussus vaccine were administered three times at 2-week intervals, and every 6 weeks thereafter. The antibody titre to quail mucin was determined by e.l.i.s.a. assays, in which purified quail mucin was used to coat e.l.i.s.a. wells (Nunc) and HRP-conjugated goat anti-rabbit antibody was used as the detection system. In cross-reactivity assays, microtitre wells were coated with purified rat, rabbit or human mucin and assayed as above. Specific antibodies to rat, rabbit or human mucin, as previously described (Mantle et al., 1984, 1989b; Mantle and Thakore, 1988), were used as positive controls, and preimmune serum as a negative control.

Intestinal lectin purification

Quail intestinal β-Gal-specific lectin was purified from mucosal scrapings by affinity chromatography on asialofetuin–Sepharose as previously described (Fang and Ceri, 1989). Affinity columns of purified quail mucin were prepared using Affi-Gel 10 (Bio-Rad) as per the manufacture's protocol. Quail mucosal scrapings were homogenized in 5 vol. (w/v) of MEPBS (PBS containing 2 mM EDTA and 4 mM 2-mercaptoethanol) containing 0.3 M lactose and subjected to centrifugation at 100000 *g* for 1 h. The supernatant was dialysed against MEPBS and absorbed to the mucin–Affi-Gel 10 affinity column. The lectin was purified from the affinity column by first washing the column with MEPBS until the absorbance at 280 nm returned to baseline, and then eluting the column with 2 column volumes of MEPBS + 0.3 M sucrose, followed by an equal volume of MEPBS + 0.3 M lactose.

Mucin immunohistochemistry

The localization of mucin and lectin in quail intestine was carried out using either rabbit anti-(quail mucin) antibody or rabbit anti-(quail lectin) antibody as previously described to localize quail intestinal lectin (Fang and Ceri, 1989). Briefly, 5 cm of quail duodenum, jejunum, ileum or colon was removed and fixed in Carnoy's solution overnight (Humason, 1972). Sections of 1 μm thickness were sequentially dehydrated in alcohol and embedded in plastic using Immunobed kits (Polyscience Inc.) according to the supplier's directions. Serial sections were then treated with either anti-mucin or anti-lectin antibody diluted 1:100 in PBS. Peroxidase detection was carried out as described for Western blots. Colour was developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma) and H_2O_2 (Fisher). Mucin distribution was confirmed by staining goblet cells with periodic acid/Schiff (Mantle et al., 1989a).

RESULTS AND DISCUSSION

Mucin purification

Endogenous lectins have been found associated with mucus secretions on the skin (Bols et al., 1986) and at mucosal surfaces (Beyer et al., 1979; Leffler et al., 1989; Fang and Ceri, 1989); however, the affinity of these secreted lectins for mucin has never been conclusively established. The quail offers an important model in which to consider lectin–mucin interactions, as only a single lectin is present in the quail intestinal tract (Fang and Ceri, 1989). However, mucins from avian systems have not been chemically defined. To isolate quail intestinal mucin, we used a standard purification procedure that has been used successfully in the past to isolate intestinal mucins from man, rabbit and rat (Mantle et al., 1984; Mantle and Thakore, 1988; Mantle, 1991), namely two consecutive density-gradient centrifugations in CsCl followed by gel filtration on Sepharose Cl-2B. Two independent quail intestinal mucin preparations run on SDS/PAGE and silver stained showed a single protein band barely entering the stacking gel and the absence of protein-staining bands in the separating gel (Figure 1, lanes C and D). This pattern is typical of purified mucins from other species, indicating that there is no non-covalently attached protein contaminating the mucin preparation. Thio-reduction of the mucin resulted in the release of a 116 kDa band that was visualized on SDS/PAGE (Figure 1, lanes B and E). This may be analogous to the so-called link protein identified previously in mammalian intestinal mucins. Carbohydrate staining, using the periodic acid/Schiff method, showed the putative mucin bands to be heavily glycosylated, as expected (Figure 1, lanes G and H). The 116 kDa band, which is not highly glycosylated and contains only N-linked sugar chains, was not easily detectable by this staining method and cannot be seen in the photographs. Reduction also affected the

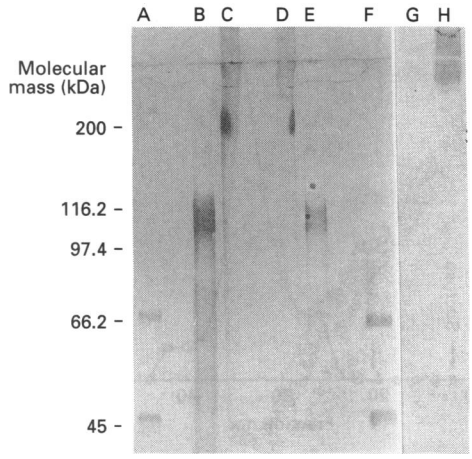

Figure 1 SDS/PAGE of quail intestinal mucin

Purified quail mucin (approx. 100 μg/lane) from two independent preparations was run on 7.5% polyacrylamide gels with 5.7% stacking gels; bands were detected by silver staining. Lanes A and F, molecular mass markers; lanes C and D, native mucin from two independent preparations; lanes B and E, the same mucin following reduction with 0.2 M 2-mercaptoethanol. Lanes G and H contain native and reduced mucin run as above, but detected by periodic/Schiff staining for carbohydrate.

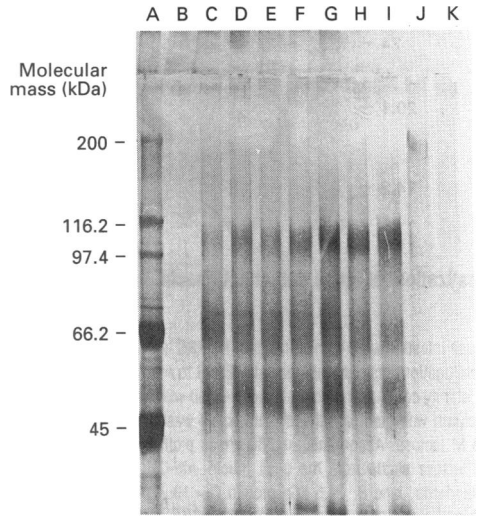

Figure 2 SDS/PAGE of quail mucin following trypsin digestion

Purified mucin was digested with trypsin (enzyme/substrate ratio 1:1000) at 37 °C for the times indicated below. Samples (~ 100 μg/lane) were then run on SDS/PAGE as described in the legend to Figure 1. Lane A, molecular mass standards; lane B, trypsin alone; lanes C–I, mucin incubated for 72, 48, 24, 16, 8, 4 and 2 h respectively in the presence of trypsin and then reduced with 0.2 M 2-mercaptoethanol prior to SDS/PAGE; lane J, mucin incubated for 72 h in trypsin but not reduced prior to SDS/PAGE; lane K, mucin incubated for 72 h without trypsin.

Sepharose-2B elution profile of the mucin, causing it to be retarded by the column and eluted in the partially included fractions (results not shown). This demonstrates that the quail mucin shares another feature in common with mammalian intestinal mucins, namely thiol-dependent polymerization. Trypsin digestion studies demonstrated the importance of non- (or poorly) glycosylated, proteinase-sensitive regions in the peptide core in mucin polymerization (Figure 2, lanes C–I). Exhaustive trypsin treatment gave a mucin which eluted in the partially included fractions of a Sepharose Cl-2B column (results not shown). Digestion at low enzyme/substrate ratios also resulted in progressive trypsin degradation of the link protein. Again, these observations are similar to those made previously

Table 1 Amino acid composition of quail intestinal mucin, expressed as mol% of total protein

Component	Composition (mol%)
Asp	7.2±0.4
Thr	19.0±1.0
Ser	15.9±0.7
Glu	9.1±0.4
Pro	8.9±0.3
Gly	6.6±0.5
Ala	5.0±0.2
Cys	1.3±0.2
Val	6.7±0.3
Met	0.5±0.1
Ile	3.3±0.1
Leu	4.8±0.3
Tyr	2.3±0.2
Phe	2.3±1.9
His	1.7±0.2
Lys	3.7±0.3
Arg	2.5±0.2

Table 2 Carbohydrate composition of quail intestinal mucin, expressed as mol% of total carbohydrate

Component	Composition (mol%)
Fuc	0
Gal	13.2±0.4
GlcNAc	3.9±0.4
GalNAc	33.3±2.0
Sialic acid	52.4±0.7
Man	0
GlcUA	0

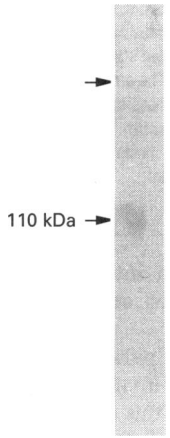

Figure 3 Immunological cross-reactivity of quail, rat, rabbit and human mucins

Purified quail mucin (~ 20 μg/lane) reduced in 0.2 M 2-mercaptoethanol and subjected to SDS/PAGE and Western blots was probed with a rabbit antibody specific for purified human intestinal mucin. Arrows mark the reactive material seen at the top of the stacking gel and at ~ 110 kDa.

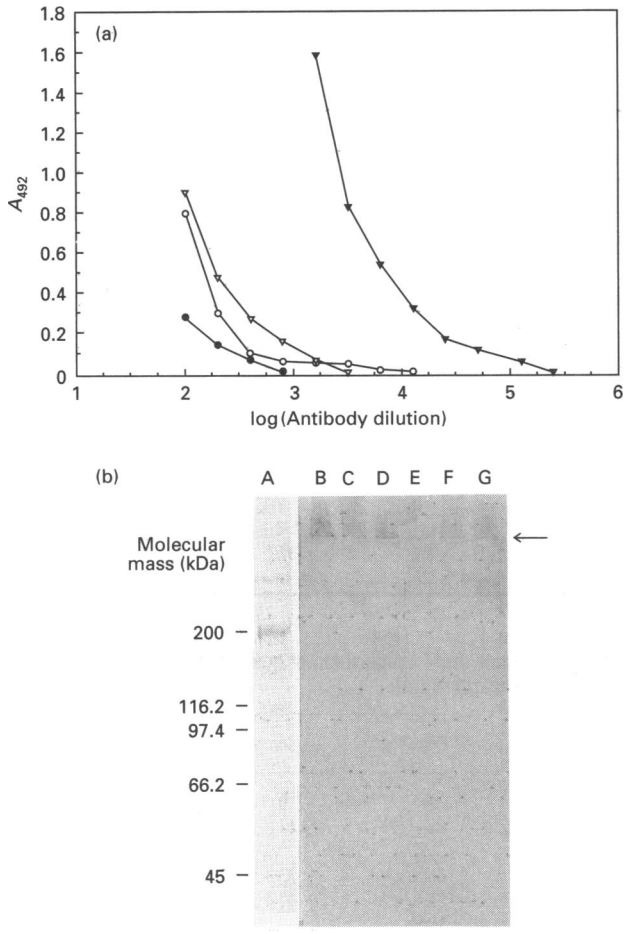

Figure 4 Interaction of quail mucin with endogenous lectin

(**a**) The specificity of the antibody raised against quail mucin was determined in e.l.i.s.a. against wells coated with quail (▼), rat (○), rabbit (●) or human (▽) mucin. Assays were read on an e.l.i.s.a. reader (Titerteck; Flow Labs) and the data were plotted as A_{492} against antibody dilution. (**b**) Purified rat, rabbit and human intestinal mucins (~ 20 μg/lane) were subjected to SDS/PAGE in both reduced and non-reduced states and Western blots were probed with the antibody specific for quail intestinal mucin. The arrow marks the reactive material seen at the top of the stacking gel. Lane A, molecular mass standards; lanes B and C, non-reduced and reduced samples respectively of purified human mucin; lanes D and E, non-reduced and reduced samples respectively of purified rat mucin; lanes F and G, non-reduced and reduced samples respectively of purified rabbit mucin.

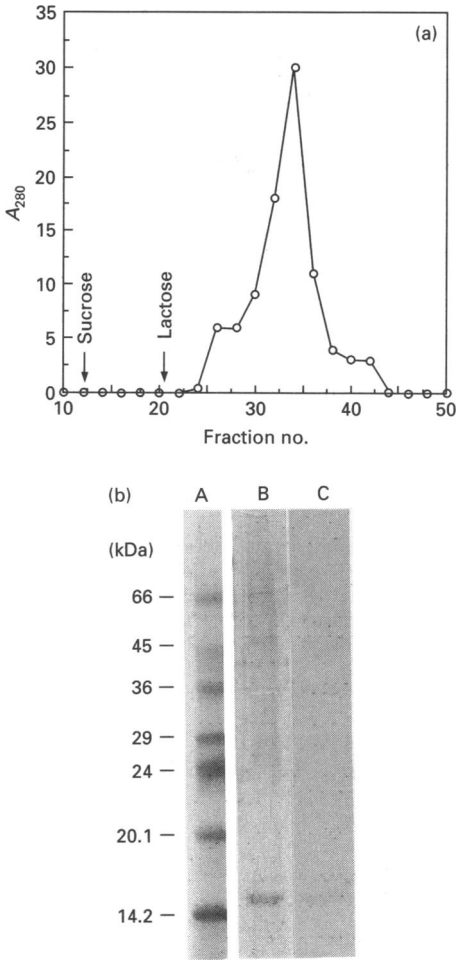

Figure 5 Localization of quail intestinal mucin and lectin by immunohistochemistry

(**a**) Scrapings of quail intestine were homogenized in MEPBS + 0.3 M lactose. The supernatant of a 100000 *g* centrifugation was dialysed against MEPBS to remove lactose and run on a quail mucin–Affi-Gel 10 affinity column. The column was washed with MEPBS until no further protein was eluted. The column was then eluted with successive washes of MEPBS + 0.3 M sucrose and MEPBS + 0.3 M lactose. Absorbance at 280 nm is plotted against fraction number. (**b**) SDS/PAGE of the lactose eluate from the quail mucin–Affi-Gel 10 affinity column. Lane A, molecular mass standards; lane B, 14.5 kDa protein (~ 10 μg/lane) eluted by lactose from the affinity column and silver-stained on an SDS/15%-PAGE gel; lane C, Western blot of the lactose-eluted fraction developed with the antibody specific for quail lectin.

in studies of rat and human intestinal mucins showing that mammalian and avian mucins are remarkably alike in gross macromolecular structure.

In addition to the physical parameters, which indicated that the isolated material from the quail was indeed intestinal mucin, the chemical composition of this material was also in keeping with that of purified mucin from other species. Amino acid analyses provided a profile characteristic of mucins with a high serine/threonine/proline content (Table 1). The sugar composition was again characteristic of mucins. Carbohydrate analysis indicated high levels of *N*-acetylgalactosamine, indicative of O-glycosidically linked polysaccharide. The absence of mannose or uronic acids would suggest no or very few N-linked sugar chains or contamination with proteoglycans (Table 2). The carbohydrate composition of quail intestinal mucin is unusual in that it suggests a majority of very simple oligosaccharides, comprising only GalNAc and sialic acid with some chains also containing β-Gal. This structure is similar to the oligosaccharides of bovine and ovine submaxillary mucins, but unlike mammalian intestinal mucins which have longer sugar chains containing *N*-acetylglucosamine and fucose. This difference in the chain length of the mucin sugar chains is also reflected in the fact that 60% of the dry weight of mucin was carbohydrate in nature, as compared with values approaching 80% in mammalian species. Sulphate was present at 3.5% dry weight, which is similar to the values reported by Xu et al. (1992) and Mantle et al. (1981). Analysis for DNA detected < 1 μmol/mg of mucin, suggesting negligible contamination of the mucin preparations.

The highly conserved nature of mucins could also be demonstrated by immunological cross-reactivity studies. Antibody raised against purified human intestinal mucin (Mantle et al., 1989b) reacted with quail mucin and its link protein on Western blots (Figure 3). Antibody produced in rabbits against purified quail intestinal mucin demonstrated strong reactivity with quail intestinal mucin and partial cross-reactivity with purified human, rat and rabbit intestinal mucins in e.l.i.s.a.s, suggesting con-

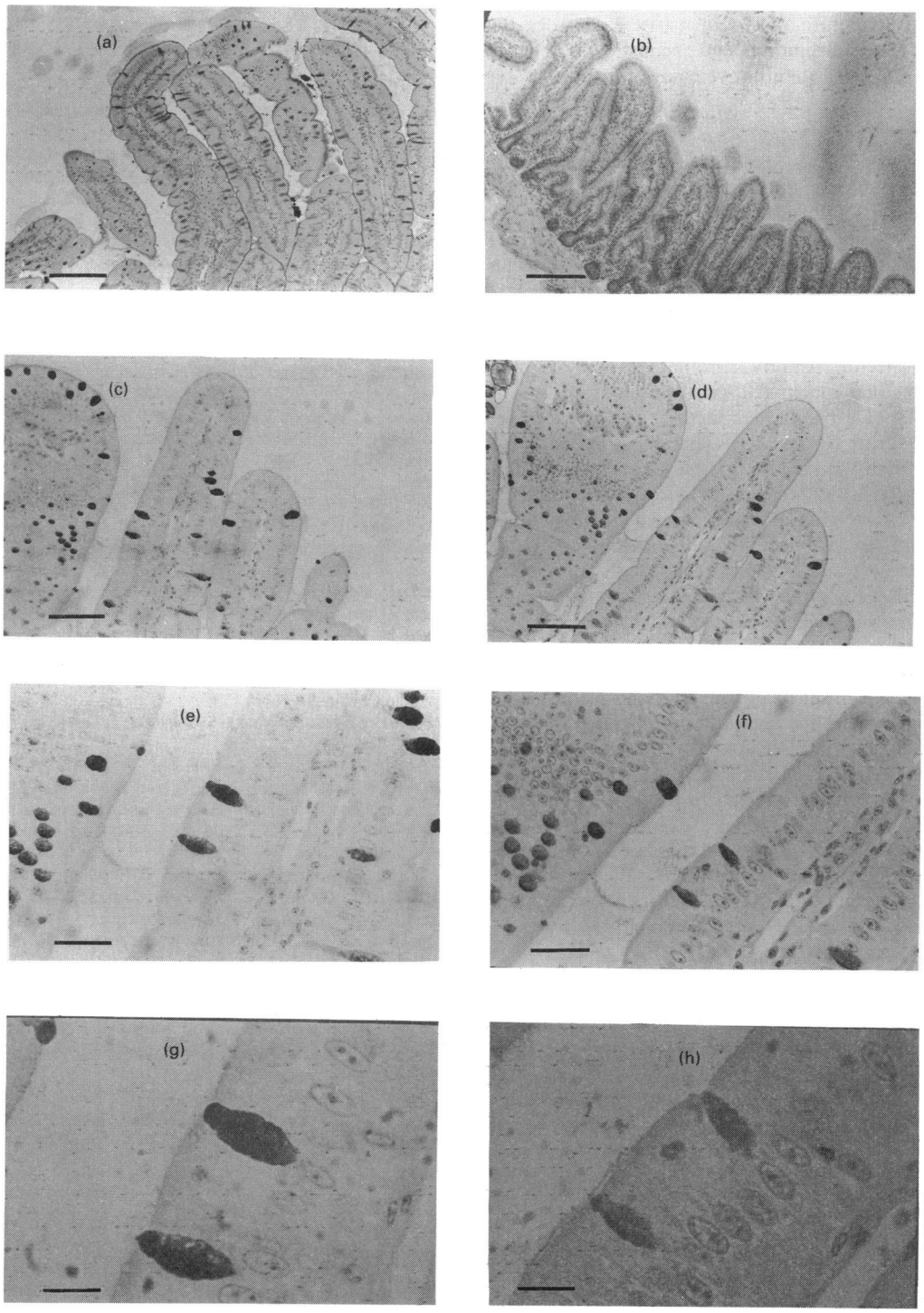

Figure 6 Localization of quail intestinal mucin and lectin by immunohistochemistry

Antibody preparations specific for either quail intestinal mucin or the 14.5 kDa quail intestinal lectin were used to localize the appropriate substrate in 1 μm serial sections of intestine. Panel (**a**) is a section stained by periodic acid/Schiff to show mucin distribution. Panel (**b**) is a control section stained with preimmune serum from the rabbit used to produce the mucin specific sera. No staining was also seen with preimmune sera taken from the rabbit used for lectin antibody production (results not shown). Panels (**c**), (**e**) and (**g**) represent sections at a low, medium and high magnification after staining with quail mucin-specific antibody. Panels (**d**), (**f**) and (**h**) represent serial sections at the same magnification as shown in (**c**), (**e**) and (**g**) respectively; however, these sections are stained with antibody to quail lectin. Note the co-localization of mucin and lectin in identifiable goblet cells. Magnification bars: (**a**) and (**b**), 4.3 μm; (**c**) and (**d**), 2.7 μm; (**e**) and (**f**), 1.0 μm; (**g**) and (**h**), 0.43 μm.

servation of antigenic structures in mucins (Figure 4a). Western blots (Figure 4b) revealed that the cross-reactivity of the anti-(quail mucin) antibody was towards the mucin glycoprotein component of the mucins from other species, as reactivity was most visible with material just entering the stacking gel. Western blots of non-reduced crude mucosal scrapings, probed with antibody to quail mucin, revealed only the mucin band in the stacking gel (results not shown), demonstrating the specificity of the antibody and the lack of cross-reactivity with the many other proteins present in the mucosal scrapings. The specificity of the

anti-mucin antibody therefore allows for its use as a probe for the distribution of mucin in immunocytochemistry (see below) and for the development of quantitative assays for mucin.

Mucin–lectin interactions

Lectin is not seen in gels of purified mucin (Figure 1), as the high-salt conditions of CsCl purification are believed to dissociate the lectin from the mucin. Lectin bands could be seen in crude mucin preparations or in mucin fractions voiding from gel-filtration columns where the mucin had not been previously purified using CsCl (results not shown). However, the purity of these preparations is then in question. To address the question of lectin–mucin interactions, we used the purified mucin as an affinity ligand for the purification of lectin. Affinity chromatography of quail intestinal extracts on quail mucin–Affi-gel 10 columns resulted in the elution of a 14.5 kDa band when lactose, but not sucrose, was added to the eluent (Figure 5a). The eluted protein was reactive with an antibody specific for quail intestinal β-Gal lectin (Fang and Ceri, 1989) in Western blots (Figure 5b). In addition, haemagglutination assays showed that the purified mucin could inhibit the activity of the quail lectin (results not shown). These findings clearly demonstrate that the quail β-Gal specific intestinal lectin can interact with the purified intestinal mucin, and supports the previously implied association of mucin and lectin in the chicken intestine (Beyer et al., 1979). The association of lectin and mucin in the intestine is further supported by the immunological co-localization of both mucin and lectin at the mucosal surface of the intestine and within goblet cells (Figure 6). Both mucin and lectin can be seen to be localized to individual goblet cells in serial sections of quail intestine. Periodic acid/Schiff staining of these sections confirms the presence of mucin in the goblet cells. Although these findings are highly suggestive of a role for endogenous lectins in the secretions of the intestinal tract, they do not yet clearly define a function for the lectin in the secretion process. Attempts to define a role for lectin in mucin secretion are the focus of our current research efforts.

This study was supported by a grant to H.C. from the Natural Sciences and Engineering Research Council of Canada. R.F. was supported by a studentship from the Canadian Cystic Fibrosis Foundation and the Alberta Heritage Foundation for Medical Research.

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Received 29 January 1993/17 March 1993; accepted 24 March 1993