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Polymeric IgA are sulfated proteins

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Abstract The main sulfated proteins secreted by rabbit mammary gland tissue had M_r of ~67 000, 63 000 and 23 000, and one component which most likely corresponded to proteoglycans had a diffuse electrophoretic mobility ($M_r > 200\,000$). The sulfate groups in the 67–63 kDa proteins were mostly linked to carbohydrates. These proteins and the 23 kDa protein were co-purified and identified to heavy chains of immunoglobulin A (IgA) and J chain, respectively. Sulfation of α-chains also occurred in rat mammary and rabbit lacrimal glands. We conclude that polymeric IgA which are produced by plasma cells and released in secretion fluids after transcytosis through epithelia are sulfated.

Key words: Polymeric IgA; Sulfation; Secretory protein; Milk protein; Mammary gland; Epithelial cell

1. Introduction

During lactation, mammary epithelial cells (MEC) synthesize and secrete huge quantities of proteins and other milk components such as lipids and lactose. In addition, proteins originating from blood or produced by cells in the interstitium of the mammary gland tissue are actively transported into milk by transcytosis across the MEC epithelium. Thereby, most milks contain several tenth grams of protein per liter. Although there has been considerable research dedicated to the mammary gland and the biochemistry of milk proteins, much is still to be learn about the cell biology of protein secretion by the MEC. The study of milk protein secretion is not only of interest due to its physiological and economical importance, but also offers an opportunity for the study of a highly developed secretory pathway.

To monitor the transport of milk proteins from the *trans*-Golgi network (TGN) and to study the molecular machinery involved in the formation of secretory vesicles from this compartment, we wished to precisely label markers for the secretory pathway of MEC in the TGN. Since sulfation is an ubiquitous TGN specific post-translational modification of proteins passing through the secretory pathway (for review, see [1,2]), short pulse labeling with [^{35}S]sulfate can be used to label proteins selectively in this compartment [3–6]. This approach has proved invaluable to study protein sorting to the regulated secretory pathway of neuroendocrine cells [7,8] (for review, see [9]). In an attempt to develop such an assay to investigate secretory vesicle formation in MEC, we have characterized the sulfated proteins produced by rabbit mammary explants, with the aim of identifying secretory markers of the MEC that would be subjected to sulfation.

2. Materials and methods

2.1. Preparation of explants and metabolic labeling

Mammary gland explants were prepared from either New Zealand rabbits or from Fischer 344 rats, both in the 14th day of first lactation, as previously described [10]. Lacrimal glands obtained from New Zealand rabbits were dissected free from connective tissue and lobules were manually cut into ~1 mm^3 fragments. Prior to labeling with either [^{35}S]sulfate or [^{3}H]leucine, explants were pre-incubated for 30 min with sulfate-free [9] or leucine-free DMEM, respectively. To analyze the secretion of sulfated proteins from rabbit mammary gland tissue, explants were then either pulse-labeled for 5 min with fresh sulfate-free DMEM containing 37 MBq/ml (1 mCi/ml) [^{35}S]sulfate (Amersham Pharmacia Biotech) or pulse-labeled, and chased for 90 min with complete DMEM containing twice the normal concentration of sulfate. To accumulate [^{35}S]sulfate or [^{3}H]leucine-labeled proteins in the medium, mammary gland explants were labeled for 30 min with either 18.5 MBq/ml (0.5 mCi/ml) [^{35}S]sulfate or 1.85 MBq/ml (50 μCi/ml) [4,5-^{3}H]leucine (Amersham) and chased for 1 or 2 h. To analyze the sulfated proteins released from either rat mammary gland or rabbit lacrimal gland, explants were continuously labeled for 2.5 h. Typically, for pulse labeling, ~100 mg of explants was used per experimental condition, while up to 10 g was used for preparative isolation of sulfated proteins from medium. All incubations were performed at 37°C, under an atmosphere of 95% O_2/5% CO_2. To prepare a fraction enriched in [^{35}S]sulfate-labeled granins, PC12 cells were grown as previously described [7], pre-incubated as above and labeled for 6 h with fresh sulfate-free DMEM containing 3.7 MBq/ml (0.1 mCi/ml) [^{35}S]sulfate.

2.2. Analysis of secretion

At the end of the pulse or the chase, explants were separated from the incubation medium, extensively washed with ice-cold Tris buffer saline (25 mM Tris-HCl pH 7.4, 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4) and homogenized at 4°C as previously described [11]. In some experiments, washed explants were resuspended in 10 mM HEPES buffer pH 6.7 containing 0.25 M sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM magnesium acetate and 0.5 mM phenyl methylsulfonyl fluoride (PMSF) and homogenized with three strokes of a tissue grinder (AA2 Teflon/glass, Thomas Scientific). The homogenate was centrifuged for 10 min at 1000×*g* at 4°C and the resulting supernatant, referred to as post-nuclear supernatant (PNS), was collected. The medium was centrifuged for 5 min at 1200×*g* at 4°C and the proteins contained in an aliquot of the resulting supernatant were precipitated with TCA. The medium and an aliquot of the tissue were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography [12].

2.3. Acidic hydrolysis of sulfate from [^{35}S]sulfate-labeled proteins

Proteins contained in the medium obtained from either [^{35}S]sulfate or [^{3}H]leucine-labeled rabbit mammary gland explants were precipitated with 50% ethanol at −20°C. In addition, a heat-stable protein fraction highly enriched in granins was prepared from [^{35}S]sulfate-labeled PC12 cells as previously described [13]. Proteins from the

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Abbreviations: HC, heavy chain; Ig, immunoglobulin; LC, light chain; MEC, mammary epithelial cells; TGN, *trans*-Golgi network

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above samples were separated by SDS-PAGE and subjected to in gel acidic hydrolysis with HCl [14].

2.4. Purification of sulfated proteins

For purification of sulfated proteins at the preparative scale, secretion medium obtained from several gram of [^{35}S]sulfate-labeled rabbit mammary gland explants was centrifuged for 30 min at 1800×*g* to pellet casein micelles and the supernatant was concentrated using a Centriprep 10 (Amicon). This and all subsequent manipulations were performed at 0–4°C. The resulting medium was adjusted to 20% glycerol and run through a Sephacryl S-200 column (Amersham) using 0.1 M Tris-HCl pH 7.5, 0.1 M NaCl. [^{35}S]Sulfate-labeled proteins were recovered in the flow-through, loaded onto a concanavalin A Sepharose column (Amersham) and bound proteins were eluted with 0.5 M α-methyl-mannose in the above buffer. Fractions containing [^{35}S]sulfate-labeled proteins were concentrated using a Centricon 10 and pooled eluate was either stored at −20°C or subjected to TCA precipitation. Samples were analyzed by either SDS-PAGE or two-dimensional electrophoresis (2D-PAGE) [10] followed by fluorography.

To analyze the potential sulfation of the 63–67 kDa protein in other tissues and species, the proteins contained in the medium obtained from [^{35}S]sulfate-labeled explants prepared from either rat mammary or rabbit lacrimal glands were purified on a protein L agarose column following the manufacturer's instructions (Clontech Laboratories). Both proteins in the flow-through and affinity-purified proteins were analyzed by SDS-PAGE followed by fluorography. An aliquot of purified human polymeric immunoglobulin (Ig) A1-kappa was loaded as a control for the electrophoretic mobilities of IgA chains.

2.5. Identification of sulfated proteins

Prior to N-terminal sequencing, the 23 kDa protein, which, as compared to total protein, was in low amount in the concanavalin A eluate, was further purified by reversed-phase high performance liquid chromatography on a Vydac RP-18 column eluted with a 5–100% acetonitrile gradient. Proteins in the relevant fractions were resolved by 2D-PAGE and transferred to ProBlott membrane (Applied Biosystems) according to the manufacturer's recommendations. To identify the 63 kDa protein, proteins eluted from concanavalin A Sepharose were separated by 2D-PAGE and the spot of interest, located according to the Coomassie blue staining, was cut from the gel. The 63 kDa protein was electroeluted from the gel piece, concentrated using a Centricon 10 and subjected to cleavage using cyanogen bromide. Peptides were separated by SDS-PAGE using Tris-Tricine as the running buffer and transferred to ProBlott membrane. Pieces of the membranes containing the proteins of interest were excised and automated Edman degradation was performed by using a A94A Procise Edman sequencer (Applied Biosystems). The 63–67 kDa protein was further identified by immunoblotting [15], using a goat antiserum against IgA from rabbit colostrum (Sigma Aldrich) at a 1:10 000 dilution.

3. Results and discussion

3.1. Characterization of the sulfated proteins secreted by rabbit mammary gland explants

To identify the subset of secretory proteins of the milk that are sulfated, we used metabolic sulfate labeling of rabbit mammary explants. As shown in Fig. 1, incubation of explants for 5 min with [^{35}S]sulfate revealed the presence of three major newly synthesized sulfated proteins in the tissue. The component with diffuse electrophoretic mobility between the top of the gel and M_r of ∼200 000 most likely corresponded to sulfated glycosaminoglycans, consistent with the presence of such molecules in the basement membranes of MEC [16,17]. The two other main sulfated proteins had M_r of ∼67 000 and 63 000. Consistent with the notion that sulfation is a post-translational modification which occurs on secretory proteins, all sulfate-labeled proteins were found in the medium after chase (Fig. 1, 90 min). In addition, a protein with M_r of ∼23 000 was faintly labeled. Compared to caseins

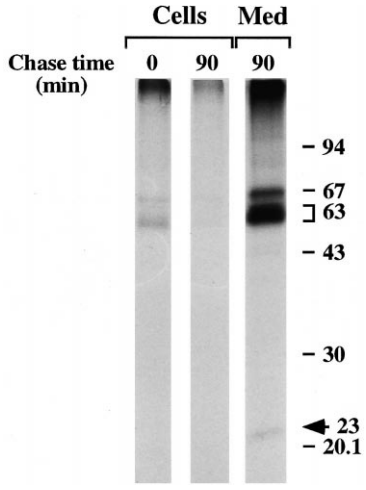

Fig. 1. Rabbit mammary gland explants secrete sulfated proteins. Rabbit mammary gland explants were pulse-labeled for 5 min with [^{35}S]sulfate and either collected or chased for 90 min. Aliquots (1/66th) of the cell lysate (Cells) and the medium (Med) were analyzed by SDS-PAGE followed by fluorography.

(M_r of ∼26 000–32 000), the main proteins in rabbit milk (∼100 mg/ml), these proteins were poorly represented in secretory medium. They neither corresponded to significant Coomassie blue-stained bands (data not shown) nor matched major radioactive proteins secreted from [^{3}H]leucine-labeled explants (see Fig. 2A). The 67 and 63 kDa proteins were secreted with identical kinetics (data not shown), ∼50% of these being recovered in the medium after 90 min chase. In contrast, the kinetics of secretion of the proteoglycans appeared much slower, only ∼6% of the molecules were found in the medium after 90 min chase. Consistent with this, the majority of sulfate-labeled proteoglycans was not extracted with carbonate at pH 11 (data not shown). Therefore, although these latter components can be selectively labeled in the TGN and might, at least in part, be expressed in MEC, they cannot serve as secretion markers. Finally, it is noteworthy that in non-reducing gels (see below and Fig. 4B), the [^{35}S]sulfate-labeled 67, 63 and 23 kDa proteins disappeared and most likely migrated as a high M_r form in the region of the gel containing the proteoglycans. As further demonstrated below, this indicated that these proteins formed homo- or hetero-oligomeric complexes of high molecular mass involving intermolecular disulfide bonds. However, when caseins were eliminated from culture medium by centrifugation (caseins are found in milk as large aggregates, the casein micelles), the 67, 63 and 23 kDa proteins were not recovered in the casein pellet (data not shown), indicating that these proteins belong to the whey fraction of milk. These features were used in the purification procedure of these molecules.

3.2. Sulfation of the main sulfated proteins secreted in milk mostly occurs on carbohydrates

Sulfation of a protein can occur on either tyrosine residues, carbohydrate moieties or both. In the first type of modification, the sulfate is linked to the tyrosine residue as an O-sulfate ester which was found to be largely more acid labile than most carbohydrate sulfate esters. Hence, short acid treatment of sulfated proteins allows to identify those proteins likely to contain tyrosine sulfate [14]. Fig. 2A shows that in

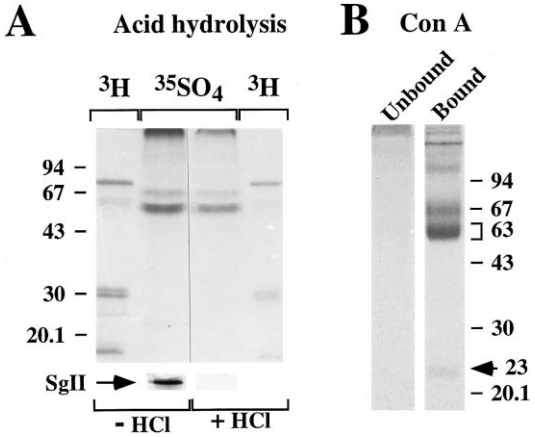

Fig. 2. Sulfation of the main sulfated proteins produced by rabbit mammary gland mostly occurs on carbohydrate residues. A: The medium obtained from either [^{3}H]leucine (^{3}H) or [^{35}S]sulfate-labeled ($^{35}SO_4$) explants and a heat-stable protein fraction prepared from [^{35}S]sulfate-labeled PC12 cells (SgII) were analyzed by SDS-PAGE and the gel was fixed. Gel pieces were incubated in the absence (−HCl) or the presence of 1 M HCl (+HCl) and processed for fluorography. Only the region of the fluorogram containing the secretogranin II band (SgII) is shown. B: A fraction enriched in sulfated proteins was prepared from the culture medium of [^{35}S]sulfate-labeled explants by chromatography and was applied to a column of concanavalin A Sepharose (Con A). Proteins recovered in the flow-through (Unbound) or eluted from the column (Bound) were analyzed by SDS-PAGE followed by fluorography.

gel HCl treatment of proteins from [^{35}S]sulfate-labeled explants resulted in a small reduction of the signal in both the 67 and 63 kDa bands in the resulting autoradiograms. This decrease in radioactivity, however, was not greater than that observed for the secreted [^{3}H]leucine-labeled proteins which were treated in an identical manner (Fig. 2A, ^{3}H). In contrast, HCl treatment of secretogranin II, a 87 kDa protein known to be primarily sulfated on tyrosine residues [12], did lead to an apparently complete loss of [^{35}S]sulfate from the protein (Fig. 2A, SgII). These comparisons strongly suggested that the 63–67 kDa proteins are mainly sulfated on carbohydrates and indicated thereby that they are glycoproteins. Consistent with this, partially purified [^{35}S]sulfate-labeled proteins were retained on a concanavalin A column and were specifically eluted with α-methyl-mannose (Fig. 2B, Bound), except proteoglycans (Fig. 2B, Unbound). As judged by Coomassie blue staining, the above procedure allowed the purification of the 63–67 kDa proteins from rabbit milk (data not shown).

3.3. The main sulfated proteins produced by rabbit mammary gland belong to polymeric IgA

In order to identify unambiguously the 63–67 kDa and 23 kDa proteins by N-terminal sequencing, [^{35}S]sulfate-labeled proteins prepared as described above were further separated by 2D-PAGE. This revealed that these proteins had a similar isoelectric point (Fig. 3, Fluorography) which was estimated to ∼4.6. The sulfated 23 kDa protein was resolved into two distinct spots of slightly different M_r, but only the polypeptide numbered 3 could be superimposed with a Coomassie blue-stained spot (data not shown). On the other hand, the main protein in the 23 kDa area of the 2D-PAGE gel, which was located within the circle in Fig. 3, was not sulfate-labeled (Fig. 3, Fluorography, spot no. 2). However, an identical N-terminal amino acid sequence was obtained from spots number 2 and 3 (Table 1). Database searches revealed that this sequence was only present in rabbit Ig J chain [18]. These observations indicated that the sulfated 23 kDa protein (spot 3) was a minor Ig J chain variant which was sulfated. This was consistent with the observation that the sulfated protein was more acidic than the predominant form (spot 2). In this context, it is worth noting that no tyrosine residues of the rabbit Ig J chain fulfilled the consensus features of tyrosine sulfation sites (for review, see [2]). While three aspartic acids precede Tyr-96 and a glutamic acid is found at position +2, the two cysteine residues at positions −5 and +4 are engaged in disulfide bound formation. The latter feature prevents tyrosine sulfation. This suggests that sulfation of the Ig J chain most likely occurs on the carbohydrate moieties of the protein.

As to spot number 1, no sequence information was obtained. This was due to the presence of a glutamine residue at its N-terminal end, as confirmed by its identity (see below). The protein was therefore subjected to cleavage with BrCN. Edman sequencing of two of the resulting peptides yielded the sequences shown in Table 1. A BLAST search [19] revealed the presence of these, or of highly homologous sequences (identity better than 65%), in a single class of molecules, the Ig heavy chain (HC). Moreover, they had 90–100% identity with rabbit Ig HC (allotype a1 or a2). These sequences were found in the variable region of Ig HC, the sequence from the second polypeptide being present in the C-terminal part of the variable region and ending three residues upstream the switch region. From these data and given the fact that IgA, in the

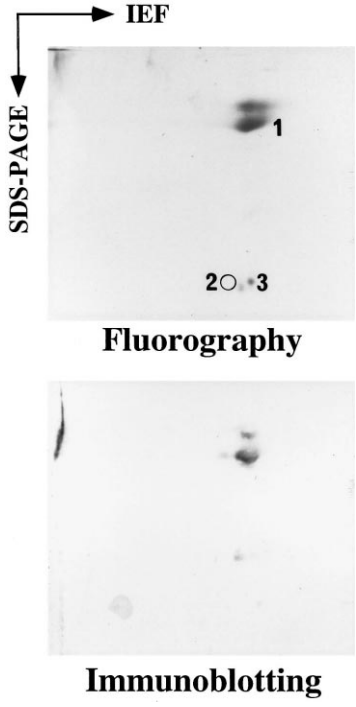

Fig. 3. The main sulfated proteins produced by rabbit mammary gland belong to polymeric IgA. Sulfated proteins contained in the medium obtained from [^{35}S]sulfate-labeled explants were purified and analyzed by 2D-PAGE followed by fluorography (Fluorography) or immunoblotting (Immunoblotting) using a goat antiserum against rabbit IgA. Numbered spots were also identified by N-terminal sequencing.

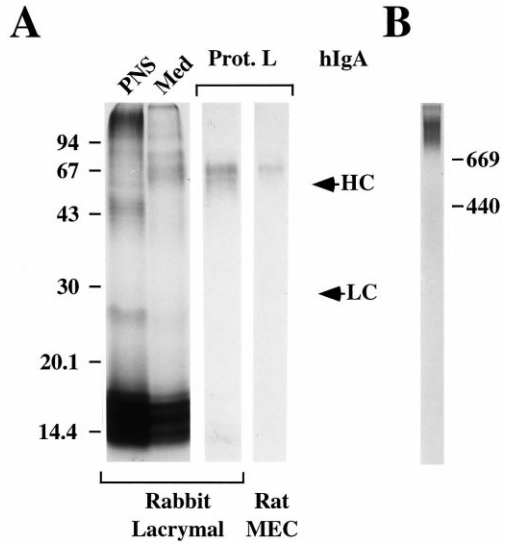

Fig. 4. Sulfation of polymeric IgA is not restricted to rabbit mammary gland. Explants from rabbit lacrimal gland or rat mammary gland were labeled with [^{35}S]sulfate. Proteins contained in an aliquot of either the PNS (PNS) or the medium (Med) of lacrimal gland explants and purified from the medium of lacrimal or mammary gland explants using protein L agarose (Prot. L) were analyzed by SDS-PAGE followed by fluorography. The positions of hIgA HC and LC are indicated on the right. B: [^{35}S]Sulfate-labeled proteins from rabbit lacrimal gland eluted from protein L agarose were analyzed by SDS-PAGE in non-reducing conditions using a 6% gel.

form of secretory IgA, is the predominant Ig in rabbit milk, it was tempting to speculate that the 63 kDa protein corresponded to rabbit IgA HC. However, since variable regions are shared among the different classes of HC and there are better than 90% homologies among the HC in the switch region, it was important to determine further to which class of Ig the 63 kDa protein belonged. As shown by immunoblotting, both the 63 kDa and the 67 kDa proteins reacted with an antiserum against rabbit IgA (Fig. 3). Similar results were obtained with proteins purified from rabbit milk (data not shown). From this, we concluded that these sulfated proteins were rabbit IgA HCs. In addition, the antibody detected spots with M_r of ~30 kDa which were not sulfated (Fig. 3, compare Immunoblotting and Fluorography) and which most likely corresponded to the IgA light chain (LC). The fact that sulfation of the α-chain mostly occurred on oligosaccharides was consistent with previous reports on the sulfation of IgG and IgM HC [3,20]. The presence of sulfate in carbohydrates of mouse IgA HC has also been mentioned (Baeuerle and Huttner, unpublished results; see [1]). These results, however, were obtained with hybridoma or myeloma cell lines.

As compared to the 63 kDa protein, the signal obtained for the 67 kDa protein was lower. This was in agreement with the relatively smaller amount of protein in the latter spot, as determined by Coomassie blue staining (data not shown). This observation suggests that the sulfated 67 kDa protein was a minor IgA HC variant. In this context, it is noteworthy that there are as much as 13 IgA isotypes in rabbit [21], in contrast to most mammals in which only one or two isotypes have been identified. At least 10 of these genes were found to be expressed in rabbit mammary gland [22].

Altogether, these data demonstrated that the main sulfated proteins of rabbit milk belong to polymeric IgA. It is well known that polymeric IgA are produced by plasma cells in the interstitium of mucosal or glandular tissues and are transported into secretion fluids by receptor-mediated transcytosis across epithelial cells [23] to function as the first line of immunological defense against infections. In line with this, we have observed that the kinetics of IgA secretion from plasma cells prepared from mammary gland was faster than that observed with mammary gland explants (data not shown). It is tempting to speculate that this difference reflected the time required for IgA transcytosis through MEC. To investigate this issue further, we attempted to show that, at least part of, the secreted IgA was in the form of secretory IgA, i.e. associated with the proteolytically cleaved extracellular portion of the polymeric Ig receptor that is responsible for the transcytosis of polymeric IgA and pentameric IgM across epithelial monolayer (for review, see [24]). However, these experiments were hampered by the fact that available anti-rabbit IgA cross-reacted with the secretory component and vice versa. On the other hand, confirming previous observations (see [2] and references therein), we noticed that inhibition of sulfation by chlorate [25] did not impair polymeric IgA secretion from plasma cells (data not shown).

3.4. Sulfation of polymeric IgA is not restricted to the rabbit mammary gland

Having established that the polymeric IgA produced by plasma cells of the rabbit mammary gland are sulfated, we then wished to investigate whether this post-translational modification would also occur in the mammary gland of other species or in other tissues known to produce high amounts of these proteins. In these experiments, we tried to directly purify IgA from the culture medium of [^{35}S]sulfate-labeled explants using protein L affinity chromatography. First, we analyzed the polymeric IgA produced by rabbit lacrimal gland for sul-

Table 1
Proteins identified by N-terminal sequencing

Spot no.	M_r	N-terminal sequence	Homologous sequences in rabbit proteins	Accession no.	Identity
1-peptide 1	55 000	G/Q/SWVRQAPGKG	^{33}MGWVRQAPGKG^{43}	P01826	Ig HC V-A1
			^{33}MSWVRQAPGKG^{43}	P01828	Ig HC V-A2
1-peptide 2	45 000	DLWGPGTLVT	^{102}MDVWGPGTLVT^{112}	P01827	Ig HC V-A2
			^{104}VDVWGPGTLVT^{114}	P01826	Ig HC V-A1
			^{105}VDVWGPGTLVT^{115}	P01828	Ig HC V-A2
			^{125}IWGPGTLVT^{133}	P01829	Ig HC V-A2
2	23 000	EDESTVLVDN	^{1}EDESTVLVDN	P23108	Ig J chain
3	23 000	EDESTVLVDN	^{1}EDESTVLVDN	P23108	Ig J chain

Underlined letters indicate the amino acid in homologous sequences which precedes the sequence recovered from peptide 1 or 2. Numbers indicate the location of the determined sequences in the homologous sequences. Ig: immunoglobulin; HC: heavy chain; V: variable region.

fation. This revealed that at least part of the purified proteins were sulfated (Fig. 4A, Prot. L, Rabbit Lacrimal). It is worth noting that while these sulfated proteins were not quantitatively retained on the protein L column (data not shown), the affinity chromatography was very specific for this material (Fig. 4A, compare Med vs. Prot. L). Several lines of evidence further indicated that these proteins corresponded to the HC of polymeric IgA. First, the purified material had an identical M_r to the IgA HC previously identified in rabbit milk. Second, the 63 kDa form of the purified [^{35}S]sulfate-labeled proteins co-migrated with hIgA HC (Fig. 4A, hIgA). Third, both the 63 kDa and 67 kDa sulfated proteins reacted with an antibody against rabbit IgA (data not shown), as previously observed (see Fig. 3). In contrast to the mammary gland, however, the radioactivity contained in the 67 kDa band was greater than that observed in the 63 kDa form (Fig. 4A, compare with Fig. 1). Fourth, in non-reducing conditions, purified [^{35}S]sulfate-labeled proteins had an electrophoretic mobility (M_r around 800 000) consistent with secretory IgA in their tetrameric form (Fig. 4B). As previously observed in the rabbit mammary gland, no sulfate label was detected in the LC (Fig. 4A). Finally, analysis of the polymeric IgA purified from the culture medium of [^{35}S]sulfate-labeled rat mammary gland explants revealed that the IgA HC produced in this tissue was also sulfated (Fig. 4A, Prot. L, Rat MEC).

We conclude from these results that sulfation of polymeric IgA is not restricted to rabbit mammary gland and is a general feature of IgA HC maturation. However, the functional significance of HC sulfation is not known. It would be interesting to investigate whether sulfation of polymeric IgA is related to their transcytosis through epithelia.

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