Polarized secretion of tyrosine-sulphated proteins and free tyrosine *O*-sulphate by filter-grown Madin–Darby canine kidney (MDCK) cells

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Filter-grown Madin–Darby canine kidney (MDCK) cells labelled for 24 h with [³⁵S]sulphate were found to secrete macromolecules [³⁵S]sulphated on their carbohydrate moieties predominantly into the basolateral medium, whereas the tyrosine-[³⁵S]sulphated proteins synthesized were predominantly secreted into the apical medium. In contrast with the predominant apical secretion of tyrosine-[³⁵S]sulphated proteins, the free tyrosine *O*-[³⁵S]sulphate (Tyr[³⁵S]) was released mostly into the basolateral medium. A time-lapse study using prelabelled MDCK cells incubated in fresh medium revealed that, during the 48 h time course monitored, the release of tyrosine-[³⁵S]sulphated proteins into the apical medium was faster and quantitatively greater than that into the basolateral medium. During the same time there was a concomitant release, predominantly into the basolateral medium, of the free Tyr[³⁵S] derived from the degradation of tyrosine-[³⁵S]sulphated proteins. An endocytotic degradation experiment was performed to demonstrate the endocytosis of tyrosine-sulphated proteins and their degradation to generate free TyrS. It was found that free Tyr[³⁵S] was generated and released when an apically secreted (or basolaterally secreted) tyrosine-[³⁵S]sulphated protein preparation was added to the apical medium (or the basolateral medium) of unlabelled filter-grown MDCK cells. In both cases, the free Tyr[³⁵S] generated was predominantly released into the basolateral medium similar to the results obtained in the time-lapse study.

INTRODUCTION

Post-translational modification by tyrosine sulphation has been shown to have a widespread occurrence among proteins in multicellular eukaryotic organisms [1]. Despite a considerable number of phenomenological observations made in recent years, the functional role of this unique protein modification still remains unclear. Based on the facts that the vast majority of the tyrosine-sulphated proteins identified are secretory proteins [1] and that tyrosylprotein sulphotransferase, the enzyme catalysing the sulphation reaction, is located in the trans-Golgi [2,3], a linkage between tyrosine sulphation and the secretion of the modified proteins has been proposed [4]. A role for protein-bound tyrosine *O*-sulphate (TyrS) residues serving as a sorting signal for the targeting and intracellular transport of tyrosine-sulphated proteins was further elaborated [5]. For this to be the case, a receptor capable of binding tyrosine-sulphated proteins through their TyrS residues would be required. Our discovery of a bovine liver membrane-bound TyrS-binding protein [6] and its existence in complex forms with representative tyrosine-sulphated proteins in freshly prepared bovine liver membrane lysate [7] seems to lend support to this hypothesis. On the other hand, free TyrS has been reported to be present in human [8] and other mammalian [9] urine. The large amount of free TyrS excreted is generally thought to be derived from the intracellular degradation of tyrosine-sulphated proteins [10]. For some cells, in particular epithelial cells, a vectorial transport mechanism may be necessary to facilitate urinary excretion of free TyrS. Filter-grown Madin–Darby canine kidney (MDCK) cells, with their cell membrane segregated by the tight junction structure, are widely used for studying mechanisms involved either in directing intracellular transport of proteins toward the apical or basolateral domain of the plasma membrane or in their vectorial solute

transport processes [11]. These cells have been shown to synthesize and deliver/secrete both endogenous and exogenous plasma membrane/secretory proteins in a polarized fashion [12]. It is speculated that these proteins carry in their molecular structures sorting signals that are recognized by specific receptor systems responsible for their intracellular transport [13], and that such signals may have been derived following their post-translational modifications. In view of the fact that MDCK cells are capable of synthesizing and secreting fibronectin [14] and laminin [15], among others, which in several mammalian species have been shown to be subjected to modification by tyrosine sulphation [1], we decided to use filter-grown MDCK cells as a model system to examine the synthesis and polarized secretion of tyrosine-sulphated proteins, as well as the polarized release of their degradation product, free TyrS.

In this paper, we report on (i) the production and polarized secretion of tyrosine-sulphated proteins, and (ii) the vectorial release of free TyrS by filter-grown MDCK cells. A time-lapse study showed that the release of tyrosine-sulphated proteins into the apical medium was faster and quantitatively greater than that into the basolateral medium, whereas the opposite situation was found for free TyrS. An endocytotic degradation experiment clearly demonstrated that free TyrS was generated as an end product of the intracellular degradation of tyrosine-sulphated proteins.

MATERIALS AND METHODS

Materials

Protein molecular mass markers were products of Sigma Chemical Company. Pronase was from Calbiochem–Boehringer. Carrier-free sodium [³⁵S]sulphate and [³⁵S]methionine (1039 Ci/mmol) were from ICN Radiochemicals. Millicell-HA (10 mm or

Abbreviations used: TyrS, tyrosine *O*-sulphate; Tyr[³⁵S], tyrosine *O*-[³⁵S]sulphate; MDCK cells, Madin–Darby canine kidney cells; MEM, minimum essential medium; PBS, phosphate-buffered saline (NaCl, 8g/l; KCl, 0.2 g/l; Na₂HPO₄·2H₂O, 1.15 g/l; KH₂PO₄, 0.2 g/l, pH 7.4).

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30 mm) culture plate inserts were obtained from Millipore Corporation. Pre-coated cellulose t.l.c. plates were products of Eastman Kodak Company. TyrS standard was synthesized according to the method of Jevons [16]. All other reagents were of the highest grades commercially available.

Cell culture and radioactive labelling

MDCK cells (strain II) were routinely maintained in minimum essential medium (MEM) supplemented with 10 % fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), penicillin G (30 µg/ml) and streptomycin sulphate (100 µg/ml) in a 5 % CO₂ atmosphere at 37 °C. For studying polarized protein secretion, the cells were grown on the apical side [17] of Millicell-HA 30 mm inserts. The Millicell-HA inserts, before being used for growing the cells, were incubated in 80 % fetal bovine serum (in MEM) overnight to block all possible protein-binding sites [18]. After this pretreatment, 2.5 ml portions of an MDCK cell suspension in MEM/10 % fetal bovine serum were then seeded on to the inserts at a density of approx. 10⁶ cells/ml. The seeded inserts were in turn placed in individual wells of a Corning six-well plate, with 2.5 ml of MEM/10 % fetal bovine serum added to the wells (on the basolateral side of the inserts). After 3 days (or longer) of growth under standard conditions (MEM/10 % fetal bovine serum at 37 °C under 5 % CO₂), the cells would grow to confluency, forming a cell monolayer on the HA membrane filter of the insert. Transepithelial electrical resistance (TER) across the cell monolayer grown on the insert was monitored using a Millicell-ERS apparatus. Inserts with TER values greater than 250 Ω·cm², which indicates a confluent monolayer [19], were used in the radioactive labelling experiments. Media used in the following radioactive labelling experiments were all supplemented with 2 % dialysed fetal bovine serum. For [³⁵S]methionine labelling, filter-grown confluent cells pre-incubated for 4 h with methionine-deficient MEM were labelled with [³⁵S]methionine (0.1 mCi/ml) in methionine-deficient MEM added on the basolateral side. Following a 24 h labelling, the apical and basolateral medium fractions were collected and the membrane filter carrying the cells, after thorough rinsing with ice-cold phosphate-buffered saline (PBS), was excised from the insert and boiled at 100 °C for 3 min in Laemmli SDS sample buffer [20] for the preparation of the cell-associated fraction. The three fractions thus prepared were subjected to the subsequent analyses. For [³⁵S]sulphate labelling, filter-grown cells, pre-incubated for 4 h with sulphate-free MEM (prepared by omitting streptomycin sulphate and replacing magnesium sulphate with magnesium chloride), were labelled basolaterally with [³⁵S]sulphate (0.3 mCi/ml) in sulphate-free MEM. Following a 24 h labelling, the apical medium and the basolateral medium fractions were collected and the membrane filter carrying the cells, after thorough rinsing with ice-cold PBS, was excised from the insert and boiled at 100 °C for 3 min in Laemmli SDS sample buffer [20] for the preparation of the cell-associated fraction. The three fractions thus prepared were subjected to the subsequent analyses. For the time-lapse study on the secretion of Tyr[³⁵S]-containing compounds into the apical and basolateral medium fractions, confluent cells grown on five inserts prepared under identical conditions were labelled basolaterally with [³⁵S]sulphate (0.3 mCi/ml). After a 24 h labelling, the labelling media were removed and the free radioactive compounds were rinsed away with MEM. The labelled cells on the five inserts were incubated in fresh MEM without added serum for 0, 3, 9, 24 and 48 h respectively. At the end of each incubation, the apical and basolateral medium fractions were collected, and the membrane filters carrying the cells, after extensive rinsing with ice-cold PBS, were boiled at 100 °C for 3 min in Laemmli SDS sample buffer

[20] for the preparation of the cell-associated fraction. The three fractions thus prepared were subjected to the subsequent analyses.

Endocytotic degradation of apically and basolaterally secreted tyrosine-[³⁵S]sulphated proteins

To prepare apically and basolaterally secreted tyrosine-[³⁵S]sulphated proteins, the [³⁵S]sulphate-labelled apical and basolateral medium fractions obtained following a 24 h labelling of filter-grown MDCK cells, as described above, were dialysed extensively against PBS to remove small ³⁵S-labelled compounds. For the experiment on the endocytotic degradation of basolaterally secreted tyrosine-[³⁵S]sulphated proteins, 250 µl of the dialysed preparation was mixed with 2.25 ml of fresh MEM without added serum. Aliquots (0.5 ml) of this mixture were used as the basolateral medium for the MDCK cells grown on the five Millicell-HA 10 mm inserts individually placed in different wells of a Corning 24-well plate. Fresh MEM only (0.5 ml) was used as the apical medium. For the experiment on the endocytotic degradation of apically secreted tyrosine-[³⁵S]sulphated proteins, 250 µl of the dialysed preparation was mixed with 2.25 ml of fresh MEM without added serum. Aliquots of 0.5 ml of this mixture were used as the apical medium for the MDCK cells grown on the five Millicell-HA 10 mm inserts placed in different wells of a Corning 24-well plate. Fresh MEM only (0.5 ml) was used as the basolateral medium. For both experiments, the five identically prepared inserts were incubated for 0, 24, 48, 72 and 96 h respectively. At the end of each incubation, the apical and basolateral medium fractions were collected and the membrane filter carrying the cells, after thorough rinsing with ice-cold PBS, was excised from the insert and boiled at 100 °C for 3 min in Laemmli SDS sample buffer [20] for the preparation of the cell-associated fraction. The three fractions thus prepared were subjected to the subsequent analyses.

Determination of Tyr[³⁵S]-containing compounds

Total Tyr[³⁵S]-containing compounds. For the determination of total Tyr[³⁵S]-containing compounds, 0.5 ml aliquots of the non-dialysed apical medium and basolateral medium and the cell-associated fractions, prepared as in the previous experiments, were individually placed in 1 ml ampoules. To each ampoule was added 0.5 ml of 0.4 M-Ba(OH)₂. The ampoules thus prepared were sealed and incubated at 110 °C for 24 h. Hydrolysis of proteins under these conditions appeared to be complete by comparison with a control with a prolonged incubation period (48 h). Furthermore, the TyrS ester bond was found to be stable under these conditions, since no detectable free tyrosine was generated when synthetic TyrS was incubated under the same conditions. After the incubation, the alkaline hydrolysates in individual ampoules were removed and placed in 1.5 ml Eppendorf microcentrifuge tubes. Approx. 0.2 ml of 0.5 M-H₂SO₄ was added to each tube to neutralize the alkaline hydrolysate. Following centrifugation to remove the precipitates formed, the clear supernatants in individual tubes were transferred to new microcentrifuge tubes and lyophilized. Lyophilized material in each tube was dissolved in 20 µl of water containing 5 µg of synthetic TyrS standard, spotted on a cellulose t.l.c. plate (10 cm × 10 cm) and analysed for Tyr[³⁵S] based on a two-dimensional separation procedure [21]. Briefly, the plate was first subjected to high-voltage electrophoresis (500 V, 45 min) in 5 % (v/v) acetic acid/0.5 % (v/v) pyridine, pH 3.5. After electrophoresis, the plate was air-dried and subjected in the second dimension to ascending chromatography in butan-1-ol/formic acid/propan-2-ol/water (3:1:1:1, by vol.). Upon completion of the chromatography, the plate was dried and sprayed with ninhydrin solution (0.5 % in acetone). The ninhydrin spot of TyrS was then scraped off, suspended in 0.5 ml of water and 5 ml

of scintillation cocktail (Ecolume, ICN Radiochemicals), and counted for ^{35}S radioactivity using a scintillation counter.

Tyrosine- ^{35}S sulphated proteins. For the determination of tyrosine- ^{35}S sulphated proteins, aliquots of the apical and basolateral medium fractions were mixed with equal amounts of $2 \times$ Laemmli SDS sample buffer. After boiling at 100°C for 3 min, the samples, together with those of the cell-associated fractions, were subjected to SDS/PAGE [20]. After electrophoresis, the gel was stained with Coomassie Blue, destained and dried. Gel electrophoretic lanes corresponding to different samples of the apical medium, the basolateral medium and the cell-associated fractions were excised, sliced into small pieces and placed in individual tubes. Aliquots (8 ml) of a solution containing Pronase (150 $\mu\text{g}/\text{ml}$) and NH_4HCO_3 (50 mM) were added to each tube and the tubes were incubated for 24 h at 37°C with constant shaking. ^{35}S sulphated proteins fixed in the gel pieces were hydrolysed by Pronase and eluted from the gel. Under these conditions, elution of ^{35}S sulphated proteins fixed in the polyacrylamide gel appeared to be complete when the samples were compared with controls that either were incubated with Pronase for an extended period (48 h) or had the polyacrylamide gel pieces completely liquefied by 30 % H_2O_2 . Pronase eluates were then subjected to the alkaline hydrolysis followed by two-dimensional thin-layer separation for the analysis of Tyr ^{35}S as described above.

Free Tyr ^{35}S . For the determination of free Tyr ^{35}S , the non-dialysed apical medium, the basolateral medium and the cell-associated fractions prepared in the previous experiments and supplemented with marker TyrS were spotted directly on to cellulose thin-layer plates and subjected to two-dimensional thin-layer separations, as for the analysis of Tyr ^{35}S described above.

RESULTS

Amounts of ^{35}S sulphated macromolecules synthesized and secreted into apical medium and basolateral medium fractions by ^{35}S sulphate-labelled filter-grown MDCK cells

When the apical and basolateral medium fractions of filter-grown MDCK cells were analysed after a 24 h labelling with

^{35}S sulphate, it was found that 25–35 % and 65–75 % of the total secreted ^{35}S sulphated macromolecules were released into the apical medium and the basolateral medium respectively. Fig. 1 shows the SDS/PAGE patterns of the ^{35}S sulphated macromolecules, in comparison with the ^{35}S methionine-labelled proteins, that were synthesized and secreted into the two medium fractions by radiolabelled filter-grown MDCK cells. Different band patterns were observed for the apical medium and basolateral medium fractions, reflecting the polarized secretion of ^{35}S sulphated macromolecules and ^{35}S methionine-labelled proteins by MDCK cells.

Amounts of Tyr ^{35}S -containing compounds synthesized and secreted into apical medium and basolateral medium by ^{35}S sulphate-labelled MDCK cells

Tyr ^{35}S -containing compounds secreted into the two medium fractions could be broadly divided into three groups: (i) non-dialysable tyrosine- ^{35}S sulphated proteins, (ii) dialysable Tyr ^{35}S -containing peptides, and (iii) free Tyr ^{35}S .

Total Tyr ^{35}S -containing compounds. When the apical and basolateral medium fractions of filter-grown MDCK cells were analysed after a 24 h labelling with ^{35}S sulphate, the distribution of the total Tyr ^{35}S -containing compounds was found to be 48–51 % in the apical medium and 49–52 % in the basolateral medium. These data indicated that comparable amounts of the total Tyr ^{35}S -containing compounds were released into the two medium fractions. Fig. 2 shows a typical autoradiograph taken from the cellulose thin-layer plate used in the Tyr ^{35}S analysis of alkaline hydrolysates of either the apical medium or the basolateral medium samples.

Tyrosine- ^{35}S sulphated proteins. When proteins in the apical and basolateral medium fractions were subjected to SDS/PAGE and analysed for Tyr ^{35}S content, it was found that the distribution of secreted tyrosine- ^{35}S sulphated proteins was 62.5 % and 37.5 % in the apical medium and the basolateral medium respectively. Filter-grown MDCK cells therefore secreted quantitatively more tyrosine- ^{35}S sulphated proteins into the apical medium than in the basolateral medium.

Free Tyr ^{35}S . When the apical medium and the basolateral medium fractions, without alkaline hydrolysis treatment, were analysed directly for Tyr ^{35}S , it was found that the distribution of free Tyr ^{35}S was 17.2 % and 82.8 % in the apical medium and the basolateral medium respectively.

The results on the analysis of Tyr ^{35}S -containing compounds are compiled in Table 1. It is clear that close to two-thirds of tyrosine- ^{35}S sulphated proteins secreted were delivered to the apical medium, with the rest being released into the basolateral medium. On the other hand, nearly five times as much free Tyr ^{35}S , presumably a degradation product of tyrosine- ^{35}S sulphated proteins [10], was recovered in the basolateral medium compared with that in the apical medium. Subtracting the combined amount of protein-bound Tyr ^{35}S and free Tyr ^{35}S from the total amount of Tyr ^{35}S -containing compounds, very few (0.03 % of total) of the small dialysable tyrosine- ^{35}S sulphated peptides were calculated to be present in the basolateral medium, and virtually none were present in the apical medium.

Time-lapse changes in the amounts of Tyr ^{35}S -containing compounds synthesized and secreted by ^{35}S sulphate-labelled filter-grown MDCK cells

To monitor more closely the polarized secretion of tyrosine- ^{35}S sulphated proteins and to obtain further evidence for free Tyr ^{35}S being derived from the degradation of tyrosine- ^{35}S sulphated proteins, the time-lapse changes in the amounts of the three groups of Tyr ^{35}S -containing compounds secreted into

Fig. 1. Autoradiograph of ^{35}S sulphate-labelled and ^{35}S methionine-labelled macromolecules following SDS/PAGE

Lanes 1 and 3, ^{35}S sulphate-labelled macromolecules secreted by ^{35}S sulphate-labelled filter-grown MDCK cells into the apical medium and the basolateral medium respectively. Lane 2, sample of ^{35}S sulphate-labelled macromolecules remaining associated with the labelled cells at the end of the 24 h labelling period. Lanes 4 and 6, ^{35}S methionine-labelled macromolecules secreted by ^{35}S methionine-labelled filter-grown MDCK cells into the apical medium and the basolateral medium respectively. Lane 5, ^{35}S methionine-labelled macromolecules remaining associated with the labelled cells at the end of the 24 h labelling period.

Fig. 2. Autoradiograph of the alkaline hydrolysate of [^{35}S]sulphate-labelled MDCK cell basolateral medium sample following the two-dimensional thin-layer separation

High-voltage electrophoresis (500 V, 45 min) in 5% (v/v) acetic acid/0.5% (v/v) pyridine, pH 3.5, was performed in the first dimension. In the second dimension, t.l.c. was carried out with butan-1-ol/formic acid/propan-2-ol/water (3:1:1:1, by vol.) as the solvent system. The dashed line indicates the position of synthetic TyrS standard detected by ninhydrin staining.

the apical medium and the basolateral medium, as well as those present in the cell-associated fractions, were monitored using filter-grown MDCK cells pre-labelled for 24 h with [^{35}S]sulphate. The following results are presented separately for each of the three groups of Tyr[^{35}S]-containing compounds.

Total Tyr[^{35}S]-containing compounds. As shown in Fig. 3(a), when pre-labelled filter-grown MDCK cells were incubated in fresh medium without [^{35}S]sulphate, the decrease in the amount of total Tyr[^{35}S]-containing compounds in the cell-associated fraction was rapid during the early phase of the incubation. An approx. 23% decrease was observed after only 3 h of incubation.

Table 1. Distribution of different groups of Tyr[^{35}S]-containing compounds secreted by [^{35}S]sulphate-labelled filter-grown MDCK cells between the apical medium, the basolateral medium and the cell-associated fraction

Data represent average values derived from three independent experiments. The results show the ^{35}S radioactivity associated with the Tyr[^{35}S]-containing compounds present in the total volume (2 ml) of the apical medium, the basolateral medium and the cell-associated fractions collected at the end of the 24 h labelling period. Counting efficiency = 86.7%.

	Total Tyr[^{35}S]-containing compounds (c.p.m.)	Protein-bound Tyr[^{35}S] (c.p.m.)	Free Tyr[^{35}S] (c.p.m.)
Apical medium	29 680	27 020	2 660
Basolateral medium	29 020	16 210	12 800
Cell-associated	26 500	18 470	1 800

Thereafter the rate of decrease slowed down and, at the end of the 48 h incubation, an amount equivalent to 30.6% of the total Tyr[^{35}S]-containing compounds originally present remained in the cell-associated fraction. Some of these Tyr[^{35}S]-containing compounds remaining at the end of the incubation might actually have been present in the basal lamina [11] or adsorbed on to the membrane filter. In terms of the kinetics of secretion, the release of total Tyr[^{35}S]-containing compounds from the labelled cells into the basolateral medium was found to be more rapid than that into the apical medium. At the end of the incubation, the amounts accumulated in the apical medium and the basolateral medium were calculated to be equivalent to 17.66% and 52.91% respectively of the total Tyr[^{35}S]-containing compounds originally present in the pre-labelled cells.

Fig. 3. Distribution of (a) total Tyr[^{35}S]-containing compounds, (b) tyrosine-[^{35}S]sulphated proteins, (c) small Tyr[^{35}S]-containing peptides and (d) free Tyr[^{35}S] in the apical medium (◆), the basolateral medium (■) and the cell-associated fraction (□) at different time points after the incubation of filter-grown MDCK cells pre-labelled with [^{35}S]sulphate

Data represent average values derived from three independent experiments. Number of c.p.m. corresponds to ^{35}S radioactivity associated with a particular group of Tyr[^{35}S]-containing compounds present in the whole sample (2 ml) of the apical medium, the basolateral medium, or the cell-associated fraction prepared from each insert for a particular time point (counting efficiency = 86.7%). In (b), the data for the cell-associated fraction shown are one-tenth of the actual values.

Table 2. Distribution of free Tyr[³⁵S] released into the apical medium and the basolateral medium upon endocytotic degradation of apically or basolaterally secreted tyrosine-[³⁵S]sulphated proteins by filter-grown MDCK cells

Incubations were for 96 h as indicated. Data represent average values derived from three independent experiments. Total ³⁵S-labelled compounds refer to the non-dialysable [³⁵S]sulphate-labelled macromolecules prepared from either the apical or the basolateral medium of [³⁵S]sulphate-labelled filter-grown MDCK cells. Counting efficiency = 86.7 %.

Fraction	Basolaterally secreted tyrosine-[³⁵ S]sulphated proteins				Apically secreted tyrosine-[³⁵ S]sulphated proteins			
	0 h		96 h		0 h		96 h	
	10 ⁻³ × Total ³⁵ S- compounds (c.p.m.)	Free Tyr[³⁵ S] (c.p.m.)	10 ⁻³ × Total ³⁵ S- compounds (c.p.m.)	Free Tyr[³⁵ S] (c.p.m.)	10 ⁻³ × Total ³⁵ S- compounds (c.p.m.)	Free Tyr[³⁵ S] (c.p.m.)	10 ⁻³ × Total ³⁵ S- compounds (c.p.m.)	Free Tyr[³⁵ S] (c.p.m.)
Basolateral medium	940.5	0	754.4	664	0	0	240.9	547
Apical medium	0	0	10.7	167	523.8	0	120.2	120
Cell-associated	0	0	275.4	57	0	0	151.2	42
Control	954.0	0	952.8	0	529.8	0	523.3	0

Tyrosine-[³⁵S]sulphated proteins. Data on the time-lapse changes in the amounts of protein-bound Tyr[³⁵S] in the three fractions examined are shown in Fig. 3(b). In contrast to the situation with the total Tyr[³⁵S]-containing compounds (Fig. 3a), the release of tyrosine-[³⁵S]sulphated proteins from the pre-labelled cells into the basolateral medium appeared to be slower and quantitatively smaller than the release into the apical medium. At the end of the 48 h incubation, the net amounts of tyrosine-[³⁵S]sulphated proteins released into the apical medium and the basolateral medium were calculated to be equivalent to 10.8 % and 3.86 % respectively of the tyrosine-[³⁵S]sulphated proteins originally present. Therefore, quantitatively, nearly three times as many tyrosine-[³⁵S]sulphated proteins were secreted into the apical medium as into the basolateral medium. An amount equivalent to 43.8 % of the tyrosine-[³⁵S]sulphated proteins remained in the cell-associated fraction at the end of the incubation. Based on these data, 41.54 % of the tyrosine-[³⁵S]sulphated proteins present at the beginning of the incubation was not recovered after the 48 h incubation. This indicated a rapid degradation of tyrosine-[³⁵S]sulphated proteins in the labelled cells during the incubation. Fig. 3(c) shows the calculated data on the amounts of the small dialysable products that are presumably derived from the degradation of tyrosine-[³⁵S]sulphated proteins. These data were obtained by subtracting the amounts of tyrosine-[³⁵S]sulphated proteins in the samples taken at different time points (Fig. 3b) from those of total Tyr[³⁵S]-containing compounds in the samples taken at the corresponding time points (Fig. 3a). It appeared that a considerable pool (approx. 30.2 % of the total) of such small dialysable Tyr[³⁵S]-containing compounds was initially present in the pre-labelled cells. During the 48 h incubation there was a rapid decrease in the amount of these compounds in the cells, and a concomitant release of such compounds from the pre-labelled cells into the two medium fractions was observed. The net amount of these small Tyr[³⁵S]-containing compounds released into the basolateral medium at the end of the 48 h incubation reached a level equivalent to 50.2 % of the tyrosine-[³⁵S]sulphated proteins originally present in the cell-associated fraction. Their release into the apical medium, however, was slower and quantitatively smaller than the release into the basolateral medium. It should be pointed out that these small Tyr[³⁵S]-containing compounds include the free Tyr[³⁵S] and a presumably heterogeneous group of tyrosine-[³⁵S]sulphated peptides.

Free Tyr[³⁵S]. Data on the amount of the free Tyr[³⁵S] present

in the two medium fractions examined at different time points are shown in Fig. 3(d). A predominant release into the basolateral medium was again observed. It appeared clear that the release of the free Tyr[³⁵S] into the apical medium and basolateral medium fractions resembled the release of the total dialysable Tyr[³⁵S]-containing compounds (Fig. 3c) with respect to both the secretion kinetics and the quantitative distribution. However, the calculated amounts of dialysable Tyr[³⁵S]-containing compounds shown in Fig. 3(c) could not be quantitatively accounted for by the amounts of free Tyr[³⁵S] determined. This discrepancy may imply that small Tyr[³⁵S]-containing peptides were also released. It is worthwhile mentioning that the pre-labelled filter-grown MDCK cells used in this time-lapse study were incubated in medium without added serum.

Endocytotic degradation of apically and basolaterally secreted tyrosine-[³⁵S]sulphated proteins

To obtain conclusive evidence that the free Tyr[³⁵S] was derived from the intracellular degradation of tyrosine-[³⁵S]sulphated proteins, a sample of dialysed [³⁵S]sulphate-labelled MDCK basolateral medium was mixed with fresh MEM and placed on the basolateral side of an insert on to which a confluent monolayer of MDCK cells had been grown. The release of free Tyr[³⁵S] was then monitored over a time course of 96 h. A significant amount of free Tyr[³⁵S] had already appeared in the basolateral medium after 24 h of incubation (results not shown). The amount continued to increase thereafter until the end of the 96 h incubation (Table 2). At the end of the incubation, the free Tyr[³⁵S] was also detected in both the cell-associated fraction and the apical medium. In the cell-associated fraction, the free Tyr[³⁵S] detected accounted for only a small portion of the total ³⁵S-labelled macromolecules endocytosed. This implied that the former (i) represented a product derived from the ongoing degradation of the endocytosed tyrosine-[³⁵S]sulphated proteins, and (ii) was rapidly released into the medium once it was generated. Quantitatively speaking, the distribution of the free Tyr[³⁵S] released was calculated to be 20.1 % and 79.9 % in the apical medium and the basolateral medium respectively. These results were in good agreement with those obtained from the study on the distribution of the free Tyr[³⁵S] generated and released by [³⁵S]sulphate-labelled filter-grown MDCK cells. In a control experiment in which dialysed [³⁵S]sulphate-labelled MDCK basolateral medium was mixed and incubated with non-radioactive conditioned MDCK basolateral medium, no detectable free Tyr[³⁵S] was generated during a 96 h incubation

Fig. 4. Flow diagram summarizing the experimental design of the time-lapse study

This study investigated the distribution of different Tyr^[35S]-containing compounds in the apical medium, the basolateral medium and the cell-associated fraction following the incubation of filter-grown MDCK cells pre-labelled with ^[35S]sulphate.

period. This suggested that the degradation of tyrosine-^[35S]sulphated proteins generating the free Tyr^[35S] indeed occurred intracellularly. A similar experiment was carried out in which a sample of dialysed ^[35S]sulphate-labelled MDCK apical medium was mixed with MEM and placed on the apical side of an insert covered with a confluent monolayer of MDCK cells. Free Tyr^[35S] was generated and released into both the apical medium and the basolateral medium following a 96 h incubation. The distribution of the released free Tyr^[35S] was calculated to be 18.0% and 82.0% in the apical medium and the basolateral medium respectively (Table 2).

DISCUSSION

By employing radioactive labelling techniques, we have observed in the present study a polarized secretion of ^[35S]sulphate- and ^[35S]methionine-labelled macromolecules by filter-grown MDCK cells. The results showed that quantitatively more macromolecules sulphated on their carbohydrate moieties were secreted into the basolateral medium, whereas tyrosine-sulphated proteins were predominantly secreted into the apical medium. The predominant apical secretion of tyrosine-sulphated proteins would appear even more dramatic if the reported surface area ratio (4:1) between the basolateral membrane domain and the apical membrane domain [23] is taken into account. Free Tyr^[35S], presumably a degradation product of tyrosine-^[35S]sulphated proteins [10], on the other hand, was found to be predominantly released into the basolateral medium. The ratio between the amount of the free Tyr^[35S] released into the basolateral medium and that released into the apical medium was greater than 4:1.

The time-lapse study was conducted to monitor more closely the polarized secretion of tyrosine-^[35S]sulphated proteins and to obtain evidence that the free Tyr^[35S] was indeed generated as an end product of the degradation of tyrosine-^[35S]sulphated proteins. The flow diagram shown in Fig. 4 summarizes the experimental design of this time-lapse study. The results indicated that, during the 48 h time course monitored, the release of tyrosine-^[35S]sulphated proteins from the pre-labelled cells into the apical medium was faster and quantitatively greater than the release into the basolateral medium. Quantitatively speaking, the release of tyrosine-^[35S]sulphated proteins from the apical surface could be calculated to be more than 10 times greater than the

release from the basolateral surface if the surface area ratio between the apical membrane domain and the basolateral membrane domain is taken into consideration. The fact that an amount equivalent to 43.8% of the tyrosine-^[35S]sulphated proteins originally present remained in the cell-associated fraction at the end of the 48 h incubation may imply that a considerable amount of tyrosine-^[35S]sulphated proteins was already present in the basal lamina [11] or was adsorbed onto the basolateral side of the membrane filter at the beginning of the incubation. Based on the results obtained, it was calculated that an amount equivalent to 41.54% of the tyrosine-^[35S]sulphated proteins originally present was degraded during the 48 h incubation. This is supported by the fact that free Tyr^[35S] was being generated and released from the cells (Fig. 3d), and, quantitatively, the amount of the free Tyr^[35S] generated was comparable with the amount of tyrosine-^[35S]sulphated proteins calculated to have been degraded (cf. Fig. 3b). Here again, the free Tyr^[35S] was predominantly released into the basolateral medium. The mechanism of release of free Tyr^[35S] from the labelled MDCK cells is at present unknown. It would be of interest to investigate whether a transport protein predominantly present in the basolateral membrane domain is responsible for the preferential release of the free TyrS into the basolateral medium. MDCK cells have previously been reported to display the morphological and enzymic properties of renal distal tubule cells [23]. The fact that the free TyrS is released from the basolateral surface, which presumably is the side facing the blood circulation, may imply that MDCK cells are not the ones in the kidney which are responsible for the trans-epithelial transport and excretion of free TyrS from the blood into the urine [8,9]. Rapid catabolism of tyrosine-sulphated proteins, generating free TyrS as an end product, has also been reported for rat embryo fibroblasts [10]. Although the reason for the rapid degradation of tyrosine-sulphated proteins in these tissue culture cells is still unclear, the present results provide strong support for the hypothesis that the large amounts of the free TyrS present in mammalian urines [8,9] are most likely derived from the intracellular degradation of tyrosine-sulphated proteins. In a multicellular eukaryotic organism, however, the majority of the tyrosine-sulphated proteins are secretory proteins [1,24]. It therefore remains to be clarified whether the tyrosine-sulphated proteins degraded are the ones diverted to, for example, lysosomes during their biosynthetic transport, or the ones already secreted which for unknown

reasons become endocytosed and delivered to, for example, lysosomes for degradation. In an attempt to test this latter possibility, an endocytotic degradation experiment was performed. Both the apically and the basolaterally secreted tyrosine- ^{35}S sulphated proteins were found to be endocytosed and degraded to form free Tyr ^{35}S . It is at present unknown whether there is a difference between the rates of endocytotic degradation for apically secreted and basolaterally secreted tyrosine-sulphated proteins. The difference in the rate of endocytotic degradation, if it indeed exists, may explain the discrepancy in the data concerning the ratio between the amount of apically secreted tyrosine-sulphated proteins and the amount of basolaterally secreted tyrosine-sulphated proteins shown in Table 1 and Fig. 3(b). Previous studies have also shown that MDCK cells may undergo endocytosis processes, of which some are receptor-mediated [25]. It would be interesting to find out whether the endocytosis of tyrosine-sulphated proteins is a non-specific phenomenon or a specific process involving a receptor capable of recognizing and binding to the TyrS residues of the tyrosine-sulphated proteins to be endocytosed.

In conclusion, we have obtained evidence in the present study that filter-grown MDCK cells indeed secrete tyrosine-sulphated proteins in a polarized fashion. The tyrosine-sulphated proteins appeared to be released predominantly from the apical surface. This polarized secretion of tyrosine-sulphated proteins may imply a role for protein-bound TyrS residues in the routing of such modified proteins toward the apical side. Tyrosine-sulphated proteins produced by MDCK cells, including fibronectin, laminin and a 80 kDa glycoprotein, could be used as models for studying their polarized secretion in the presence or absence of sulphation inhibitors, tunicamycin and acidotropic compounds. It will be important to clarify whether a receptor capable of binding tyrosine-sulphated proteins, and thereby mediating their intracellular transport, does exist in MDCK cells. It was also observed that tyrosine-sulphated proteins, once secreted, could be endocytosed and subjected to degradation, generating free TyrS as an end product. In contrast with the secretion of tyrosine-sulphated proteins, the free TyrS generated was found to be predominantly released into the basolateral medium. The free TyrS released by various cells *in vivo* could contribute to the high concentration of free TyrS in blood, which would subsequently be excreted in the urine [8,9].

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