Special Issue

Role of a putative tyrosine-O-sulfate receptor in the targeting and/or intracellular transport of tyrosine-sulfated proteins

Ming-Cheh Liu¹, Yoichi Sakakibara¹ & Masahito Suiko²

¹ Department of Biochemistry, University of Texas Health Center at Tyler, Tyler, TX 75710 USA; ² Department of Biological Resource Sciences, Miyazaki University, Miyazaki 889–21 Japan

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Abstract

By employing the affinity gel fraction technique, we have detected a 175 kDa tyrosine-O-sulfate (TyrS)-binding protein in sodium cholate extracts of the microsomal membrane fractions of bovine liver and pancreas, as well as canine liver and pancreas. Western blot analysis revealed the presence of the bovine liver TyrS-binding protein in complexes with tyrosine-sulfated proteins both *in vivo* and *in vitro*, suggesting the putative role of the former being the receptor for the latter. Using filter-grown Madin-Darby canine kidney (MDCK) cells as a model, it was demonstrated that the tyrosine-sulfated proteins synthesized were predominantly secreted into the apical medium. The results further indicate the production and differential polarized secretion of different sulfated forms of the two major secretory proteins produced by MDCK cells, fibronectin (FN) and an 80 kDa glycoprotein (gp 80), with their tyrosine-sulfated forms being predominantly secreted from the apical surface. Treatment of filter-grown MDCK cells with glycosylation inhibitors, swainsonine and 1-deoxymannojirimycin, appeared to enhance the apical secretion of tyrosine-sulfated FN and gp 80. A similar 175 kDa membrane-bound 'TyrS receptor', cross-reactive toward antiserum against the canine liver TyrS receptor, was shown to be present in MDCK cells. Pulse-chase experiments revealed its presence in complexes with newly synthesized FN and gp 80. A hypothetical model for TyrS residues serving as an apical targeting signal during the biosynthetic transport of tyrosine-sulfated proteins, as mediated by the TyrS receptor, in MDCK cells is proposed.

Post-translational protein modification by tyrosine sulfation, first discovered in bovine fibrinogen (Bettelheim, 1954), is now known to have a widespread occurrence among proteins of multicellular eukaryotic organisms (Huttner, 1988). It has been estimated that as much as 1% of the total proteins in an organism may be tyrosine-sulfated (Baeuerle and Huttner, 1985). In view of that the vast majority of tyrosine-sulfated proteins identified are secretory proteins (Huttner, 1984) and that the tyrosylprotein sulfotransferase, which catalyzes the protein tyrosine sulfation reaction, is located in the Golgi (Lee and Huttner, 1983), the initial speculation on the functional relevance of this unique protein modification was focused on the aspect of protein secretion (Hille, *et al.*, 1984). A hypothetical role of tyrosine sulfation in the packaging of secretory proteins into secretory granules was further elaborated (Herzog, 1985; Rosa *et al.*, 1985). Along the same

line, we have been investigating the presence of a putative TyrS receptor involved in mediating the targeting and/or intracellular transport of tyrosine-sulfated proteins.

Identification of a putative 'TyrS receptor'

In an earlier study (Liu *et al.*, 1988), we have used an affinity gel covalently bonded with TyrS to fractionate a 175 kDa protein from the sodium cholate extract of bovine liver membrane fraction. The protein appeared to be specific for TyrS as it bound neither the unmodified tyrosine nor the structurally similar tyrosine-O-phosphate. Furthermore, the binding of the protein to TyrS-Sepharose was found to be strong from pH 8.0 down through pH 6.0, and became dramatically weaker at pH 5.5. A similar 175 kDa TyrS-binding

protein was also detected in the choleate extracts of the microsomal membrane fractions prepared from bovine pancreas and from canine liver and pancreas. We have subsequently purified this 175 kDa 'TyrS-binding protein' from bovine liver using the TyrS-Affi-Gel affinity chromatography in combination with other chromatography techniques (Lu *et al.*, 1991). The purified protein, present in monomeric form, was found to bind concanavalin A-Sepharose and yielded a positive reaction toward periodic acid-Schiff staining, indicating its glycoprotein nature. The purified bovine liver TyrS-binding protein similarly displayed strong binding to TyrS, but not the unmodified tyrosine, covalently bonded to Sepharose. Using a tyrosine-sulfated cholecystokinin octapeptide (CCK-8) as the ligand in a radioimmunoassay, the purified TyrS-binding protein also exhibited a pH-dependent binding, being strong from pH 8.0 down through 6.5 and becoming dramatically weaker at pH below 6.0. Divalent cations, added to the assay mixture, exerted significant enhancement effects on the binding being in the order $Mn^{2+} > Ca^{2+} > Mg^{2+}$. Using a rabbit antiserum against the bovine liver TyrS-binding protein as the probe, Western blot analysis for the presence of TyrS-binding protein/tyrosine-sulfated protein complexes in bovine liver membrane lysates was performed (Liu *et al.*, 1991). It was found that the TyrS-binding protein co-precipitated with three tyrosine-sulfated proteins (fibronectin, fibrinogen and complement C4) immunoprecipitated by their respective antibodies. In contrast, for the two non-tyrosine-sulfated proteins (haptoglobin and transferrin) tested, co-precipitation of the TyrS-binding protein was not observed. On employing the affinity gel fractionation technique, it was shown that the purified TyrS-binding protein exhibited binding affinity toward Sepharose gels covalently bonded to fibronectin or fibrinogen, but not toward Sepharose gels bonded to albumin or transferrin. These results indicate that the TyrS-binding protein formed complexes with tyrosine-sulfated proteins both *in vivo* and *in vitro*, and thus provide support for the putative role of the former being the receptor for the latter.

Polarized secretion of tyrosine-sulfated proteins by filter-grown Madin-Darby canine kidney (MDCK) cells

We have previously shown that polarized filter-grown MDCK cells, labeled for 24 h with [^{35}S]sulfate, secreted macromolecules [^{35}S]sulfated on their carbohy-

drate moieties predominantly into the basolateral medium, whereas the tyrosine-[^{35}S]sulfated proteins synthesized were predominantly secreted into the apical medium (Han and Liu, 1991). A time-lapse study using prelabeled MDCK cells incubated in fresh medium revealed that, during the 48 h time course monitored, the release of tyrosine-[^{35}S]sulfated proteins into the apical medium was kinetically faster and quantitatively greater than that into the basolateral medium. These results, have prompted our interest in investigating the possible involvement of tyrosine sulfation in the polarized (apical) targeting and secretion of tyrosine-sulfated proteins produced by MDCK cells.

Since the polarized apical secretion of tyrosine-sulfated proteins produced by MDCK cells was not found to be absolute, we decided to first carry out an experiment to investigate the differential polarized secretion of different sulfated forms of secretory proteins produced by MDCK cells. MDCK cells were doubly labeled with [^{14}C]leucine and [^{35}S]sulfate. After a 24-hour labeling, the apical and basolateral medium fractions were collected. The proteins secreted into the two medium fractions were analyzed with respect to their constituent Tyr[^{35}S], carbohydrate-bound [^{35}S]sulfate and [^{14}C]leucine. The results obtained indicate that the Tyr[^{35}S] content of the apically secreted proteins on average was 4.7 times that of the basolaterally secreted proteins. In contrast, the carbohydrate-bound [^{35}S]sulfate content of the basolaterally secreted proteins was more than two times higher than that of the apically secreted proteins (data not shown). Both fibronectin (FN) and an 80 kDa glycoprotein (gp 80), two major tyrosine-sulfated proteins previously shown to be produced by MDCK cells (Han *et al.*, 1991), were secreted at different proportions into the two medium fractions. Interestingly, as shown in Table 1, the sub-population secreted into the apical medium had a much higher (2.6 and 4.7 times, respectively, for FN and gp 80) Tyr[^{35}S] content than the sub-population released into the basolateral medium. These results demonstrate clearly the production and differential polarized secretion of different sulfated forms of FN and gp 80, with the tyrosine-sulfated forms being predominantly secreted from the apical surface of MDCK cells (Liu *et al.*, manuscript in preparation).

To probe further the possible role of protein-bound TyrS residues and carbohydrate or carbohydrate-bound sulfate groups as signals for polarized protein targeting, we have subsequently carried out an experiment examining the effects of sulfation and glycosylation inhibitors on the polarized secretion of (the normally

Table 1. Differential polarized secretion of different sulfated forms of fibronectin (FN) and gp 80 into apical medium and basolateral medium by filter-grown MDCK cells doubly labeled with [35 S]sulfate and [14 C]leucine

		Apically secreted	Basolaterally secreted
FN	Tyr[35 S] ^a	9 130	16 870
	[14 C]Leucine ^a	21 623	104 375
	[35 S]/[14 C]	0.422	0.162
gp 80	Tyr[35 S] ^a	97 660	22 823
	[14 C]Leucine ^a	25 865	1 287
	[35 S]/[14 C]	0.265	0.056

^a Data shown represent cpm values for the [35 S]- or [14 C]-radioactivity determined for FN or gp 80 immunoprecipitated from the apical medium or the basolateral medium. For the determination of Tyr[35 S] content, the two-dimensional thin-layer separation procedure previously developed (Liu and Lipmann, 1984) was employed.

tyrosine-sulfated and N-glycosylated) FN and gp 80, produced by filter-grown MDCK cells. Identically prepared confluent MDCK cells grown on the filters of individual Millicell-HA inserts were labeled with [35 S]methionine in the presence or absence of either the inhibitor for the processing of N-linked oligosaccharides (swainsonine (SWSN) (Elbein, 1991) or 1-deoxymannojirimycin (DMM) (Elbein, 1991)) or the sulfation inhibitor, sodium chlorate (Baeuerle and Hutter, 1986). Upon an 18-hour labeling, the labeled apical medium and basolateral medium fractions were collected, mixed with equal volumes of a 2X SDS sample buffer, and subjected to SDS-PAGE. After electrophoresis, the gel was stained, destained, and dried. The autoradiograph taken from the dried gel, as shown in Figure 1, revealed that FN (indicated by the upper arrowhead) was secreted predominantly into the basolateral medium under normal growth conditions (lanes 1a and 1b). In the presence of SWSN (lanes 2a and 2b), the polarized secretion of FN remained virtually unchanged. In the presence of DMM (lanes 3a and 3b), however, FN became predominantly secreted into the apical medium. When the cells were treated with sodium chlorate (lanes 4a and 4b), FN was equally secreted into the two medium fractions. In the case of gp 80 (indicated by the three lower arrowheads), the protein was found to be predominantly apically secreted under normal growth conditions (lanes 1a and 1b). In the presence of SWSN (lanes 2a and 2b) or DMM (lanes 3a and 3b), gp 80 became even more predominantly apically secreted. When the cells were treated with

Figure 1. Effects of swainsonine (SWSN), 1-deoxymannojirimycin (DMM), and sodium chlorate on the polarized secretion of fibronectin and gp 80 produced by filter-grown MDCK cells labeled with [35 S]methionine. Aliquots of the labeled apical or basolateral medium fraction collected at the end of a 20-hour labeling were mixed with 2X SDS sample buffer and subjected to SDS-PAGE, followed by autoradiography. Samples analyzed were: 1, normal conditions; 2, with SWSN; 3, with DMM; 4, with sodium chlorate. Lanes a and b correspond to, respectively, apical and basolateral medium of MDCK cells labeled under above-mentioned conditions. The upper arrowhead indicates the position of the fibronectin band, and the three lower arrowheads indicate the positions of the three subunits of gp 80.

sodium chlorate, gp 80, similar to FN, started losing its polarized manner of secretion. These results appear to be compatible with the putative role of TyrS residues being an apical targeting signal for tyrosine-sulfated proteins (see the model proposed below) and the role of N-linked oligosaccharides and/or their bound sulfate being a (dominant) basolateral protein targeting signal. Similar experiments with the apical medium and basolateral medium samples being further subjected to immunoprecipitation using antibodies against FN or gp 80, prior to SDS-PAGE, have been carried out, and similar electrophoretic patterns for FN or gp 80 under different labeling conditions were observed.

Identification of a putative TyrS receptor possibly functioning for the biosynthetic transport of tyrosine-sulfated proteins in MDCK cells

We then attempted to investigate the presence of a TyrS receptor responsible for mediating the intracel-

Figure 2. Affinity gel fractionation of sodium cholate-extracted MDCK cellular protein(s) exhibiting immunologic cross-reactivity toward antiserum against canine liver 175 kDa TyrS receptor. Samples analyzed were: lane 1, sodium cholate extract of MDCK cells; lane 2, glycine-Sepharose-fractionated sample; lane 3, tyrosine-Sepharose-fractionated sample; lane 4, TyrS-Sepharose-fractionated sample; lane 5, tyrosine-O-phosphate-Sepharose-fractionated sample; and lane 6, gelatin-Sepharose-fractionated sample. Samples analyzed were subjected to SDS-PAGE, electrotransferred onto an Immobilon-P membrane, probed with anti-canine liver TyrS receptor antiserum, and treated with [125 I]-protein A, followed by autoradiography. (Reprinted, with permission, from *Biochem. J.* (1993) 294: 407–417).

lular transport of tyrosine-sulfated proteins in MDCK cells. By employing the Western blotting technique, we have detected, in sodium cholate extract of MDCK cells, a 175 kDa protein exhibiting cross-reactivity toward antiserum against canine liver 175 kDa TyrS receptor (Figure 2). Affinity gel fractionation revealed that the protein displayed binding affinity toward TyrS, but not the free tyrosine or tyrosine-O-phosphate, covalently bonded to Sepharose gels through the α -amino group. N-glycanase treatment showed this 175 kDa TyrS-binding protein to be a glycoprotein with N-linked carbohydrate accounting for ca. 10% of its molecular mass. To clarify the membrane-bound nature of this 175 kDa TyrS-binding protein being either integral or peripheral, Triton X-114 extraction of proteins from MDCK cells was performed followed by a temperature-induced phase separation. Subsequent analysis revealed the identity of the extracted 175 kDa TyrS-binding protein as an integral membrane protein. Furthermore, in MDCK cells, this 175 kDa TyrS-binding protein was present in complex with a major tyrosine-sulfated protein, gp 80, as indicated by the co-precipitation of the former when the latter was immunoprecipitated from MDCK cell lysate. The binding of the 175 kDa TyrS-binding protein to gp 80 being through the TyrS residues of the latter was indicated by the finding that when protein tyrosine sulfation was inhibited by treatment of cultured

MDCK cells with 20 mM sodium chlorate, a metabolic sulfation inhibitor (Baeuerle and Huttner, 1986), co-precipitation of the 175 kDa TyrS-binding protein with gp 80 immunoprecipitated from the cell lysate was no longer observed. These results suggest the identity of the 175 kDa TyrS-binding protein as a TyrS receptor for tyrosine-sulfated proteins in MDCK cells.

Trying to discern if the function of the TyrS receptor is in the biosynthetic transport of newly synthesized tyrosine-sulfated proteins or in the receptor-mediated endocytosis of exogenous tyrosine-sulfated proteins, we have performed a series of studies. One study employed the cell-surface biotinylation technique (Sargiacomo *et al.*, 1989) to determine the cell-surface vs. intracellular location of the TyrS receptor. In the experiments, cell-surface proteins of confluent MDCK cells were subjected to biotinylation. After the reaction, excess biotinylation reagent was removed and the cells were lysed in a radioimmune precipitation (RIPA) buffer (Gilead *et al.*, 1976). Biotinylated proteins in the RIPA buffer cell lysate were fractionated using streptavidin-agarose gel and centrifuged. The fractionated proteins bound on the pelleted streptavidin-agarose gel beads and the non-biotinylated proteins present in the supernatant were subjected to SDS-PAGE, followed by the Western blot analysis for the presence of the TyrS receptor. Results showed that the 175 kDa TyrS receptor was detected only in the

Figure 3. Radioactive pulse-chase study for investigating the presence of newly synthesized tyrosine-sulfated proteins in complexes with the 175 kDa TyrS receptor. For Part (A), identically prepared dishes of MDCK cells were pulse labeled with [^{35}S]methionine and chased for different lengths of time in high-methionine (10 X) medium. At the end of each chase time period, the cells were lysed in RIPA buffer. Cell lysates prepared, upon immunoprecipitation by anti-TyrS receptor antiserum, were subjected to SDS-PAGE, followed by autoradiography. The lengths of time of chase for the immunoprecipitated samples prepared from different cell lysates shown on the autoradiograph were: lane 1, 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 45 min; lane 6, 60 min; lane 7, 90 min; and lane 8, 120 min. Lane 9 corresponds to a preimmune serum control sample prepared at 25 min into the chase period. For Part (B), aliquots of unlabeled MDCK cell lysate were immunoprecipitated using preimmune serum (lane 1), anti-canine fibronectin antiserum (lane 2), or anti-canine liver TyrS receptor antiserum (lane 3). Immunoprecipitated samples prepared were subjected to SDS-PAGE, electrotransferred onto an Immobilon-P membrane, probed with anti-canine fibronectin antiserum, and treated with [^{125}I]-protein A, followed by autoradiography. In Part (C), identically prepared dishes of MDCK cells were pulse labeled with [^{35}S]Sulfate and chased for different lengths of time in high-sulfate (10 X) medium. At the end of each chase time period, the cells were lysed in RIPA buffer. Cell lysates prepared, upon immunoprecipitation by antiserum against canine liver TyrS receptor, were subjected to SDS-PAGE, followed by autoradiography. The lengths of time of chase for the immunoprecipitated samples prepared from different cell lysates shown on the autoradiograph were: lane 1, 0 min; lane 2, 1 min; lane 3, 3 min; lane 4, 10 min; lane 5, 30 min, and lane 6, 60 min. (Reprinted, with permission, from *Biochem. J.* (1993) 294: 407–417).

non-biotinylated protein fraction, indicating its predominant, if not exclusive, intracellular location. Cell-surface trypsinization technique was also employed to examine the cell-surface vs. intracellular location of the TyrS receptor. In a time-course study, it was found that, following digestion of intact MDCK cells with trypsin for as long as two hours, no cleavage of the TyrS receptor was observed. These results again indicated the predominant, if not exclusive, intracellular location of the TyrS receptor. Another study employed a radioactive pulse-chase technique. Identically prepared dishes of MDCK cells were pulse-labeled with [^{35}S]methionine or [^{35}S]sulfate. At the end of the 5 min pulse period, pulse media were removed and replaced by chase media containing a 10 fold excess of methionine or sulfate. At 0, 5, 15, 30, 45, 60, 90, and 120 min after the chase period began, the chase media were removed and the cells were lysed in the RIPA buffer. RIPA buffer lysates were subjected to immunoprecipitation using anti-canine liver TyrS receptor antiserum. Immunoprecipitated samples were subjected to SDS-PAGE. After electrophoresis, the gel was dried and autoradiographed. Results shown in Figure 3 revealed that a high-molecular weight [^{35}S]methionine-labeled protein (indicated by the upper arrowhead in part A) co-precipitated with the TyrS receptor immunoprecipitated from the cell lysate prepared at approximately 15 min into the chase period. The identity of this high-molecular weight protein as FN was indicated by both its immunologic cross-reactivity toward anti-FN antibodies (part B) and its disulfide-bonded dimeric 220 kDa subunit structure (Yamada, 1983) (not shown). The 175 kDa radioactive band indicated by the lower arrowhead in part A was that of the [^{35}S]methionine-labeled immunoprecipitated TyrS receptor. When [^{35}S]sulfate was used for the pulse labeling, [^{35}S]sulfate-labeled FN (indicated by the upper arrowhead in part C) was found to co-precipitate with the immunoprecipitated TyrS receptor as soon as the chase period began. Upon prolonged exposure of the autoradiograph, three fainter bands (located respectively at 45, 40 and 35 kDa positions) characteristic of gp 80 (Kondor-Koch *et al.*, 1985; Gottlieb *et al.*, 1986; Han *et al.*, 1991) could also be detected (as indicated by the three small arrowheads). These results indicate clearly the presence of newly synthesized tyrosine-sulfated FN and gp 80 in complexes with the 175 kDa TyrS receptor, and therefore suggest the possible involvement of the latter in the biosynthetic transport of the former (Liu *et al.*, 1993).

Figure 4. A schematic diagram illustrating the hypothetical model for protein-bound TyrS residues functioning as an apical targeting signal in filter-grown MDCK cells.

A hypothetical model

Putting together the findings presented above, we have proposed a hypothetical model for TyrS residues serving as an apical targeting signal during the intracellular transport of tyrosine-sulfated proteins, as mediated by the TyrS receptor, in MDCK cells (Figure 4). The important features of the proposed model and the supporting evidence for different steps involved are summarized below.

1. Newly synthesized, tyrosine-sulfatable proteins transported through the *trans* Golgi compartment are subjected to tyrosine sulfation by the tyrosylprotein sulfotransferase (TPST) using 3'-phosphoadenosine, 5'-phosphosulfate (PAPS) as the sulfate donor. Supporting Evidence: the *trans* Golgi location of TPST (Baeuerle and Huttner, 1987).
2. Tyrosine-sulfated proteins thus formed are recognized and bound by the TyrS receptor, and packaged into the transport vesicle budded off from the *trans* Golgi membrane compartment. Supporting evidence: the existence of the membrane-bound TyrS receptor and its presence in complexes with newly synthesized tyrosine-sulfated proteins in MDCK cells (Liu *et al.*, 1993), in bovine liver (Liu *et al.*, 1991), bovine pancreas and canine liver and pancreas (Liu *et al.*, 1988).
3. The transport vesicle carrying tyrosine-sulfated proteins bound to the TyrS receptor fuses with a CURL (compartment of uncoupling of receptor and ligand)-like membrane compartment in which tyrosine-sulfated proteins become dissociated from the TyrS receptor due to the low pH environment presumably generated through the action of ATPase/H⁺ pump. Supporting evidence: i) pH-dependence of the ligand binding of the TyrS receptor (Lu *et al.*, 1991; Liu *et al.*, 1993), and ii) intracellular membrane location of the TyrS receptor in MDCK cells (Liu *et al.*, 1993).
4. The vesicle carrying free tyrosine-sulfated proteins buds off from the CURL-like membrane compartment and migrates toward the apical domain of the plasma membrane. Upon fusion with the apical plasma membrane, tyrosine-sulfated proteins become secreted from the apical surface. Supporting evidence: i) predominantly apical secretion of tyrosine-sulfated proteins in general by filter-grown MDCK cells (Han and Liu, 1991), ii) predominantly apical secretion of tyrosine-sulfated forms of FN and gp 80 by filter-grown MDCK cells (Liu *et al.*, unpublished data), and iii) intracellular membrane location of the TyrS receptor in MDCK cells (Liu *et al.*, 1993).
5. A distinct type of vesicle carrying unbound TyrS receptor buds off from the CURL-like membrane

compartment and migrates toward the *trans* Golgi membrane compartment. Upon fusion with the *trans* Golgi membrane, the recycled TyrS receptor may participate in another round of the transport of tyrosine-sulfated proteins. Supporting evidence: intracellular membrane location of the TyrS receptor in MDCK cells (Liu *et al.*, 1993).

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Address for Correspondence: Ming-Cheh Liu, Department of Biochemistry, University of Texas Health Center at Tyler, P.O. Box 2003, Tyler, TX 75710 USA.