Identification of a putative tyrosine-*O*-sulphate (TyrS) receptor possibly functioning in the biosynthetic transport of tyrosine-sulphated proteins in Madin–Darby canine kidney cells

Jianwei LIU,* Jian R. HAN,* Chau-Ching LIU,† Masahito SUIKO‡ and Ming-Cheh LIU*§

* Department of Chemistry and Biochemistry, The University of Oklahoma, Norman, OK 73019, U.S.A.,

† The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A. and ‡ Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

By employing an affinity-gel fractionation technique coupled to Western-blot analysis, we have identified a 175 kDa tyrosine-*O*-sulphate (TyrS)-binding protein present in Madin–Darby canine kidney (MDCK) cells. The binding of this TyrS-binding protein to TyrS covalently bonded to Sepharose gel was found to be pH-dependent, being strong from pH 8.0 down to pH 6.5 and increasingly weak at pH 6.0 and below. Results obtained from Triton X-114 temperature-induced phase separation and sodium carbonate buffer (pH 11) extraction experiments indicated that the TyrS-binding protein is an integral membrane protein. This 175 kDa TyrS-binding protein was found to be present in association with a major tyrosine-sulphated protein, the apically secreted 80 kDa glycoprotein (gp 80), in cell lysate prepared from MDCK cells maintained under normal growth conditions. When the cell lysate used was prepared from MDCK cells pretreated with 20 mM sodium chlorate, a metabolic sulphation

inhibitor, the complex formed between the two proteins could no longer be detected, indicating that the binding of the TyrS-binding protein is through the TyrS residue(s) of gp 80. Both cell-surface biotinylation and cell-surface trypsinization studies demonstrated the predominantly, if not exclusively, intracellular location of the TyrS-binding protein. Furthermore, radioactive pulse-chase experiments revealed that the newly synthesized radiolabelled fibronectin and gp 80 were present in complexes with the TyrS-binding protein in MDCK cells pulse-labelled with [³⁵S]methionine or [³⁵S]sulphate. Exogenous [³⁵S]methionine-labelled gp 80 added to the medium, on the other hand, was not found to be present in association with the TyrS-binding protein in MDCK cells over a 2-h time course. These results strongly suggested the identity of the 175 kDa TyrS-binding protein as a putative ‘TyrS receptor’, possibly functioning in the biosynthetic transport of tyrosine-sulphated proteins in MDCK cells.

INTRODUCTION

Tyrosine sulphation has been shown to have a widespread occurrence among proteins in multicellular eukaryotic organisms (Huttner and Baeuerle, 1988). The enzyme responsible for this unique post-translational protein modification, tyrosylprotein sulphotransferase, was reported to be located intracellularly in trans Golgi network (Baeuerle and Huttner, 1987). Therefore, only those proteins whose biosynthetic transport involves passage through this particular intracellular membrane compartment may be subjected to sulphation on tyrosine residues. Indeed, the vast majority of the tyrosine-sulphated proteins identified to date are secretory proteins (Huttner and Baeuerle, 1988). Despite a considerable amount of effort made in recent years, the functional relevance of protein tyrosine sulphation remains poorly understood. Among a number of possible functional roles that have been proposed, the involvement of tyrosine sulphation in intracellular protein sorting and transport received strong support from our recent finding of a bovine liver 175 kDa membrane-bound tyrosine-*O*-sulphate (TyrS)-binding protein (Liu et al., 1988; Lu et al., 1991). Similar 175 kDa membrane-bound TyrS-binding proteins have also been detected in bovine pancreas and canine liver and pancreas (Liu et al., 1988). Furthermore, the discovery that the bovine liver 175 kDa TyrS-binding protein was present in association with several representative tyrosine-sulphated proteins in freshly prepared bovine liver membrane

lysates (M.-C. Liu et al., 1991) strongly suggested the identity of the TyrS-binding protein as a putative ‘TyrS receptor’ involved in mediating the intracellular transport of tyrosine-sulphated proteins in bovine liver cells. Whether this putative ‘TyrS receptor’ functions *in vivo* in the biosynthetic transport of newly synthesized tyrosine-sulphated proteins or the endocytosis of exogenous tyrosine-sulphated proteins, however, remains to be clarified.

Filter-grown Madin–Darby canine kidney (MDCK) cells, with their plasma membrane segregated into the apical and the basolateral domains, have been widely used in the study of the mechanisms involved in polarized protein sorting (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Caplan et al., 1987; Lisanti et al., 1989). These cells have been shown to synthesize and deliver/secrete both endogenous and exogenous plasma membrane/secretory proteins to or from their apical or basolateral surface (Bartles and Hubbard, 1988). It is generally conceived that newly synthesized proteins, while being transported, carry in their molecular structures the sorting signals which are recognized by specific receptors responsible for their intracellular transport (Simons and Wandinger-Ness, 1990). Such signals may have been generated after specific post-translational modifications occurring during the biosynthetic transport of newly synthesized proteins. In a preliminary study on the possible involvement of tyrosine sulphation in polarized protein sorting, we found that filter-grown MDCK cells secreted tyrosine-

Abbreviations used: gp 80, 80 kDa glycoprotein; MDCK cells, Madin–Darby canine kidney cells; RIPA buffer, radio-immune precipitation buffer; TyrS, tyrosine-*O*-sulphate; MEM, minimal essential medium; CMC, critical micellar concentration; sulpo-NHS-biotin, sulpo-*N*-hydroxysuccinimidobiotin.

§ To whom correspondence should be addressed at Department of Biochemistry, University of Texas Health Center at Tyler, P.O. Box 2003, Tyler, Texas 75710, U.S.A.

sulphated proteins predominantly from their apical surface (Han and Liu, 1991). This phenomenological observation alone, however, is insufficient for justifying a role for TyrS residues as polarized (apical) protein sorting signals. Additional evidence, in particular the presence of a specific 'TyrS receptor' involved in the intracellular transport of newly synthesized tyrosine-sulphated proteins, is necessary for unequivocally proving this putative role of protein-bound TyrS residues.

In this paper, we report the presence in MDCK cells of a 175 kDa TyrS-binding protein exhibiting cross-reactivity toward antiserum raised against canine liver membrane-bound TyrS-binding protein. Western-blot analysis revealed the presence of the TyrS-binding protein in association with an 80 kDa glycoprotein (gp 80), a major tyrosine-sulphated protein known to be secreted predominantly from the apical surface by filter-grown MDCK cells (Kondor-Koch et al., 1985; Gottlieb et al., 1986). Experiments using sodium chlorate, a metabolic sulphation inhibitor, further indicated that binding of the TyrS-binding protein occurred through the TyrS residue(s) of gp 80. Studies employing cell-surface biotinylation and cell-surface trypsinization revealed the predominantly, if not exclusively, intracellular location of this putative 'TyrS receptor'. These results, in combination with those showing the protein to be present in complexes with newly synthesized, radiolabelled fibronectin and gp 80, as obtained from the radioactive pulse-chase study, indicated the possible functional involvement of the 'TyrS receptor' in the intracellular transport of newly synthesized tyrosine-sulphated proteins.

MATERIALS AND METHODS

Materials

Sulpho-*N*-hydroxysuccinimidobiotin (sulpho-NHS-biotin) and streptavidin were products of Pierce Chemical Company. Amberlite XAD-2 was from Aldrich Chemical Company. Protein A-Sepharose CL-4B, gelatin-Sepharose, and 6-aminohexanoic acid-activated Sepharose-4B were purchased from Pharmacia. TyrS was synthesized according to the procedure developed by Jevons (Jevons, 1963). Immobilon-P membranes were obtained from Millipore Corporation. Carrier-free sodium [³⁵S]sulphate, [³⁵S]methionine (1039 Ci/mmol), and ¹²⁵I-labelled protein A (7.2 µCi/mg) were from ICN Biomedicals. Affinity Sepharose gels bonded with streptavidin, glycine, tyrosine, TyrS, or tyrosine-*O*-phosphate were prepared according to the procedure recommended by the manufacturer. Canine liver 175 kDa membrane-bound TyrS-binding protein was purified using the procedure previously developed for the purification of bovine liver TyrS-binding protein (Lu et al., 1991). Antisera raised against canine liver 175 kDa membrane-bound TyrS-binding protein and gp 80 were prepared as described previously (Han et al., 1991; Lu et al., 1991). [³⁵S]Methionine-labelled gp 80 was purified from the [³⁵S]methionine-labelled spent medium of MDCK cells based on the procedure described previously (J. Liu et al., 1991). All other chemicals were of the highest grades commercially available.

Cell culture

MDCK cells (stain II) were routinely maintained in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories), penicillin G (30 µg/ml) and streptomycin sulphate (50 µg/ml), in a 5% CO₂ atmosphere at 37 °C. Confluent cells grown on 35-mm or 100-mm diam. dishes were used in the following experiments.

Examination of the presence of TyrS-binding protein(s) exhibiting immunological cross-reactivity toward antiserum raised against canine liver 175 kDa membrane-bound TyrS-binding protein

To examine the presence of protein(s) exhibiting cross-reactivity toward the antiserum, confluent MDCK cells, grown on a 100-mm-diam. dish, were rinsed three times with PBS and extracted at 4 °C with 6 ml of a sodium cholate solution (2 mg/ml in 10 mM Tris/HCl buffer, pH 7.4) supplemented with 10 µg/ml aprotinin. Following a 2-h extraction procedure, the sodium cholate extract was collected, and centrifuged to remove insoluble material. A 50 µl aliquot of the clear supernatant obtained was mixed with an equal vol of a 2× Laemmli's SDS sample buffer, heated at 100 °C for 3 min, and subjected to SDS/PAGE (Laemmli, 1970), followed by the Western-blot analysis described below. To examine the ligand specificity of the protein(s) cross-reacting with the antiserum against canine liver TyrS-binding protein, aliquots (1 ml) of the cholate extract, prepared as described above, were placed in 1.5-ml capacity Eppendorf microcentrifuge tubes. To different tubes, 50 µl of a suspension (in a 1:1 ratio of packed gel beads to PBS) of glycine-Sepharose, tyrosine-Sepharose, TyrS-Sepharose, tyrosine-*O*-phosphate-Sepharose, or gelatin-Sepharose was added. The contents of the tubes were agitated for 20 min at room temperature and centrifuged. The pelleted gel beads were washed five times with 10 mM Tris/HCl buffer (pH 7.4), placed in Laemmli's SDS sample buffer, heated for 3 min at 100 °C and subjected to SDS/PAGE, followed by Western-blot analysis as described below.

To examine the pH-dependence of the binding of the TyrS-binding protein to TyrS-Sepharose gel, 0.5 ml aliquots of the sodium cholate extract were placed in 1.5 ml Eppendorf microcentrifuge tubes containing 0.5 ml of a 0.2 M Hepes buffer (pH 8.0, 7.5 or 7.0) supplemented with 0.15 mM Triton X-100, or a 0.2 M Mes buffer (pH 6.5, 6.0 or 5.5) supplemented with 0.15 mM Triton X-100. To different tubes, 50 µl aliquots of a TyrS-Sepharose-gel suspension (in a 1:1 ratio of packed gel beads to H₂O) were added. The contents of the tubes were agitated for 20 min at room temperature and then centrifuged. The pelleted gel beads were washed three times with the respective buffer solutions used during fractionation, placed in Laemmli's SDS sample buffer, heated for 3 min at 100 °C and subjected to SDS/PAGE, followed by Western-blot analysis as described below.

Examination of the integral membrane protein nature of the 175 kDa TyrS-binding protein

Two methods, Triton X-114 temperature-induced phase separation and sodium carbonate buffer (pH 11) extraction, were employed for examining the integral membrane protein nature of the TyrS-binding protein.

Triton X-114 temperature-induced phase separation

Confluent MDCK cells grown on a 100-mm-diam. dish were washed three times with ice-cold PBS and lysed by treatment with 1 ml of a Triton X-114 lysis buffer [containing 1% (v/v) Triton X-114, 10 mM Hepes buffer (pH 7.4), 5 mM EDTA, 150 mM NaCl and 5 µg/ml aprotinin] for 1 h at 4 °C (Pryde, 1986). The lysate, upon removal of insoluble material, was loaded over 300 µl of a sucrose cushion [containing 6% (w/v) sucrose, 0.06% Triton X-114, 10 mM Hepes buffer (pH 7.4), 5 mM EDTA and 150 mM NaCl] placed in a 1.5 ml Eppendorf microcentrifuge tube. The tube was incubated at 30 °C until clouding of the solution occurred (approx. 3 min), and then

centrifuged at 13000 *g* for 5 min at room temperature. The aqueous (upper) phase (still containing approx. 0.06 % Triton X-114) was separated and subjected to exhaustive dialysis against 10 mM Hepes buffer, pH 7.4, containing 5 mM EDTA, 150 mM NaCl and 1 % (w/v) Amberlite XAD-2 at 4 °C for 3 days. The dialysed fraction was diluted with 3 vol of 10 mM Hepes buffer, pH 7.4, and centrifuged at 144000 *g* for 2 h at 4 °C. The supernatant collected was subjected to immunoprecipitation using antiserum against canine liver TyrS-binding protein and the pellet was solubilized in Laemmli's SDS sample buffer. The immunoprecipitated supernatant sample and the solubilized pellet sample, as well as the initial detergent-phase sample, were subjected to SDS/PAGE, followed by Western-blot analysis described below.

Sodium carbonate buffer (pH 11) extraction

Confluent MDCK cells grown on 100-mm-diam. dishes were scraped off, suspended in 10 mM Hepes buffer (pH 7.4) containing 10 µg/ml aprotinin and 5 mM EDTA and homogenized. The homogenized preparation was centrifuged at 1000 *g* for 5 min at 4 °C. The supernatant obtained was further centrifuged at 144000 *g* for 1 h at 4 °C. The pelleted membrane was twice subjected to extraction with 100 mM sodium carbonate buffer (pH 11) according to the established procedure (Howell and Palade, 1982). The extract obtained was neutralized and subjected to immunoprecipitation using antiserum against canine liver TyrS-binding protein. The extracted membrane was lysed in Laemmli's SDS sample buffer. The immunoprecipitated sample and the membrane lysate were subjected to SDS/PAGE, followed by Western-blot analysis as described below.

Examination of the presence of 175 kDa TyrS-binding protein in association with gp 80, a major tyrosine-sulphated protein produced by MDCK cells, and the effect of sodium chlorate, a metabolic sulphation inhibitor, on complex formation

To examine the presence of the complex between the TyrS-binding protein and gp 80, confluent MDCK cells grown on a 100-mm diam. dish and rinsed free of extracellular soluble proteins, were lysed in 3 ml of a modified radioimmune precipitation (RIPA) buffer [containing 0.05 M Tris/HCl buffer (pH 7.4), 0.15 M NaCl, 1 % (v/v) Triton X-100, 1 % (v/v) sodium cholate and 5 µg/ml aprotinin] (Gilead et al., 1976). RIPA buffer lysate (1 ml) was placed in a 1.5 ml Eppendorf microcentrifuge tube and 15 µl of anti-(gp 80) antiserum was added to the tube. The mixture in the tube was incubated at room temperature for 1 h. At the end of the incubation period 30 µl of protein A–Sepharose-gel suspension (in a 1:1 ratio of packed gel beads to PBS) was added to the tube and the contents of the tube were agitated at room temperature. After a 20-min agitation period, protein A–Sepharose-gel beads bound with antibodies or antibody–antigen complexes were pelleted by centrifugation, washed three times with the modified RIPA buffer, placed in Laemmli's SDS sample buffer and subjected to SDS/PAGE, followed by the Western-blot analysis as described below. To study the effect of the sulphation inhibitor on the formation of complex, sub-confluent cells grown on 100-mm-diam. dishes were incubated in sulphate-free MEM (prepared by omitting streptomycin sulphate and substituting MgSO₄ with MgCl₂) supplemented with 5 mM or 20 mM sodium chlorate. After a 24-h incubation period, the medium was removed and cells were lysed and analysed for the presence of the complex, based on the same procedure described above.

Double radioactive labelling for the determination of the inhibitory effect of sodium chlorate on the tyrosine sulphation of proteins

To examine the level of protein-tyrosine sulphation under normal growth conditions, confluent MDCK cells grown on a 100-mm-diam. dish were pre-incubated in low-methionine (20 % of the normal concentration), sulphate-free MEM. After 4 h, the medium was removed and the cells were labelled with [³⁵S]sulphate (0.3 mCi/ml) and [³⁵S]methionine (50 µCi/ml) in the same medium. After a 24-h labelling period, the medium was collected for the analysis of [³⁵S]TyrS and [³⁵S]methionine associated with the secreted labelled proteins. To determine the effect of the sulphation inhibitor on the extent of protein-tyrosine sulphation, confluent MDCK cells were pre-incubated and labelled in the low-methionine, sulphate-free MEM supplemented with 5 mM or 20 mM sodium chlorate, based on the procedure described above. After a 24-h labelling period, the medium was collected for the analysis of [³⁵S]TyrS and [³⁵S]methionine associated with the secreted labelled proteins. The analysis of protein-bound [³⁵S]TyrS and [³⁵S]methionine was based on the procedure developed previously (Liu and Lipmann, 1984).

Examination of an intracellular versus a cell-surface location for the 175 kDa TyrS-binding protein

Two different techniques, cell-surface biotinylation and cell-surface trypsinization, were employed to examine the intracellular versus the cell-surface location of the TyrS-binding protein.

Cell-surface biotinylation

Confluent MDCK cells, grown on a 100-mm-diam. dish, were labelled with sulpho-NHS-biotin, based on the established procedure (Sargiacomo et al., 1989). The biotinylated cells were washed to remove unreacted sulpho-NHS-biotin and lysed in 1 ml of a lysis buffer [containing 1 % (v/v) Triton X-100 and 5 µg/ml aprotinin in PBS]. The lysate, upon removal of insoluble material, was placed in a 1.5 ml Eppendorf microcentrifuge tube and 50 µl of a streptavidin–Sepharose-gel suspension (in a 1:1 ratio of packed gel beads to PBS) was added. The contents of the tube were agitated for 1 h at room temperature and the gel beads bound with biotinylated proteins were pelleted by centrifugation, washed three times with PBS containing 1 % (v/v) Triton X-100 and placed in Laemmli's SDS sample buffer. The supernatant, containing non-biotinylated proteins, was subjected to immunoprecipitation using antiserum against canine liver TyrS-binding protein. The streptavidin–Sepharose-fractionated sample, together with the immunoprecipitated sample, were subjected to SDS/PAGE, followed by Western-blot analysis as described below.

Cell-surface trypsinization

Confluent MDCK cells, grown on 100-mm-diam. dishes, were washed five times with PBS and incubated in 4 ml of PBS containing 0.5 mg/ml trypsin and 2.7 mM EDTA for 0, 3, 30, 60, or 120 min at 37 °C with constant shaking. At the end of each incubation, the trypsinized cells were pelleted by centrifugation, washed five times with ice-cold PBS to remove residual trypsin and lysed in 1 ml of RIPA buffer containing 10 µg/ml aprotinin, 5 µg/ml soybean trypsin inhibitor, and 5 mM EDTA. RIPA buffer lysates were subjected to immunoprecipitation using antiserum raised against canine liver TyrS-binding protein. Immunoprecipitated samples were subjected to SDS/PAGE, followed by Western-blot analysis as described below.

Western-blot analysis

Samples prepared in the above-mentioned experiments, after separation by SDS/PAGE, were electroblotted on Immobilon-P membrane. The blotting was performed at 350 mA for 15 h in a buffer solution containing 25 mM Trizma base and 192 mM glycine (Towbin et al., 1979). The blotted membrane was blocked with 1% (w/v) haemoglobin in PBS for 1 h and probed with 25 μ l of anti-(canine liver membrane-bound TyrS-binding protein) antiserum. After a 1-h incubation period, the Immobilon-P membrane was washed five times with PBS to remove unbound antibodies. The washed membrane was treated for 45 min with PBS containing 1 μ Ci of 125 I-labelled protein A plus 1% (w/v) haemoglobin, washed five times with PBS and dried. Autoradiography was performed on the dried membrane. Alternatively, for demonstrating the presence of immunoprecipitated gp 80 in the sulphation-inhibitor-treatment experiment, the blotted Immobilon-P membrane, after blocking with haemoglobin, was probed with antiserum against gp 80, followed by 125 I-protein A treatment.

Radioactive pulse-chase experiment

Confluent MDCK cells grown on 35-mm-diam. dishes, pre-incubated for 4 h in methionine-deficient MEM, were pulse-labelled with [35 S]methionine (0.1 mCi/ml) in methionine-free MEM for 5 min. At the end of the labelling period, the labelling medium was removed and the labelled cells, after three quick rinses to remove residual free radioactive material, were incubated in MEM containing 10 times the normal concentration of methionine. The labelled cells in different dishes were incubated in this chase medium for 0, 5, 15, 30, 45, 60, 90 and 120 min. At the end of each incubation, the chase medium was removed and the cells, after three quick rinses with fresh MEM, were lysed in 1 ml of the modified RIPA buffer. Cell lysates prepared at different time points were subjected to immunoprecipitation using antiserum against canine liver TyrS-binding protein. Alternatively, confluent MDCK cells grown on 35-mm-diam. dishes, pre-incubated for 4 h in sulphate-deficient MEM, were pulse-labelled with [35 S]sulphate (0.25 mCi/ml) in sulphate-free MEM for 10 min. At the end of the labelling period, the labelling media were removed and the labelled cells, after three quick rinses to remove residual free radioactive material, were incubated in MEM containing 10 times the normal concentration of MgSO_4 . The labelled cells in different dishes were incubated in this chase medium for 0, 1, 3, 10, 30 and 60 min. At the end of each incubation, the chase medium was removed and the cells, after three quick rinses with fresh MEM, were lysed in 1 ml of modified RIPA buffer. Cell lysates prepared at different time points were subjected to immunoprecipitation using antiserum raised against canine liver TyrS-binding protein. Immunoprecipitated [35 S]methionine- or [35 S]sulphate-labelled samples were subjected to SDS/PAGE, followed by fluorography to reveal radioactive bands corresponding to [35 S]methionine- or [35 S]sulphate-labelled proteins co-precipitated with the TyrS-binding protein immunoprecipitated from the pulse-labelled MDCK cell lysates.

Endocytosis experiment

Confluent MDCK cells grown on 35-mm-diam. dishes were incubated in MEM containing added [35 S]methionine-labelled gp 80 (approx. 20000 c.p.m./dish). The cells in different dishes were incubated for 1, 3, 10, 30, 60, 90 and 120 min. At the end of each incubation, the medium was removed and the cells, after

three quick rinses with fresh MEM, were lysed in 1 ml of the modified RIPA buffer. Cell lysates were subjected to immunoprecipitation using antiserum against canine liver TyrS-binding protein. Immunoprecipitated samples were subjected to SDS/PAGE, followed by fluorography to reveal radioactive bands corresponding to [35 S]methionine-labelled gp 80, possibly co-precipitated with the immunoprecipitated TyrS-binding protein.

Miscellaneous methods

SDS/PAGE was performed in 5–10% (w/v) polyacrylamide gradient gels according to the method of Laemmli (Laemmli, 1970). Conditions for the immunoprecipitation experiments were as previously described (M.-C. Liu et al., 1991). Partial V8 protease hydrolysis during electrophoresis was carried out, based on the procedure of Cleveland et al. (1977). The *N*-linked carbohydrate content of the TyrS-binding protein was determined by treatment with *N*-Glycanase (Hirani et al., 1987), followed by SDS/PAGE. Fluorography was performed according to the procedure developed by Chamberlain (1979).

RESULTS

Presence of a 175 kDa TyrS-binding protein in MDCK cell lysate

In examining the presence of a putative 'TyrS receptor' in MDCK cells, we first set out to look for detergent-extractable protein(s) which exhibit(s) both the immunological cross-reactivity toward anti-(canine liver 175 kDa membrane-bound TyrS-binding protein) antiserum and the ligand-binding affinity for TyrS. Indeed, as shown in Figure 1, when the proteins present in the sodium cholate extract of MDCK cells were separated by SDS/PAGE, electroblotted on to an Immobilon-P membrane, and probed with the antiserum, a distinct 175 kDa protein (indicated by the arrowhead on lane 1) exhibiting the immunological cross-reactivity was detected. Affinity-gel fractionation, using Sepharose gels bonded with different ligands, was performed to test the ligand-binding specificity of this 175 kDa protein. Of the five kinds of affinity Sepharose gels tested, only TyrS-Sepharose (lane 4) was capable of fractionating this 175 kDa protein from the sodium cholate extract of MDCK

Figure 1 Affinity-gel fractionation of sodium cholate-extracted MDCK cellular protein(s) exhibiting immunological cross-reactivity toward antiserum against canine liver 175 kDa membrane-bound TyrS-binding protein

Samples shown on the autoradiograph taken from the Immobilon-P membrane after Western-blot analysis 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 analysed were subjected to SDS/PAGE, electrotransferred to an Immobilon-P membrane, probed with anti-(canine liver TyrS-binding) antiserum, and treated with 125 I-labelled protein A, followed by autoradiography.

Figure 2 pH-dependence of binding between the 175 kDa TyrS-binding protein and TyrS-Sepharose

(a) This shows the autoradiograph taken from the Immobilon-P membrane after the Western-blot analysis. Lanes 1, 2 and 3 were samples prepared in 0.1 M Mes buffer at pH values of 5.5, 6.0 and 6.5 respectively, and lanes 4, 5 and 6 were samples prepared in Hepes buffer prepared at pH values of 7.0, 7.5 and 8.0 respectively. (b) shows the graph obtained by plotting the ^{125}I radioactivity associated with the 175 kDa radioactive bands detected for samples against the respective pH values at which the TyrS-Sepharose fractionations were carried out. Data represent average values derived from three independent experiments.

cells. Neither tyrosine-Sepharose (lane 3) nor tyrosine-*O*-phosphate-Sepharose (lane 5) was capable of fractionating the 175 kDa protein. These results indicated clearly the TyrS-binding affinity of the 175 kDa protein exhibiting immunological cross-reactivity toward antiserum against canine liver TyrS-binding protein. When aliquots of the sodium cholate extract were adjusted to different pH environments (Hepes buffer, pH 8.0–7.0; Mes buffer, pH 6.5–5.5) and tested for the binding of the 175 kDa TyrS-binding protein to TyrS-Sepharose gel, results shown in Figure 2 revealed that the binding was strong from pH 8.0 down to pH 6.5. At pH 6.0 and below, the binding became dramatically weaker. These results therefore indicated a pH-dependence of the binding of the 175 kDa TyrS-binding protein to TyrS covalently bonded to the Sepharose gel.

Integral membrane protein nature of the 175 kDa TyrS-binding protein

Triton X-114 temperature-induced phase separation and sodium carbonate buffer (pH 11) extraction techniques were employed to examine whether the TyrS-binding protein is an integral membrane protein.

Triton X-114 temperature-induced phase separation

Previous studies have shown that membrane proteins extracted from the cells with Triton X-114, following the temperature-induced phase separation, would partition between the aqueous

Figure 3 Triton X-114 temperature-induced phase separation and sodium carbonate buffer (pH 11) extraction experiments for investigating the integral membrane protein nature of the 175 kDa TyrS-binding protein

In the Triton X-114 temperature-induced phase separation experiment, membrane proteins extracted from MDCK cells using Triton X-114 were subjected to temperature-induced phase separation. Proteins present in the aqueous phase, upon extensive dialysis in the presence of Amberlite XAD-2, were subjected to ultracentrifugation. Supernatant immunoprecipitated using anti-(canine TyrS-binding protein) antiserum and pellet solubilized in Laemmli's SDS sample buffer were subjected to SDS/PAGE, followed by the Western-blot analysis for the presence of the 175 kDa TyrS-binding protein. Samples shown on the autoradiograph taken from the Immobilon-P membrane following the analysis were: lane 1, immunoprecipitated sample; and lane 2, solubilized ultracentrifugation pellet. In the sodium carbonate buffer (pH 11) extraction experiment, MDCK membrane fraction was twice subjected to extraction using sodium carbonate buffer. The extracted fraction immunoprecipitated by antiserum against canine liver TyrS-binding protein, and the extracted membrane solubilized in Laemmli's SDS sample buffer were subjected to SDS/PAGE, followed by Western-blot analysis for the presence of the 175 kDa TyrS-binding protein. Samples shown on the autoradiograph taken from the Immobilon-P membrane after the analysis were: lane 3, immunoprecipitated sample; and lane 4, solubilized, extracted membrane sample.

phase, containing the hydrophilic peripheral membrane proteins, and the detergent phase, containing the amphiphilic integral membrane proteins (Bordier, 1981). In a preliminary experiment employing Triton X-114 temperature-induced phase separation technique, it was somewhat puzzling to find that the 175 kDa TyrS-binding protein was detected in the aqueous phase, but not the detergent phase (results not shown). However, it was then noted that the aqueous phase collected after the temperature-induced phase separation still contained approx. 0.7 mM Triton X-114, which is more than three times the critical micellar concentration (CMC) of the detergent. This concentration of Triton X-114 is still sufficient for solubilizing some integral membrane proteins. Indeed, during Triton X-114 temperature-induced phase separation, a number of integral membrane proteins had been reported to partition in the aqueous phase (Maher and Singer, 1985; Pryde, 1986). These proteins, however, formed precipitates upon exhaustive dialysis against the buffer solution without Triton X-114. Since Triton X-114 has a low CMC, a hydrophobic resin is normally included in the dialysis buffer to facilitate removal of the detergent (Pryde, 1986). To clarify the identity of the TyrS-binding protein as being either a peripheral membrane protein or an integral membrane protein, we attempted to remove the residual detergent from the above-mentioned aqueous-phase sample by dialysis against Triton X-114-deficient buffer containing a hydrophobic resin, Amberlite XAD-2. The results shown in Figure 3 revealed that the TyrS-binding protein was present in the pellet fraction (lane 2), but not

Figure 4 Western-blot analysis for the presence of the complex between the 175 kDa TyrS-binding protein and gp 80 in RIPA buffer lysate of MDCK cells with or without treatment with 20 mM sodium chlorate

In (a), samples shown on the autoradiograph taken from the Immobilon-P membrane after the Western-blot analysis were: lane 1, pre-immune serum-immunoprecipitated sample prepared from normal cell lysate; lane 2, anti-(canine liver TyrS-binding protein) antiserum-immunoprecipitated sample prepared from normal cell lysate; lane 3, anti-(gp 80) antiserum-immunoprecipitated sample prepared from normal cell lysate; lane 4, pre-immune serum-immunoprecipitated sample prepared from sodium chlorate-treated cell lysate; lane 5, anti-(canine liver TyrS-binding protein) antiserum-immunoprecipitated sample prepared from sodium chlorate-treated cell lysate; and lane 6, anti-(gp 80) antiserum-immunoprecipitated sample prepared from sodium chlorate-treated cell lysate. In (b), samples shown on the autoradiograph taken from the Immobilon-P membrane after the Western-blot analysis were: lane 1, anti-(gp 80) antiserum-immunoprecipitated sample prepared from normal cell lysate; and lane 2, anti-(gp 80) antiserum-immunoprecipitated sample prepared from sodium chlorate-treated cell lysate. Samples analysed were subjected to SDS/PAGE, electrotransferred to an Immobilon-P membrane, probed with anti-(canine liver TyrS-binding protein) antiserum [for samples in (a)] or anti-(gp 80) antiserum [for samples in (b)], and treated with ^{125}I -labelled protein A, followed by autoradiography.

the supernatant fraction (lane 1), when the aqueous-phase sample, upon exhaustive dialysis, was subjected to centrifugation. It is therefore clear that the 175 kDa TyrS-binding protein is an integral membrane protein.

Sodium carbonate buffer (pH 11) extraction

As a separate method for examining the integral membrane protein nature of the 175 kDa TyrS-binding protein, sodium carbonate buffer (pH 11) extraction of the membrane fraction prepared from MDCK cells was performed. As shown in Figure 3, high-pH sodium carbonate buffer failed to extract the TyrS-binding protein from the membrane (lane 3). Upon repeated extraction with 100 mM sodium carbonate buffer, the TyrS-binding protein still remained tightly bound to the membrane (lane 4). These results again clearly indicated that the 175 kDa TyrS-binding protein was an integral membrane protein.

Presence of the 175 kDa TyrS-binding protein in complex with gp 80, a major tyrosine-sulphated protein, in MDCK cell lysate

For the 175 kDa TyrS-binding protein identified to function as a putative TyrS receptor *in vivo*, a requirement would be the

capacity to bind and form complexes with tyrosine-sulphated proteins in MDCK cells. In an earlier study (Han et al., 1991), we have characterized the apically secreted gp 80 as a major tyrosine-sulphated protein produced by filter-grown MDCK cells. We decided to examine the presence of the complex formed between the 175 kDa TyrS-binding protein and gp 80 in MDCK cells using a Western-blot analysis procedure previously developed (M.-C. Liu et al., 1991; see the Materials and methods section). As shown in Figure 4(a), a major radioactive band corresponding to a 175 kDa protein species was observed in the electrophoretic lane corresponding to the sample immunoprecipitated using anti-(gp 80) antiserum (lane 3), indicating the co-precipitation of the 175 kDa protein with gp 80 immunoprecipitated from the RIPA buffer lysate of MDCK cells. The identity of this 175 kDa protein being the 175 kDa TyrS-binding protein identified in the earlier study was suggested by both its immunological cross-reactivity toward anti-(canine liver membrane-bound TyrS-binding protein) antiserum and an identical electrophoretic mobility to that immunoprecipitated by anti-(canine liver membrane-bound TyrS-binding protein) antiserum (lane 2). In addition to the 175 kDa TyrS-binding protein band, a minor radioactive band corresponding to a 110 kDa protein species was also observed. The identity of this 110 kDa protein is currently unknown. In view of its immunological cross-reactivity toward anti-(canine liver TyrS-binding protein) antiserum, the 110 kDa protein could be speculated to be either a non-glycosylated precursor or a proteolytic product of the 175 kDa TyrS-binding protein generated despite the considerable amount of the protease inhibitor added to the lysis buffer. As an attempt to clarify the former possibility, de-glycosylation of the 175 kDa TyrS-binding protein by treatment with *N*-glycanase, an enzyme known to be capable of hydrolysing all types of N-linked carbohydrate side chains of N-glycosylated protein (Hirani et al., 1987), was performed. Results shown in Figure 5 revealed that, under conditions that allowed complete hydrolysis, the de-glycosylated 175 kDa TyrS-binding protein still exhibited a molecular mass (approx. 155 kDa) considerably greater than the 110 kDa protein species. As described below, the 110 kDa protein species also displayed a distinct topological distribution in contrast with that of the 175 kDa TyrS-binding protein. It is worthwhile pointing out that the 110 kDa species was not detected in the TyrS-Sepharose-fractionated sample (cf. lane 4, Figure 1), indicating its lack of binding affinity toward TyrS. These results therefore suggested that the 110 kDa protein was distinct from the 175 kDa TyrS-binding protein.

Absence of the complex on treatment of MDCK cells with sodium chlorate, a metabolic sulphation inhibitor

Although the above-mentioned results indicated rather clearly the presence of the 175 kDa TyrS-binding protein in complex with gp 80 in MDCK cells, whether or not the TyrS-binding protein in fact bound the gp 80 molecule through its TyrS residue(s) still remained unclear. To clarify this, we decided to examine the effect of sodium chlorate, a metabolic sulphation inhibitor, on formation of a complex between the 175 kDa TyrS-binding protein and gp 80 in MDCK cells. It has been reported that sodium chlorate, at a concentration as high as 30 mM, exerts only a slight inhibitory effect on the growth of cultured cells (Baeuerle and Huttner, 1986; Humphries and Silbert, 1988). To examine the inhibitory effect of sodium chlorate on protein tyrosine sulphation, MDCK cells were doubly labelled with [^{35}S]sulphate and [^{35}S]methionine in the absence or presence of sodium chlorate. Based on the results obtained from the analysis of protein-bound [^{35}S]methionine and [^{35}S]TyrS, the average

Figure 5 Electrophoretic patterns of untreated and *N*-glycanase-treated [^{35}S]methionine-labelled 175 kDa TyrS-binding protein following SDS/PAGE

[^{35}S]Methionine-labelled 175 kDa TyrS-binding protein was prepared by TyrS–Sepharose fractionation of the sodium cholate extract of [^{35}S]methionine-labelled MDCK cells, and SDS/PAGE of the fractionated proteins followed by electroelution of the 175 kDa [^{35}S]methionine-labelled TyrS-binding protein for use in the *N*-glycanase treatment experiment. Untreated (lane 1) and *N*-glycanase-treated (lane 2) samples were subjected to SDS/PAGE, followed by autoradiography.

Table 1 Inhibition of tyrosine sulphation of proteins secreted by MDCK cells treated with sodium chlorate

Sodium chlorate (mM)	Protein-bound [^{35}S]methionine (c.p.m.)*	Protein-bound [^{35}S]TyrS (c.p.m.)*	Ratio of [^{35}S]TyrS to [^{35}S]methionine†	Inhibition of tyrosine sulphation (%)‡
0	2881 500	312 828	0.1086	0
5	2807 625	100 001	0.0356	67.2
20	2889 949	54 885	0.0190	82.5

*Data shown represent ^{35}S radioactivities associated with protein-bound [^{35}S]methionine or [^{35}S]TyrS present in the total volume (7.5 ml) of the labelling medium.

†Radioactivity ratio between protein-bound [^{35}S]TyrS and [^{35}S]methionine.

‡Values were calculated based on the percentage decrease in the radioactivity ratio between protein-bound [^{35}S]TyrS and [^{35}S]methionine.

percentage inhibition of protein tyrosine sulphation for the labelled proteins secreted into the medium was calculated to be 67.2% at a 5 mM concentration of sodium chlorate (Table 1). At a concentration of 20 mM sodium chlorate, the percentage inhibition value increased to 82.5%. Under these conditions, protein synthesis in MDCK cells appeared to be virtually unaffected, as indicated by the comparable radioactivities associated with [^{35}S]methionine-labelled proteins secreted by the untreated cells and the cells treated with sodium chlorate. Therefore, in cells maintained in medium containing 20 mM sodium chlorate, tyrosine sulphation of the newly synthesized proteins was dramatically suppressed. A cell lysate prepared from MDCK cells maintained in medium containing 20 mM sodium chlorate was similarly subjected to immunoprecipitation, followed by Western-blot analysis. It was found that under this condition, which suppressed protein tyrosine sulphation below 17.5% of the normal level, co-precipitation of the 175 kDa TyrS-binding protein with gp 80 immunoprecipitated by anti-(gp 80)

Figure 6 Cell-surface biotinylation for investigating an intracellular compared with a cell-surface location of the 175 kDa TyrS-binding protein in MDCK cells

MDCK cells were subjected to cell-surface biotinylation of proteins present on the cell membrane. After biotinylation, the membrane proteins were solubilized in a lysis buffer containing 1% (v/v) Triton X-100. The cell lysate was subjected to streptavidin–Sepharose fractionation. Streptavidin–Sepharose gel beads bound with biotinylated proteins were pelleted by centrifugation. Non-biotinylated proteins present in the supernatant were subjected to immunoprecipitation by antiserum raised against canine liver TyrS-binding protein. The streptavidin–Sepharose-fractionated and the immunoprecipitated samples were subjected to SDS/PAGE followed by the Western-blot analysis for the presence of the 175 kDa TyrS-binding protein. Samples shown on the autoradiograph taken from the Immobilon-P membrane after the analysis were: lane 1, immunoprecipitated sample; lane 2, streptavidin-fractionated sample; and lane 3, TyrS–Sepharose-fractionated TyrS-binding protein biotinylated under the same experimental conditions.

antiserum no longer occurred [Figure 4(a), lane 6]. Control experiments showed that both the 175 kDa TyrS-binding protein [Figure 4(a), lane 5] and gp 80 (migrating under reducing conditions as three characteristic bands at 45, 40 and 35 kDa positions) (Kondor-Koch et al., 1985; Han et al., 1991) as indicated by the arrowheads in Figure 4(b), (lane 2), were still synthesized at levels comparable with those in cells maintained in normal medium without sodium chlorate. These results therefore suggested that binding of the 175 kDa TyrS-binding protein to gp 80 occurred through its TyrS residue(s).

The predominantly, if not exclusively, intracellular location of the 175 kDa TyrS-binding protein

As part of an effort to clarify its functional involvement in the biosynthetic transport of newly synthesized tyrosine-sulphated proteins or the endocytic transport of exogenous tyrosine-sulphated proteins, we decided to investigate the intracellular versus cell-surface location of the 175 kDa TyrS-binding protein. Two different techniques, cell-surface biotinylation and cell-surface trypsinization, were employed in this study.

Cell-surface biotinylation

To investigate whether the TyrS-binding protein appears on the cell (plasma) membrane of MDCK cells, cell-surface biotinylation, followed by the fractionation of biotinylated cell-surface proteins using streptavidin–Sepharose and the subsequent Western-blot analysis for the presence of the TyrS-binding protein were carried out. As shown in Figure 6, the 175 kDa TyrS-binding protein was present in the non-biotinylated protein

Figure 7 Time course cell-surface trypsinization study for investigating intracellular compared with cell-surface location of the 175 kDa TyrS-binding protein in MDCK cells

Identically prepared dishes of MDCK cells were subjected to trypsinization for different lengths of time. Trypsinized cells were lysed in RIPA buffer. Cell lysates prepared, upon immunoprecipitation by antiserum raised against canine liver 175 kDa TyrS-binding protein, were subjected to SDS/PAGE followed by Western-blot analysis for the presence of the 175 kDa TyrS-binding protein. The lengths of time of trypsinization for the cell lysate samples shown on the autoradiograph taken from the Immobilon-P membrane after the Western-blot analysis were: lane 1, 0 min; lane 2, 3 min; lane 3, 30 min; lane 4, 60 min; and lane 5, 120 min.

fraction (lane 1), but not the biotinylated protein fraction (lane 2). A control experiment showed that the 175 kDa TyrS-binding protein fractionated from the sodium cholate extract of MDCK cells could be subjected to biotinylation under the same experimental conditions (Figure 6; lane 3). These results suggested absence from the cell surface and an intracellular membrane location for the TyrS-binding protein. However, in view of its somewhat low efficiency of biotinylation (cf. Figure 6, lane 3), the possibility of a small proportion (not detectable by the cell-surface-biotinylation technique) of the 175 kDa TyrS-binding protein being present on the cell surface still can not be excluded. It is interesting to note that the 110 kDa protein, previously found to exhibit cross-reactivity toward antiserum raised against canine liver TyrS-binding protein, was present in both the biotinylated and non-biotinylated protein fractions. These results suggested that, unlike the 175 kDa TyrS-binding protein, the 110 kDa protein appeared to be present in both the intracellular membrane and the cell membrane. The different topological locations for the two proteins also provided supporting evidence for the two being distinct protein species.

Cell-surface trypsinization

As a separate approach for investigating the intracellular versus cell-surface location of the 175 kDa TyrS-binding protein, a cell-surface-trypsinization technique was employed. In a time-course study performed, it was found that no proteolytic cleavage of the 175 kDa TyrS-binding protein was observed upon digestion of the intact MDCK cells with trypsin for as long as 2 h (Figure 7), although a control experiment showed that the TyrS-Sephrose-fractionated 175 kDa TyrS-binding protein was rapidly cleaved by trypsin to generate smaller fragments which were still

Figure 8 Radioactive pulse-chase study for investigating the presence of newly synthesized tyrosine-sulphated proteins in complexes with the 175 kDa TyrS-binding protein

In (a), identically prepared dishes of MDCK cells were pulse-labelled with [35 S]methionine and chased for different lengths of time in high-methionine ($10\times$) medium. At the end of each chase time period, the cells were lysed in RIPA buffer. Cell lysates prepared, upon immunoprecipitation by antiserum raised against canine liver TyrS-binding protein, 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 pre-immune serum control sample prepared at 25 min into the chase period. In (b), aliquots of unlabelled MDCK cell lysate were subjected to immunoprecipitation using pre-immune serum (lane 1), anti-(canine fibronectin) antiserum (lane 2), or anti-(canine liver TyrS-binding protein) antiserum (lane 3). Immunoprecipitated samples prepared were subjected to SDS/PAGE, electrotransferred to an Immobilon-P membrane, probed with anti-(canine fibronectin) antiserum, and treated with [125 I]-labelled protein A, followed by autoradiography. In (c), identically prepared dishes of MDCK cells were pulse-labelled with [35 S]sulphate and chased for different lengths of time in high-sulphate ($10\times$) 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-binding protein, 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.

recognizable by the antiserum against canine liver TyrS-binding protein (results not shown). In contrast, the band corresponding to the 110 kDa protein grew increasingly faint, with the concomitant appearance of a new band located at approx. 95 kDa (Figure 7), indicating that the 110 kDa protein was being digested by trypsin with time. These results again indicated the predominantly, if not exclusively, intracellular location of the TyrS-

binding protein and the cell-surface location of the 110 kDa protein in MDCK cells.

Presence of the 175 kDa TyrS-binding protein in association with newly synthesized, but not exogenous (endocytosed), tyrosine-sulphated proteins

To obtain evidence supporting the possible involvement of the 175 kDa TyrS-binding protein in the biosynthetic transport of newly synthesized tyrosine-sulphated proteins, radioactive pulse–chase experiments were performed, followed by immunoprecipitation using the antiserum against canine liver TyrS-binding protein. Results obtained showed that a high-molecular-mass [³⁵S]methionine-labelled protein (indicated by the upper arrowhead in Figure 8a) co-precipitated with the TyrS-binding protein immunoprecipitated from the cell lysate prepared approx. 15 min into the chase period. The identity of this high-molecular-mass protein as fibronectin, one of the major tyrosine-sulphated proteins produced by MDCK cells (Han et al., 1991), was indicated by both its immunological cross-reactivity toward anti-fibronectin antibodies (Figure 8b) and its disulphide-bonded dimeric subunit structure and the subunit molecular mass of approx. 220 000 (Yamada, 1983) (results not shown). The 175 kDa radioactive band indicated by the lower arrowhead in Figure 8(a) was that of the [³⁵S]methionine-labelled immunoprecipitated TyrS-binding protein. When [³⁵S]sulphate was used for the pulse labelling, [³⁵S]sulphate–fibronectin (indicated by the upper arrowhead in Figure 8c) was found to co-precipitate with the immunoprecipitated TyrS-binding protein as soon as the chase period began. Upon prolonged exposure of the autoradiograph, three fainter bands (located at 45, 40 and 35 kDa positions) characteristic of gp 80 (Kondor-Koch et al., 1985; Han et al. 1991), another tyrosine-sulphated protein produced by MDCK cells (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, therefore, clearly indicated that the newly synthesized tyrosine-sulphated proteins were present in complexes with the 175 kDa TyrS-binding protein. To test the possibility of the TyrS-binding protein functioning in the endocytic transport of exogenous tyrosine-sulphated proteins, purified [³⁵S]methionine-labelled gp 80 was used in an endocytosis experiment. Results obtained revealed that no [³⁵S]methionine-labelled gp 80 co-precipitated when the TyrS-binding protein was immunoprecipitated from the cell lysate prepared from MDCK cells incubated in medium containing [³⁵S]methionine-labelled gp 80 throughout a 2-h time course monitored (results not shown).

DISCUSSION

The present study represents part of an overall effort to elucidate the mechanism of the predominantly apical secretion of tyrosine-sulphated proteins produced by filter-grown MDCK cells (Han and Liu, 1991). In an earlier study, we discovered a bovine liver 175 kDa membrane-bound TyrS-binding protein (Lu et al., 1991). The subsequent finding that this TyrS-binding protein is present in association with several representative tyrosine-sulphated proteins in bovine liver membrane lysates (M.-C. Liu et al., 1991) and the detection of similar 175 kDa membrane-bound TyrS-binding proteins in bovine pancreas and canine liver and pancreas (Liu et al., 1988) strongly suggest the identity of the TyrS-binding protein as a putative 'TyrS receptor' involved in the intracellular transport of tyrosine-sulphated proteins. The question that then arises is: what purpose does a receptor-

mediated mechanism serve for the intracellular transport of tyrosine-sulphated proteins? Two possibilities exist with respect to the functional role of the putative TyrS receptor: one being in mediating the biosynthetic transport of newly synthesized tyrosine-sulphated proteins and the other being in mediating the endocytosis of exogenous tyrosine-sulphated proteins. In searching for clues to confirm the actual functional role of the putative TyrS receptor between these two possibilities, we have examined the list of tyrosine-sulphated proteins that have been identified (Huttner and Baeuerle, 1988). It was intriguing to find that the majority of them fall into the category of proteins being secreted in a polarized manner by different polarized cell types. To investigate further in this direction, we have carried out an investigation of the distribution, between the apical medium and the basolateral medium, of tyrosine-sulphated proteins produced and secreted by the polarized filter-grown MDCK cells. Results obtained indicated that tyrosine-sulphated proteins produced were predominantly secreted from their apical surface (Han and Liu, 1991). To follow up this finding, we decided to examine whether the intracellular transport and secretion of tyrosine-sulphated proteins in MDCK cells involves a receptor-mediated mechanism.

In the present study, we have detected, in a sodium cholate extract of MDCK cells, the presence of a 175 kDa TyrS-binding protein. The protein displayed binding affinity for TyrS, but not for unmodified tyrosine or tyrosine-*O*-phosphate, when these substances were bonded to Sepharose gel. Since both tyrosine and tyrosine-*O*-phosphate bonded (through their α -amino groups) to Sepharose gels, similar to TyrS bonded to Sepharose, are also negatively charged species, a non-specific charge-interaction effect for the binding of the TyrS-binding protein to TyrS–Sepharose gel seems unlikely. A pH-dependence study showed that the binding of the TyrS-binding protein to TyrS–Sepharose gel was strong from pH values of 8.0 down to 6.5 and started becoming dramatically weaker at pH 6.0 and below. Employing Triton X-114 temperature-induced phase separation (Bordier, 1981) and sodium carbonate buffer (pH 11) extraction (Howell and Palade, 1982) techniques, the TyrS-binding protein was unequivocally demonstrated to be an integral membrane protein. Of particular interest is the fact that the 175 kDa TyrS-binding protein identified exhibited immunological cross-reactivity toward the antiserum against canine liver 175 kDa membrane-bound TyrS-binding protein. Although definite proof is still awaited from the sequence data, preliminary results obtained from V8 mapping experiments strongly suggested that the 175 kDa TyrS-binding protein from MDCK cells is homologous to the canine liver 175 kDa TyrS-binding protein (results not shown). In clarifying the functional identity of the TyrS-binding protein, we have examined its presence in complexes with tyrosine-sulphated proteins in MDCK cells. Western-blot analysis revealed that the TyrS-binding protein was present in complex with gp 80, which is a major tyrosine-sulphated protein produced and secreted by MDCK cells predominantly from their apical surface (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Han et al., 1991). These results therefore clearly indicated that the TyrS-binding protein is a putative 'TyrS receptor' for tyrosine-sulphated proteins in MDCK cells. (The 175 kDa TyrS-binding protein will henceforth be called the 'putative TyrS receptor' in the following discussion.) In view of the fact that MDCK cells and the liver cells (hepatocytes) both have a polarized cellular organization, it would be interesting to investigate whether the similar putative TyrS receptors play a similar functional role in the polarized secretion of tyrosine-sulphated proteins in these two different polarized cell types. The finding of the 110 kDa protein species,

which co-precipitated during the immunoprecipitation of the putative TyrS receptor, appeared somewhat puzzling. Although this 110 kDa protein species also exhibited immunological cross-reactivity toward antiserum against canine liver membrane-bound TyrS-binding protein, affinity-gel fractionation using TyrS-Sepharose showed that it lacked the ligand-binding affinity for TyrS. Furthermore, results obtained from *N*-glycanase treatment and cell-surface biotinylation and cell-surface trypsinization studies indicated that the 110 kDa protein species was both structurally and topologically distinct from the putative TyrS receptor.

The sulphation inhibitor experiment was designed to clarify the nature of the binding between the putative TyrS receptor and gp 80. The absence of the complex between the two proteins in MDCK cells pre-incubated in medium containing a metabolic sulphation inhibitor, sodium chlorate (Baeuerle and Huttner, 1986; Humphries and Silbert, 1988), provided supporting evidence for the binding of the putative TyrS receptor to gp 80 through the TyrS residue(s) of the latter. It should, however, be pointed out that sodium chlorate had previously been shown to be an inhibitor of ATP sulphurylase, the first of the two enzymes involved in the sulphate activation reaction (Robbins and Lipmann, 1958). Treatment of cells with sodium chlorate would result therefore in a decreased production of 'active sulphate', 3'-phosphoadenosine 5'-phosphosulphate (PAPS), which serves as the sulphate donor in various sulphation reactions taking place in animal cells (Lipmann, 1958). Thus, treatment of the cells with sodium chlorate would result in inhibition of sulphation on both the tyrosine residues of proteins and the carbohydrate moieties of glycoproteins. Therefore, whether carbohydrate-bound sulphate influences in any way the binding of gp 80 to the putative TyrS receptor still remains to be clarified. Unfortunately, there are at present no sulphation inhibitors available which inhibit specifically the sulphation on tyrosine residues, but not on the carbohydrate moieties of proteins. Such compounds, if available, would probably have to exert their actions directly on tyrosylprotein sulphotransferase, the trans Golgi-located enzyme responsible for catalysing the protein tyrosine sulphation reaction (Baeuerle and Huttner, 1987).

Following the identification of the putative TyrS receptor, we then attempted to clarify whether its functional role was in the biosynthetic transport of newly synthesized tyrosine-sulphated proteins or in the endocytic transport of exogenous tyrosine-sulphated proteins. In clarifying this issue, two different approaches were taken. The first approach involved the examination of the intracellular versus cell-surface location of the putative TyrS receptor. The underlying assumption is that the putative TyrS receptor would have to be located on the cell surface if it were to function in the endocytosis of exogenous tyrosine-sulphated proteins. Conversely, if the putative TyrS receptor were not present on the cell surface, it would not function in the endocytosis of exogenous tyrosine-sulphated proteins. The second approach we took was to carry out the radioactive pulse-chase and endocytosis studies in an attempt to examine directly the identity of the tyrosine-sulphated proteins bound with the putative TyrS receptor as being either the newly synthesized ones or the endocytosed ones. Both the cell-surface biotinylation and cell-surface trypsinization techniques were employed to examine whether the putative TyrS receptor was located on the cell surface. As a low-molecular-mass compound, sulphy-NHS-biotin is readily diffusible and has been shown to be an excellent reagent for the selective labelling of cell-surface proteins (Sargiacomo et al., 1989). The NHS group carried on sulphy-NHS-biotin is reactive toward the primary amino group of either the N-terminal amino acid or lysine residues of proteins

to be labelled. The biotin moiety, on the other hand, offers excellent selectivity and sensitivity for detection through its tight binding ($K_d = 10^{-15}$ M) with avidin or streptavidin (Hurley et al., 1985; Ingalls et al., 1986). At pH values close to neutrality, sulphy-NHS-biotin carries a net negative electrical charge and it has been shown experimentally to be incapable of penetrating the cell membrane (Sargiacomo et al., 1989). To guard against the possibility that the putative TyrS receptor lacks accessible (extracellular-domain-located) amino group-containing residues (lysine residues and/or the N-terminal residue) needed for biotinylation, another cell-surface-modifying reagent, trypsin, was employed. Both the cell-surface biotinylation and the cell-surface trypsinization studies showed that the putative TyrS receptor was not affected by either of these two modifying reagents. These results implied the absence of the putative TyrS receptor on the cell membrane and its intracellular membrane location. Nevertheless, in view of the sensitivity limits of the two techniques used, the possibility of a small proportion of the putative TyrS receptor being present on the cell surface still can not be excluded. On the other hand, the radioactive pulse-chase study showed that newly synthesized, radiolabelled fibronectin and gp 80, two tyrosine-sulphated proteins known to be produced by MDCK cells (Kondor-Koch et al., 1985; Liu and Lipmann, 1985; Gottlieb et al., 1986; Han et al., 1991), were present in complexes with the putative TyrS receptor. This finding, together with the absence of the intracellularly located putative TyrS receptor in complex with [35 S]methionine-labelled gp 80 added in the incubation medium of MDCK cells during a 2-h time course and monitored in the endocytosis study, suggested that the putative TyrS receptor might be involved in the biosynthetic transport of newly synthesized tyrosine-sulphated proteins. An important point that needs to be addressed is related to the intracellular route traversed by the putative TyrS receptor. Since tyrosine sulphation of newly synthesized proteins occurs in trans Golgi (Baeuerle and Huttner, 1987) and the putative TyrS receptor appears predominantly, if not exclusively, inside the cell, it could be speculated that the putative TyrS receptor may mediate the transport of newly synthesized tyrosine-sulphated proteins from trans Golgi to a post-Golgi intracellular membrane compartment in which the two are uncoupled. The membrane vesicle carrying the putative TyrS receptor may then be recycled back to trans Golgi, whereas the dissociated newly synthesized tyrosine-sulphated proteins carried in a distinct membrane vesicle may proceed to the cell membrane and become secreted. Alternatively, the putative TyrS receptor may migrate with the bound tyrosine-sulphated proteins in transport membrane vesicles directly to the cell membrane where the dissociated tyrosine-sulphated proteins become secreted and the unbound putative TyrS receptor poses only a transient appearance before being recycled back to trans Golgi. Further studies employing, for example, immunogold electron microscopy and subcellular fractionation techniques are needed in order to obtain supporting evidence for these hypothetical models.

This research was supported in part by funds from the American Cancer Society (grant no. BC-705) and the American Heart Association, Oklahoma Affiliate (grant no. OK-90-G-6).

REFERENCES

- Baeuerle, P. A. and Huttner, W. B. (1986) *Biochem. Biophys. Res. Commun.* **141**, 870–877
- Baeuerle, P. A. and Huttner, W. B. (1987) *J. Cell Biol.* **105**, 2655–2664
- Bartles, J. R. and Hubbard, A. L. (1988) *Trends Biochem. Sci.* **13**, 181–184
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Caplan, M. J., Stow, J. L., Newman, A. P., Mardi, J., Anderson, H. C., Farquhar, M. G., Palade, G. E. and Jamieson, J. D. (1987) *Nature (London)* **329**, 632–635

- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Gilead, Z., Jeng, Y.-H., Wold, W. S. M., Sugawara, K., Rho, H. M., Harter, M. L. and Green, M. (1976) *Nature (London)* **264**, 263–266
- Gottlieb, T. A., Beaudry, G., Rizzolo, L., Colman, A., Rindler, M., Adesnik, M. and Sabatini, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2100–2104
- Han, J. R. and Liu, M. C. (1991) *Biochem. J.* **279**, 289–295
- Han, J. R., Suiko, M., Liu, C.-C. and Liu, M.-C. (1991) *Arch. Biochem. Biophys.* **286**, 337–345
- Hirani, S., Bernasconi, R. J. and Rasmussen, J. R. (1987) *Anal. Biochem.* **162**, 485–492
- Howell, K. E. and Palade, G. E. (1982) *J. Cell Biol.* **92**, 822–823
- Humphries, D. E. and Silbert, J. E. (1988) *Biochem. Biophys. Res. Commun.* **154**, 365–371
- Hurley, W. L., Finkelstein, E. and Holst, B. D. (1985) *J. Immunol. Methods* **85**, 195–202
- Huttner, W. B. and Baeuerle, P. A. (1988) in *Modern Cell Biology* (B. Satir, ed.), vol. 6, pp. 97–140, Alan R. Liss, New York
- Ingalls, H. M., Goodloe-Holland, C. M. and Luna, E. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4779–4783
- Jevons, F. R. (1963) *Biochem. J.* **89**, 621–624
- Kondor-Koch, C., Bravo, R., Fuller, S. D., Cutler, D. and Garoff, H. (1985) *Cell* **43**, 297–306
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lipmann, F. (1958) *Science* **128**, 575–580
- Lisanti, M. P., Le Bivic, A., Sargiacomo, M. and Rodriguez-Boulan, E. (1989) *J. Cell Biol.* **109**, 2117–2127
- Liu, J., Lu, R., Han, J. R. and Liu, M.-C. (1991) *Biochem. Int.* **25**, 109–121
- Liu, M.-C. and Lipmann, F. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3695–3698
- Liu, M.-C. and Lipmann, F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 34–37
- Liu, M.-C., Suiko, M. and Tang, X. (1988) *Biochem. Biophys. Res. Commun.* **156**, 964–969
- Liu, M.-C., Lu, R., Han, J. R., Tang, X., Suiko, M. and Liu, C.-C. (1991) *Biochem. J.* **275**, 259–262
- Lu, R., Tang, X., Han, J. R., Suiko, M. and Liu, M.-C. (1991) *Arch. Biochem. Biophys.* **286**, 481–487
- Maher, P. A. and Singer, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 958–962
- Pryde, J. G. (1986) *Trends Biochem. Sci.* **11**, 160–163
- Robbins, P. W. and Lipmann, F. (1958) *J. Biol. Chem.* **233**, 681–685
- Sargiacomo, M., Lisanti, M., Graeve, L., Le Bivic, A. L. and Rodriguez-Boulan, E. (1989) *J. Membr. Biol.* **107**, 277–286
- Simons, K. and Wandinger-Ness, A. (1990) *Cell* **62**, 207–210
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Yamada, K. M. (1983) *Annu. Rev. Biochem.* **52**, 761–789