Inhibition of Tyrosine Sulfation in the \textit{trans}-Golgi Retards the Transport of a Constitutively Secreted Protein to the Cell Surface

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Abstract. The effect of tyrosine sulfation on the transport of a constitutively secreted protein, yolk protein 2 (YP2) of \textit{Drosophila melanogaster}, to the cell surface was investigated after expression of YP2 in mouse fibroblasts. Inhibition of YP2 sulfation was achieved by two distinct approaches. First, the single site of sulfation in YP2, tyrosine 172, was changed to phenylalanine by oligonucleotide-directed mutagenesis. Second, L cell clones stably expressing YP2 were treated with chlorate, a reversible inhibitor of sulfation. Pulse-chase experiments with transfected L cell clones showed that the half-time of transport from the rough endoplasmic reticulum to the cell surface of the unsulfated mutant YP2 and the unsulfated wild-type YP2 produced in the presence of chlorate was 15–18 min slower than that of the sulfated wild-type YP2. Control experiments indicated (a) that the tyrosine to phenylalanine change itself did not affect YP2 transport, (b) that the retardation of YP2 transport by chlorate occurred only with sulfatable but not with unsulfatable YP2, (c) that the transport difference between wild-type and mutant YP2 was not due to the level of YP2 expression, and (d) that transport of the endogenous secretory protein fibronectin was the same in L cell clones expressing wild-type and mutant YP2. Since the half-time of transport of wild-type YP2 from the intracellular site of sulfation, the \textit{trans}-Golgi, to the cell surface was found to be 10 min, the 15–18-min retardation seen upon inhibition of tyrosine sulfation reflected a two- to threefold increase in the half-time of \textit{trans}-Golgi to cell surface transport, which was most probably caused by an increased residence time of unsulfated YP2 in the \textit{trans}-Golgi. The results demonstrate a role of tyrosine sulfation in the intracellular transport of a constitutively secreted protein.

Secretory proteins acquire a variety of posttranslational modifications during their synthesis in the rough endoplasmic reticulum (RER)\textsuperscript{1} and their subsequent transport from the RER via the Golgi complex to the cell surface. The best-characterized modification is the addition of carbohydrate to asparagine residues followed by the specific processing of the N-linked oligosaccharide (for review see Kornfeld and Kornfeld, 1985). The role of N-glycosylation in the intracellular transport of secretory proteins has been extensively investigated (see for example Lodish and Kong, 1984; Yeo et al., 1985; and references therein). Several cases have been reported in which inhibition of either N-glycosylation as such or the early processing steps of N-linked oligosaccharides were found to affect the kinetics of transport of secretory proteins from their site of synthesis in the RER to the Golgi complex (see Lodish and Kong, 1984, and references therein). One possible interpretation of these findings is that certain secretory proteins require N-linked oligosaccharides for correct folding which in turn is the major rate-limiting factor for exit from the RER. Once included in transport vesicles leaving the RER, secretory proteins are thought to move to and through the Golgi complex by bulk flow (see Wieland et al., 1987; for review see Rothman, 1987). Regulated secretory proteins destined to secretory storage granules are actively diverted from the bulk flow pathway at the level of the \textit{trans}-Golgi, whereas constitutive secretory proteins continue to move by bulk flow to the cell surface (for review see Burgess and Kelly, 1987).

The bulk flow concept does not exclude the modulation of the kinetics of secretory protein transport by posttranslational modifications occurring after exit from the RER. A protein modification that has frequently been found in secretory proteins and that occurs late in the secretory pathway is tyrosine sulfation (Huttner, 1987; Huttner and Baeuerle, 1988). Recent work has shown that protein tyrosine sulfation occurs specifically in the \textit{trans}-Golgi (Baeuerle and Huttner,
1987). We have previously hypothesized that tyrosine sulfation may affect the intracellular transport of certain secretory proteins (Huttner et al., 1986).

Several of our previous studies have been concerned with establishing a model system that allows us to test this hypothesis for a constitutive secretory protein by site-directed mutagenesis of a tyrosine sulfation site. Yolk protein 2 (YP2) of Drosophila melanogaster, the gene of which has been cloned (Hovemann et al., 1981), was found to have all the desired features of a suitable model protein. (a) YP2 is sulfated only on tyrosine residues and does not carry additional sulfate on carbohydrate (Baeuerle and Huttner, 1985). (b) The stoichiometry of tyrosine sulfation of YP2 is close to 1 mol sulfate per mole polypeptide (Baeuerle and Huttner, 1985). (c) The sulfite is linked to the same tyrosine residue in all YP2 molecules, which has been identified as tyrosine 172 (Baeuerle et al., 1988). (d) The stoichiometric sulfation of tyrosine 172 is conserved after expression of YP2 in mammalian cells suitable for transport studies (Friederich et al., 1988). We were therefore in a position to investigate a possible role of tyrosine sulfation in the intracellular transport of YP2 by mutagenesis of tyrosine 172. In addition, to develop an alternative to the mutagenesis approach, we have searched for an inhibitor of sulfation that could be applied to intact cells and have found that the sulfite analogue chlorate is a potent sulfation inhibitor (Baeuerle and Huttner, 1986). Here we show that inhibition of tyrosine sulfation of YP2 by either mutagenesis or chlorate treatment leads to a two- to threefold retardation of YP2 transport from the trans-Golgi to the cell surface, which most likely reflects a marked increase in the residence time of this protein in the trans-Golgi.

Materials and Methods

Isotopes

[^35]Sulfate (carrier free) and L-[35]Smethionine (50 TBq/mmol) were purchased from Amersham Buchler, Braunschweig. FRG. L-[2,3,5,6-3H]tyrosine (~3 TBq/mmol) was obtained from New England Nuclear (Cambridge, MA).

Oligonucleotide-directed In Vitro Mutagenesis

To mutagenize the codon TAC at positions 633-635 of the YP2 gene (Hung and Wensink, 1983), coding for tyrosine 172 of pre-YP2, to TTC, coding for phenylalanine 172, we used a mutagenic 17-mer oligonucleotide with the sequence 5'-d(pCGTTGGAGAAGTCGGTG)-3'. The oligonucleotide was produced by a synthesizer (model 380 A; Applied Biosystems, Inc., Foster City, CA) using the phosphoramidite method, purified on PAGE, and phosphorylated (Maniatis et al., 1982). The phosphorylated oligonucleotide was separated from the nonphosphorylated form by PAGE (Maniatis et al., 1982). Wild-type YP2 DNA to be mutagenized was inserted into M13mp9 RF-DNA as follows. After digestion of pSP64-YFP2 (Friederich et al., 1988) with Pir I, a ~1 kb fragment of the YP2 gene containing the codon TAC coding for tyrosine 172 was isolated on a low melting agarose gel (Maniatis et al., 1982). The purified YFP2 gene fragment was ligated with T4 DNA ligase into Pir I-digested, dephosphorylated (calf intestinal phosphatase; Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) M13mp9 RF-DNA. The resulting M13mp9-YFP2 recombinant DNA was used to perform the oligonucleotide-directed mutagenesis using the gapped duplex technique described in detail by Kramer et al. (1984). M13mp9-YFP2mut clones, containing the mutated codon, were identified by sequencing using previously described methods (Benedum et al., 1986). One such clone, M13mp9-P-YFP2mut-13, was used for the construction of pSV2-YFP2mut by using the ~500 bp Ava I fragment of the YP2 gene containing the mutagenized codon. Sequencing of this Ava I fragment revealed no other mutations than the specific A to T change produced by oligonucleotide-directed mutagenesis.

Construction of pSV2-YFP2mut

M13mp9-YFP2mut-13 RF-DNA was digested with Ava I, and a ~500-bp fragment of the YP2 gene containing the mutagenized codon was isolated on a low melting agarose gel. The corresponding ~500-bp fragment of the wild-type YP2 gene was cut out by Ava I digestion from the previously described pSV2-YFP2 recombinant DNA (Friederich et al., 1988), referred to in this study as pSV2-YFP2wt. The digested DNA was dephosphorylated, and the large pSV2-YFP2wt DNA fragment lacking the ~500-bp Ava I fragment was isolated on a low melting agarose gel. This fragment was ligated with the ~500-bp Ava I fragment from the M13mp9-YFP2mut-13 RF-DNA with T4 DNA ligase, yielding pSV2-YFP2mut. The orientation of the inser was determined by Bgl II and Cla I digestion.

Antisera

The rabbit antiserum against yolk proteins of Drosophila melanogaster was the same as previously described (Friederich et al., 1988). Antiserum against human fibronectin was a kind gift of Dr. R. Timpl. This antiserum was found to immunoprecipitate mouse fibronectin.

Purification of Yolk Proteins from Drosophila Melanogaster Flies

Unlabeled yolk proteins were purified from Drosophila flies as previously described (Baeuerle et al., 1988; fraction D).

Cell Culture

Unless indicated otherwise, all cells were grown in DME supplemented with 10% FCS at 37°C in an atmosphere containing 10% CO2. All labeling experiments described below were performed at 37°C in an atmosphere containing 10% CO2.

Transient and Stable Expression of YP2 in L Cells

For transient expression of wild-type (tyrosine 172) and mutant (phenylalanine 172) YP2 in Ltk- cells, cells were grown on 6-cm dishes to subconfluence. Transfection of Ltk- cells with pSV2-YFP2wt or pSV2-YFP2mut DNA was carried out as described by Sussman and Milman (1984). Dishes received 3 ml of culture medium containing 100 ~g/ml of DEAE dextran (Sigma Chemical Co., St. Louis, MO) and 6~g of either pSV2-YFP2wt or pSV2-YFP2mut DNA. After 27 h, cells were used for labeling as described below.

The L cell clone stably expressing wild-type YP2 used in most experiments was the clone L-YFP2-3 described previously (Friederich et al., 1988), obtained by transfection of Ltk- cells with pSV2-YFP2wt DNA and referred to here as L-YFP2wt-3. In addition, another L cell clone isolated in the previous study (Friederich et al., 1988) was used, clone 9 which is referred to here as L-YFP2wt-9. L cell clones stably expressing mutant YP2 were obtained as described previously for the isolation of L cell clones expressing wild-type YP2 (Friederich et al., 1988), using the Ca-phosphate DNA precipitation method (Matthias et al., 1983). Each of three 10-cm dishes of Ltk- cells, grown to subconfluence, received 2 ~g of pSV2-YFP2mut DNA and 100 ~g of pFGS5 DNA carrying the herpes simplex thymidine kinae gene which allowed selection of transfected cells. 20 aminopterine-resistant colonies were picked, transferred to 3.5-cm dishes, and the cells were cultured as described previously (Friederich et al., 1988) before they were used in labeling experiments. The L cell clones stably expressing mutant YP2 used in the experiments were clones L-YFP2mut-4 and L-YFP2mut-14.

Pulse–Chase Experiments with Radiolabeled Amino Acids

Pulse–chase experiments comparing dishes of L-YFP2wt and L-YFP2mut cells or L-YFP2 cells in the absence and presence of chlorate were always performed in parallel on the same day.

Experiments without Chlorate. Pretreatment: Confluent 3.5-, 6-, or 10-cm dishes of L-YFP2wt or L-YFP2mut cells were used for labeling. Cells were washed twice with PBS at 37°C and preincubated for 2 h in DME lacking either methionine or tyrosine (for subsequent labeling with either [35]Smethionine or [3H]tyrosine, respectively) and supplemented with 1% FCS which had previously been dialyzed against 10 mM Hepes, pH 7.3, and 150 mM NaCl.

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Pulse labeling: After preincubation, the medium was removed and replaced by the same medium (0.5, 1, and 2.2 ml for 3.5-, 6-, and 10-cm dishes, respectively) but containing [35S]methionine (26–37 MBq/ml) or [3H]tyrosine (14.8-18.5 MBq/ml) which had been preequilibrated at 37°C under a 10% CO2 atmosphere. The cells were incubated for 15 min on a slowly rocking platform.

Chase: The "pulse" medium was removed and the cells were washed, in less than 30 s, twice with PBS at 20°C, followed by addition of pre-equilibrated "chase" medium (0.5, 1, and 2.2 ml for 3.5-, 6-, and 10-cm dishes, respectively). The chase medium consisted of DME containing an excess of either methionine (10-fold DME concentration) or tyrosine (three-fold DME concentration) and supplemented with 1% FCS and 23 μg/ml aprotinin. The cells were incubated on a slowly rocking platform. In most experiments, the chase medium was collected and replaced with fresh chase medium after 10, 20, 30, 40, 50, 60, 90, and 120 min. After 180 min of chase, the chase medium was collected and the cells were washed with ice-cold PBS and lysed in lysis buffer I as described below. In some experiments, the chase medium was collected and replaced with fresh chase medium after 20 min, and the chase was terminated by collection of the chase medium and lysis of cells after 90 min in lysis buffer I. Chase media and cell lysates were processed for immunoprecipitation or YP2 of fibronectin as described below.

For the determination of the recovery of amino acid-labeled YP2 over the chase period, duplicate dishes of L-YP2wt-3 cells and L-YP2mut-4 cells were preincubated and pulse-labeled with [3H]tyrosine as described above. Cells of one dish were lysed at the end of the pulse in lysis buffer II as described below. Cells of the other dish were chased for 180 min, followed by collection of the chase medium, and lysis of cells in lysis buffer II. Medium and cell lysates were processed for immunoprecipitation of YP2 as described below. To determine the recovery of amino acid-labeled fibronectin over the chase period, cells on duplicate dishes which had been pulse-labeled as those subjected to the 180-min chase were lysed at the end of the pulse in lysis buffer I, followed by immunoprecipitation of fibronectin as described below.

Experiments with Chlorate. Pretreatment: Confluent 6- or 10-cm dishes of L-YP2wt or L-YP2mut cells were preincubated for 3 h in tyrosine-free DME (for subsequent labeling with [3H]tyrosine), supplemented with 1% diazoyl FCS and containing reduced methionine and cysteine concentrations (1% of normal DME concentration) and either normal DME concentration of sulfate and no chlorate (chlorate-free medium) or no sulfate and 5 mM freshly made sodium chlorate (chlorate-containing medium).

Pulse labeling: After preincubation, the medium was removed and replaced by the same medium (1 ml) but which contained [35S]methionine (3.7 MBq/ml), [3H]tyrosine (14.8 MBq/ml), or [35S]sulfate (37 MBq/ml). The cells were incubated for 2 h on a slowly rocking platform. Chase media and cell lysates were processed for immunoprecipitation of YP2 as described below.

Long-term Labeling with Radiolabeled Amino Acids or [35S]Sulfate

Pretreatment: Pretreatment of confluent 6- or 10-cm dishes of L cells for subsequent labeling with either [35S]methionine, [3H]tyrosine, or [35S]sulfate was performed as described above in Pulse-Chase Experiments with Radiolabeled Amino Acids, Experiments without Chlorate and Pulse-Chase Experiments with [35S]Sulfate. Pretreatment for subsequent labeling with [3H]tyrosine in the presence of chlorate was performed as described for Pulse–Chase Experiments with radiolabeled Amino Acids, Experiments with Chlorate.

Labeling: After preincubation, the medium was removed and the cells received the same medium (2 and 6 ml for 6- and 10-cm dishes, respectively) but it contained [35S]methionine (3.7 MBq/ml), [3H]tyrosine (14.8 MBq/ml), or [35S]sulfate (37 MBq/ml). This was followed by incubation for 20–24 h. After labeling, dishes were placed on ice, the medium was collected, and the cells were lysed in lysis buffer III. Chase media and cell lysates were processed for immunoprecipitation of YP2 as described below.

Reversibility of Inhibition of Sulfation by Chlorate

Pretreatment: Confluent 6-cm dishes of L-YP2wt cells were washed twice with PBS at 37°C and then preincubated for 3 h in the absence or presence of 5 mM sodium chlorate in DME containing reduced methionine and cysteine concentrations (1% of normal DME concentrations), lacking sulfate, and supplemented with 1% diazoyl FCS. Two dishes, preincubated in the absence or presence of chlorate, were directly labeled as described below. Two other dishes, preincubated in the absence or presence of chlorate, were subjected to "chlorate wash-out" before being labeled: they were washed twice with PBS at 37°C, incubated for 30 min in DME containing reduced methionine and cysteine concentrations, lacking sulfate, and supplemented with 1% diazoyl FCS, and then incubated for 2 h in DME containing reduced methionine and cysteine concentrations, lacking sulfate, and supplemented with 1% diazoyl FCS.

Labeling: After the various pretreatments, the medium was removed and replaced by the same medium (1 ml) but which contained [35S]sulfate (26 MBq/ml). The cells were incubated for 2 h on a slowly rocking platform. After labeling, dishes were placed on ice, the medium was collected, and the cells were lysed in lysis buffer I. Chase media and cell lysates were processed for immunoprecipitation of YP2 as described below.

Immunoprecipitations

Unless otherwise indicated, all steps were performed at 4°C.

Preparation of Cell Lysates. In pulse–chase experiments and in the chlorate reversibility experiment, after collection of medium and washing of cells, dishes received lysis buffer I (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, pH 7.0, 1% NP-40, 23 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]; 0.4, 1, and 2 ml for 3.5-, 5-, and 10-cm dishes, respectively). The cells were scrapped off the dish and the suspensions were passed three times through a 25-gauge needle, followed by a 15-min incubation on ice. The cell lysates were centrifuged for 10 min at 15,000 g. Aliquots of the supernatants were diluted to 2 and 0.7 ml with lysis buffer I and adjusted to 0.1% SDS before immunoprecipitation of YP2 and fibronectin, respectively. In the pulse–chase experiments in which the recovery of YP2 was determined, dishes received PBS lacking calcium and magnesium and containing 0.03% trypsin and 0.02% EDTA (1 ml for a 3.5-cm dish). After 10 min on ice, this solution was removed and the cells were detached from the dish with ice-cold normal culture medium (7 ml) containing 1 mM of tosyl-l-lysine chloromethyl ketone (TLCK) and pelleted by centrifugation for 5 min at 100 g. Boiling lysis buffer II (0.5 M NaCl, 1% SDS, 1 mM PMSF; 200 μl for 3.5-cm dishes) was added to the pelleted cells which were immediately boiled for 5 min. The cell lysate was diluted to 2 ml with buffer I containing 1 mM TLCK, 0.1% α-macroglobulin, and sonicated for 30 s. The cell lysate was centrifuged for 10 min at 15,000 g and the supernatant was used for immunoprecipitation. In the long-term labeling experiment shown in Fig. 1 top, dishes received lysis buffer III (0.5 M NaCl, 2% SDS, 1 mM PMSF; 0.4 ml for 6-cm dishes). The cell lysate was boiled for 5 min and centrifuged as above. The supernatant was diluted to 13 ml with lysis buffer I and used for immunoprecipitation.

Processing of the Media. Immediately after collection, all media were cleared by centrifugation for 5 min at 100 g. Aliquots of the cleared media were adjusted to contain the ingredients of buffer I plus 0.1% SDS and brought to a volume of 2 and 0.7 ml for immunoprecipitation of YP2 and fibronectin, respectively. In the pulse–chase experiments in which the

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recovery of YP2 was determined, cleared media were brought to a volume of 2 ml, and adjusted to contain the ingredients of buffer I containing 1 mM TLCK, 0.1% α-macroglobulin, and 0.1% SDS, before immunoprecipitation. In the long-term labeling experiment shown in Fig. 1, top, cleared media (2 ml) were diluted to 13 ml with buffer I plus 0.1% SDS, before immunoprecipitation.

**Immunoprecipitation.** Each 2 ml sample of cell lysate and cleared medium received 10 μl of yolk protein antiserum (Friederich et al., 1988). Each 0.7 ml sample of cell lysate and cleared medium received 1 μl of fibronectin antiserum. After 1-2 h of incubation, 50 (2-ml sample) or 10 μl (0.7-ml sample) of a suspension of fixed *Staphylococcus aureus* cells, preincubated in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), pH 7.5, 150 mM NaCl), was added and samples were incubated overnight on a rotating wheel. The adsorbed immunocomplexes were pelleted by centrifugation for 2 min at 15,000 g. The pellets were washed once in high salt buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA, pH 7.0, 1% NP-40), once in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and once in TBS. These conditions had previously been shown to give quantitative immunoprecipitation of YP2 and fibronectin. For SDS-PAGE, these washed pellets were resuspended in Laemmli sample buffer containing 2-mercaptoethanol (Laemmli, 1970), boiled for 5 min, and loaded as suspension. For two-dimensional PAGE, the washed pellets were resuspended in O'Farrell lysis buffer (O'Farrell, 1975) containing either nonequilibrium pH gradient electrophoresis (NEPHGE) or IEF ampholytes (see below), centrifuged for 2 min at 15,000 g at 20°C, and the supernatants were loaded. Each 13-ml sample of cell lysate and cleared medium received 20 μl yolk protein antiserum. Immunocomplexes were adsorbed to protein A-Sepharose and loaded as suspensions in Laemmli sample buffer for SDS-PAGE, as previously described (Friederich et al., 1988).

### SDS-PAGE and Two-dimensional PAGE

SDS-PAGE under reducing conditions was performed according to Laemmli (Laemmli, 1970). For two-dimensional PAGE either NEPHGE (O'Farrell et al., 1977), using 1% (wt/vol) pH 3.5-9.5 (LKB, Bromma, Sweden), or IEF (O'Farrell, 1975), using 1% (wt/vol) pH 3.5-9.5, 0.5% (wt/vol) pH 5-7, and 0.5% (wt/vol) pH 7-9 ampholytes, was used as the first dimension. For quantitative analysis of pulse-chase experiments, immunoprecipitated [35S]methionine or [3H]tyrosine-labeled wild-type YP2 and mutant YP2 was subjected to a modified two-dimensional PAGE, referred to as "multi-sample 2D-PAGE." The immunoprecipitates in O'Farrell lysis buffer were mixed with an amount of unlabeled yolk proteins purified from flies which was sufficient to be detected as marker by the subsequent Coomassie Blue staining. The immunoprecipitates were then subjected to IEF or NEPHGE in the first dimension. After electrophoresis, a 4-cm-long gel piece of the tube gel, which by experience was known to contain YP2, was cut. To obtain an optimal and reproducible recovery of YP2, the cut gel pieces were directly placed on top of a 30-cm-wide SDS-polyacrylamide slab gel and embedded in 1% agarose in Laemmli sample buffer. Up to five tube gel pieces were loaded on one SDS-polyacrylamide slab gel. Proteins were then subjected to SDS-PAGE in the second dimension. Gels were fixed, stained, and destained. At this point, the correct cutting of the IEF and NEPHGE gels was verified by the presence of the unlabeled yolk protein marker. A horizontal strip comprising the whole width of the gel and containing the multiple yolk protein spots was then cut, treated with sodium salicylate, and fluorographed as previously described (Lee and Huttner, 1983). Two such multisample 2D-gel strips were used to analyze YP2 from the nine chase media and the cell lysate that were obtained from one dish in a typical pulse-chase experiment, and were exposed on one x-ray film.

### Quantitative Analyses of Radiolabeled Proteins

For the determination of [35S]methionine, [3H]tyrosine, and [35S]sulfate incorporation into YP2 and fibronectin, dried gel pieces containing these labeled proteins were incubated for 30 min in 10% acetic acid, 30% methanol, washed in water for 1 h, lyophilized, and incubated with shaking for 24 h at 37°C in 0.8 ml of 30 mM NH4HCO3 containing 20 μg pronase. After addition of 10 ml of scintillation fluid (Aquaphase; Baker Co., Frankfurt, FRG) and further shaking for 2 h, the radioactivity was determined by liquid scintillation counting. For the determination of the stoichiometry of tyrosine sulfation of YP2, [3H]tyrosine-labeled YP2 contained in dried gel pieces was subjected to pronase elution and alkaline hydrolysis, followed by separation of unmodified [3H]tyrosine and [3H]tyrosine sulfate by two-dimensional thin layer electrophoresis/ascending chromatography, as described previously (Baeuerle and Huttner, 1985), using methanol/pyridine/water (80:4:20) in the chromatography.

### Calculations of Secretion Half-Times

The half-times of secretion (ts0) were determined from a semilog plot of the percentage of protein (YP2 or fibronectin) remaining with the cell (100% minus percentage of secreted protein) as a function of time. Regression analysis showed that the ts0 was determined with a precision of 2-4 min in the case of amino acid–labeled protein and 0.6 min in the case of [35S]sulfate-labeled protein.

### Results

**Oligonucleotide-directed Mutagenesis of Tyrosine 172, the Sulfation Site of YP2, to Phenylalanine**

The first approach to inhibit tyrosine sulfation of *Drosophila* YP2 involved oligonucleotide-directed mutagenesis. As described in Materials and Methods, the wild-type *Drosophila* YP2 gene was selectively mutagenized in the codon TAC, coding for tyrosine 172 that is the sulfation site of YP2 in vivo (Baeuerle et al., 1988), to TGT which codes for phenylalanine. To express wild-type (wt) and mutant (mut) YP2 in mouse fibroblasts for subsequent studies on its intracellular transport, the recombinant DNA constructs pSV2-YP2wt and pSV2-YP2mut, respectively, were used. The pSV2-YP2wt construct has been described previously (Friederich et al., 1988). This construct is based on the eucaryotic expression vector pSV2 (Garoff et al., 1983) and contains the wild-type YP2 genomic DNA lacking its promoter under the control of the early SV-40 promoter. The pSV2-YP2mut construct was obtained by replacing a fragment of the pSV2-YP2wt construct containing the normal codon TAC with the corresponding fragment of the mutated YP2 genomic DNA containing the codon TGC (see Materials and Methods for details). The pSV2-YP2wt and pSV2-YP2mut constructs were identical to each other except for the single base pair difference. The pSV2-YP2wt and pSV2-YP2mut constructs were used for the transient transfection of mouse fibroblasts (Ltk- cells) and for the generation of mouse fibroblast (L cell) clones stably expressing wild-type and mutant YP2, respectively (see Materials and Methods for details). The clones used for most of the studies described below are referred to as L-YP2wt-3 and L-YP2mut-4.

**Mutant Drosophila YP2 Produced by Mouse Fibroblasts Is Unsulfated**

It was important to determine whether YP2 rendered unsulfatable in position 172 was indeed produced in unsulfatable form or whether a compensatory sulfation of other tyrosine residues occurred. For this purpose, mouse fibroblasts (L cells) were transiently transfected with pSV2-YP2wt or pSV2-YP2mut DNA and labeled with [35S]sulfate or [35S]methionine. Wild-type and mutant YP2 was immunoprecipitated from the medium and cell lysate and analyzed by SDS-PAGE followed by fluorography (Fig. 1, top). Cells transfected with pSV2-YP2wt DNA produced and secreted wild-type YP2 in sulfated form (Fig. 1, top, left). We have shown previously that sulfation of wild-type YP2 in L cells occurs on tyrosine 172 (Friederich et al., 1988). In contrast, cells transfected with pSV2-YP2mut DNA produced and...
secreted mutant YP2 in completely unsulfated form, as indicated by the absence of any $[^{35}\text{S}]$sulfate incorporation (Fig. 1 top, right). The lack of tyrosine sulfation of mutant YP2 was confirmed when $[^{3}\text{H}]$tyrosine-labeled mutant YP2 secreted by the L cell clone L-YP2mut-4 was used to determine the stoichiometry of sulfation (Table I). Quantitation of the amount of unmodified $[^{3}\text{H}]$tyrosine and of $[^{3}\text{H}]$tyrosine sulfate in an alkaline hydrolyzate of mutant YP2 showed that none of its 16 tyrosine residues were sulfated (Table I, mutant YP2). Parallel analysis of $[^{3}\text{H}]$tyrosine-labeled wild-type YP2 secreted by the L cell clone L-YP2wt-3 revealed that 4.4% of its 17 tyrosine residues were recovered in sulfated form, which corresponded to 0.75 mol tyrosine sulfate per mole polypeptide (Table I, wild-type YP2), a value very similar to the previously reported value of 0.83 mol per mole (Friederich et al., 1988) and indicative of sulfation of most YP2 molecules produced by L-YP2wt-3 cells. The lack of tyrosine sulfation of mutant YP2 was not due to a general inhibition of the cellular tyrosine sulfation machinery since fibronectin, an endogenous fibroblast protein known to undergo tyrosine sulfation (Paul and Hynes, 1984; Liu and Lipmann, 1985), which was coprecipitated with YP2 due to its affinity for protein A, was sulfated (Fig. 1 top, arrowhead). Lack of sulfate incorporation into mutant YP2 and sulfation of endogenous fibronectin were also observed when individual L cell clones stably expressing mutant YP2 were analyzed (data not shown). Thus, mutagenesis of tyrosine 172 caused the specific inhibition of sulfation of YP2.

**Tyrosine Sulfation of Wild-type Drosophila YP2 Produced by Mouse Fibroblasts Can Be Reversibly Inhibited by Chlorate**

In the case of mutant YP2, the lack of sulfation resulted from a structural change in the protein. We wanted to use a second, distinct approach to inhibit tyrosine sulfation of YP2. We therefore exploited the previous observation that chlorate is a potent, nontoxic inhibitor of sulfation in intact cells

172 to phenylalanine. Wild-type YP2: L cells transiently transfected with pSV2-YP2wt; mutant YP2: L cells transiently transfected with pSV2-YP2mut. L cells were labeled for 22 h with $[^{35}\text{S}]$sulfate or $[^{35}\text{S}]$methionine. Wild-type and mutant YP2 were immunoprecipitated from the cell lysate (C) and culture medium (M) and analyzed by SDS-PAGE followed by fluorography. The amino acid sequences of the wild-type and the mutated tyrosine sulfation site of YP2 are shown in the boxes above the fluorogram. (Bottom) Reversible inhibition of tyrosine sulfation of wild-type YP2 by chlorate treatment of cells. Four dishes with cells of clone L-YP2wt-3, stably expressing wild-type YP2, were preincubated for 3 h in the absence (−) or presence (+) of 5 mM chlorate. Two dishes were then labeled for 2 h with $[^{35}\text{S}]$sulfate in the continued absence or presence of chlorate. The other two dishes were subjected to chlorate wash-out (see Materials and Methods), followed by $[^{35}\text{S}]$sulfate labeling for 2 h in the absence of chlorate. YP2 was immunoprecipitated from the cell lysate (C) and culture medium (M) and analyzed by SDS-PAGE followed by autoradiography. The treatment of cells and the resulting effect on the sulfation of tyrosine 172 are summarized above the autoradiogram. (Top and bottom) The positions of YP2 and fibronectin (Fn; present in the YP2 immunoprecipitates due to its affinity for protein A) are indicated by arrows and the arrowhead, respectively. Molecular mass standards are given in kilodaltons.

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**Figure 1.** Inhibition of tyrosine sulfation of *Drosophila* YP2 by either site-specific mutagenesis or chlorate. (Top) Abolishment of tyrosine sulfation of *Drosophila* YP2 by mutagenesis of tyrosine

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Table I. Stoichiometry of Tyrosine Sulfation of Wild-type YP2, Mutant YP2, and Wild-type YP2 from Chlorate-treated Cells

| Protein                     | \[^{3}H\]Tyrosine | \[^{3}H\]Tyrosine sulfate | \[^{3}H\]Tyrosine sulfate per total \[^{3}H\]tyrosine | Number of tyrosine sulfate residues per polypeptide | Tyrosine sulfate per polypeptide |
|-----------------------------|-------------------|---------------------------|------------------------------------------------------|--------------------------------------------------|---------------------------------|
| Wild-type YP2               | 9,827             | 448                       | 4.4                                                  | 17                                               | 0.75                            |
| Mutant YP2                  | 10,280            | 0                         | 0                                                    | 16                                               | 0                               |
| Wild-type YP2 + chlorate    | 11,529            | 31                        | 0.27                                                 | 17                                               | 0.05                            |

\[^{3}H\]Tyrosine-labeled wild-type YP2, mutant YP2, and wild-type YP2 produced by chlorate-treated cells, contained in two-dimensional gels, were obtained as described in the legend to Fig. 2. The YP2 spots were cut out and subjected to pronase elution followed by alkaline hydrolysis. \[^{3}H\]Tyrosine and \[^{3}H\]tyrosine sulfate were determined after separation by two-dimensional cellulose thin-layer electrophoresis/ascending chromatography. The \[^{3}H\]tyrosine values shown are the original counted values multiplied by a factor of 1.2 to account for the slightly lower recovery of \[^{3}H\]tyrosine standard than \[^{3}H\]tyrosine sulfate standard. The \[^{3}H\]tyrosine and \[^{3}H\]tyrosine sulfate values were used to calculate the percentage of \[^{3}H\]tyrosine sulfate per total \[^{3}H\]tyrosine (sum of \[^{3}H\]tyrosine plus \[^{3}H\]tyrosine sulfate), which corresponded to 0.05 mol of tyrosine sulfate per mole polypeptide and thus indicated 95% inhibition of tyrosine sulfation (Table I, wild type YP2 + chlorate). In agreement with the previous observation that chlorate does not inhibit protein synthesis in cell cultures (Baeuerle and Huttner, 1986), the inhibition of tyrosine sulfation of YP2 was not caused indirectly by an inhibition of YP2 synthesis since almost identical amounts of \[^{3}H\]tyrosine-labeled YP2 were produced in the absence as well as the presence of chlorate (data not shown). The inhibitory effect of chlorate on the tyrosine sulfation of YP2 was completely reversed by chlorate wash-out before \[^{3}S\]sulfate-labeling (Fig. 1 bottom, right). It is worth noting that after chlorate wash-out, the proportion of YP2 in the cells and medium after the 2-h sulfation labeling was virtually identical to that found without chlorate. Thus, chlorate was not only a reversible inhibitor of tyrosine sulfation of wild-type YP2 but also did not appear to damage the secretory machinery of L cells.

Lack of the Sulfate in Mutant YP2 and Wild-type YP2 from Chlorate-treated Cells Is Not Compensated by Another Acidic Modification

When \[^{3}H\]tyrosine-labeled wild-type YP2, immunoprecipitated from the medium of L-YP2wt-3 cells, was analyzed by two-dimensional PAGE, two isoelectric variants of sulfated YP2 (indicated by the double arrow in Fig. 2) were observed. These two variants are not caused by different degrees of sulfation (Baeuerle and Huttner, 1985) but probably result from variations in other posttranslational modifications, e.g., phosphorylation (Brennan and Mahowald, 1982; Minoo and Postlethwait, 1985). In addition, a small amount of \[^{3}H\]tyrosine-labeled wild-type YP2 less acidic than the two major variants was observed, which may represent a small amount of unsulfated wild-type YP2 secreted from L-YP2wt-3 cells, in line with the stoichiometry of tyrosine sulfation determined in Table I. In comparison with the \[^{3}H\]tyrosine-labeled sulfated wild-type YP2 variants, the unsulfated mutant YP2 variants secreted from L-YP2mut-4 cells and the unsulfated wild-type YP2 variants secreted from chlorate-treated L-YP2wt-3 cells had less acidic isoelectric points (Fig. 2, center and bottom). The shift in isoelectric point confirmed the lack of the sulfate and, more importantly, showed that no other acidic modification such as phosphorylation occurred to compensate for the lack of sulfate. Furthermore, the isoelectric points of mutant YP2 variants and wild-type YP2 variants from chlorate-treated cells were indistinguishable from one another, showing that the two distinct manipulations to inhibit tyrosine sulfation resulted in the same charge phenotype of YP2.

Sulfated Wild-type YP2 Is Transported to the Cell Surface Faster than Unsulfated Mutant YP2

Before analyzing the kinetics of secretion of wild-type and mutant YP2 by pulse-chase experiments, we investigated whether amino acid–labeled wild-type and mutant YP2 were equally stable in such experiments. The recovery of \[^{3}H\]tyrosine-labeled wild-type and mutant YP2 found in the sum of medium and cells at the end of a 180-min chase was 116 and 114%, respectively, of that found in L-YP2wt-3 and L-YP2mut-4 cells after a 15-min pulse label. (The slight overrecovery was probably due to the fact that \[^{3}H\]tyrosine incorporation continued during the first few minutes of the chase.) This showed that wild-type and mutant YP2 were equally stable under the present experimental conditions.

Fig. 3, A and B show the kinetics of secretion of wild-type and mutant YP2 produced by L-YP2wt-3 and L-YP2mut-4 cells, respectively, analyzed by pulse-chase experiments. Immunoprecipitates of amino acid–labeled YP2 were subjected to a multi-sample 2D-PAGE before quantitation of YP2. The latter form of separation was necessary since the immunoprecipitates of YP2 were variably contaminated with an amino acid–labeled protein that comigrated with YP2 in SDS-PAGE (see Fig. 1 top) but was clearly distinct from YP2 by peptide mapping (data not shown). The combination
The lack of the sulfate in Drosophila YP2 results in a less acidic isoelectric point of the protein. L cells expressing wild-type YP2 (clone L-YP2wt-3) were labeled for 20 h with $[^{3}H]$tyrosine in the absence (top pair of panels) or presence (bottom pair of panels) of 5 mM chlorate. L cells expressing mutant YP2 (clone L-YP2mut-4) were labeled in the absence of chlorate (center pair of panels). $[^{3}H]$tyrosine-labeled wild-type and mutant YP2 were isolated from the culture medium by immunoprecipitation. Unlabeled yolk proteins, purified from Drosophila flies, were added to the immunoprecipitates before two-dimensional PAGE. The gels were stained with Coomassie Blue (CB, top panels of each pair) and fluorographed ($[^{3}H]$tyr, bottom panels of each pair). The horizontal brackets (panels CB) indicate the position of Coomassie Blue-stained fly YP2. After drying the gels, two pieces of radioactive paper were glued as markers to the left and right of the Coomassie Blue-stained fly YP2 spot, at a distance of 1 cm from the center of the spot (circles in panels CB). These marker spots are visible in the fluorograms (dotted circles in panels $[^{3}H]$tyr) and have been used to align the Coomassie Blue staining and the fluorogram (see dashed lines). The positions of the two isoelectric variants of the $[^{3}H]$tyrosine-labeled wild-type YP2, mutant YP2, and wild-type YP2 produced by chlorate-treated cells are indicated by the double arrows. The solid lines mark the center of the Coomassie Blue-stained fly YP2 spot. Note the shift in isoelectric point of the unsulfated mutant YP2 (center panels) and the unsulfated chlorate-treated wild-type YP2 (bottom panels) toward the basic side of the IEF gel.

**Figure 2.**

The lack of the sulfate in Drosophila YP2 results in a less acidic isoelectric point of the protein. L cells expressing wild-type YP2 (clone L-YP2wt-3) were labeled for 20 h with $[^{3}H]$tyrosine in the absence (top pair of panels) or presence (bottom pair of panels) of 5 mM chlorate. L cells expressing mutant YP2 (clone L-YP2mut-4) were labeled in the absence of chlorate (center pair of panels). $[^{3}H]$tyrosine-labeled wild-type and mutant YP2 were isolated from the culture medium by immunoprecipitation. Unlabeled yolk proteins, purified from Drosophila flies, were added to the immunoprecipitates before two-dimensional PAGE. The gels were stained with Coomassie Blue (CB, top panels of each pair) and fluorographed ($[^{3}H]$tyr, bottom panels of each pair). The horizontal brackets (panels CB) indicate the position of Coomassie Blue-stained fly YP2. After drying the gels, two pieces of radioactive paper were glued as markers to the left and right of the Coomassie Blue-stained fly YP2 spot, at a distance of 1 cm from the center of the spot (circles in panels CB). These marker spots are visible in the fluorograms (dotted circles in panels $[^{3}H]$tyr) and have been used to align the Coomassie Blue staining and the fluorogram (see dashed lines). The positions of the two isoelectric variants of the $[^{3}H]$tyrosine-labeled wild-type YP2, mutant YP2, and wild-type YP2 produced by chlorate-treated cells are indicated by the double arrows. The solid lines mark the center of the Coomassie Blue-stained fly YP2 spot. Note the shift in isoelectric point of the unsulfated mutant YP2 (center panels) and the unsulfated chlorate-treated wild-type YP2 (bottom panels) toward the basic side of the IEF gel.

**Transport to the Cell Surface of Fibronectin, an Endogenous L Cell Protein, Is Identical in L-YP2wt-3 and L-YP2mut-4 Cells**

In view of the difference in the secretion kinetics between wild-type and mutant YP2, it was important to exclude that transport of secretory proteins to the cell surface in general was slower in L-YP2mut-4 cells than in L-YP2wt-3 cells, either because of intrinsic differences in the secretory properties of these clones or as a result of the perturbation of the secretion process in one of the clones by insertional mutagenesis caused by DNA transfection. We therefore compared the secretion of fibronectin, an endogenous L cell protein, in L-YP2wt-3 and L-YP2mut-4 cells by using the same pulse-chase protocol as with YP2. The kinetics of $[^{35}S]$methionine-labeled fibronectin secretion from L-YP2wt-3 cells and L-YP2mut-4 cells were indistinguishable; the half-time of secretion was 47 min for both clones (Fig. 3, C and C').

**Faster Transport to the Cell Surface of Sulphated Wild-type YP2 Rather than Un sulphated Mutant YP2 Occurs in Several L Cell Clones Expressing Various Amounts of YP2**

In addition to the clones L-YP2wt-3 and L-YP2mut-4 studied above, we investigated the secretion kinetics of wild-type and mutant YP2 in two other L cell clones, L-YP2wt-9 and L-YP2mut-14 cells, which produced sulfated YP2 and unsulfated YP2, respectively (data not shown). These four clones varied with respect to the amount of radioactive YP2 produced during a 15-min $[^{3}H]$tyrosine pulse followed by a 90-min chase (Fig. 4 left). Since the overall $[^{3}H]$tyrosine incorporation into cellular proteins was very similar between the clones (data not shown), the differences in the amounts of $[^{3}H]$tyrosine-labeled YP2 could be taken to reflect the differences in YP2 synthesis between these four clones. Quantitation of the $[^{3}H]$tyrosine-labeled YP2 secreted after 20 and 90 min showed that, independently of the clone analyzed, wild-type YP2 was secreted faster than mutant YP2 (Fig. 4 right). Only a very small difference in the kinetics of secretion of wild-type YP2 was observed between the two L-YP2wt clones (wt-3 and wt-9). Likewise, the kinetics of secretion of mutant YP2 from the two L-YP2mut clones (mut-4 and mut-14) were almost identical. Thus, the more rapid transport of sulfated wild-type YP2 relative to unsulfated mutant YP2 was seen in distinct clones producing different amounts of YP2.
Figure 3. Mutagenesis of tyrosine 172 of Drosophila YP2 results in a slower transport to the cell surface. (A) Fluorograms showing the secretion of wild-type and mutant YP2. L-YP2wt-3 and L-YP2mut-4 cells (one 10-cm dish each) were pulse-labeled for 15 min with [35S]methionine. The labeling medium was removed and replaced with chase medium (0 min). The chase medium was collected and replaced by fresh chase medium after 10, 20, 30, 40, 50, 60, 90, and 120 min. After 180 min of chase, the medium was collected and the cells were lysed. [35S]Methionine-labeled wild-type and mutant YP2 were immunoprecipitated from the media and the cell lysate and subjected to multi-sample 2D-PAGE. The individual squares showing the wild-type YP2 spots are sections from one fluorogram. The individual squares showing the mutant YP2 spots are sections from another fluorogram. Wild-type YP2 and mutant YP2 spots represent the accumulation of [35S]methionine-labeled wild-type and mutant YP2 in the medium during the indicated chase intervals. The thick line between 50-60 and 60-90 indicates the transition from 10-min chase intervals to longer chase intervals. (B) Kinetics of secretion of wild-type and mutant YP2. L-YP2wt-3 cells (open squares) and L-YP2mut-4 cells (solid squares) were pulse-labeled and chased, and wild-type and mutant YP2 was purified, as in A, except that [3H]tyrosine was used. The radioactivity in the YP2 spots was determined. Total YP2 is defined as the radioactive YP2 in the sum of the chase media plus that recovered from the cells at the end of the 180-min chase. YP2 secreted at the various times of chase was calculated by summing up the amounts in the individual chase intervals and is expressed as
Inhibition of Tyrosine Sulfation by Chlorate Has the Same Effect on the Transport of YP2 to the Cell Surface as Mutagenesis of Tyrosine 172

If the faster transport to the cell surface of wild-type YP2 than mutant YP2 was due to the sulfation of tyrosine 172, one would expect that inhibition of tyrosine sulfation of wild-type YP2 by chlorate treatment of cells should have the same effect on its transport as the mutagenesis of tyrosine 172. We therefore investigated the secretion kinetics of sulfated and unsulfated wild-type YP2 produced by L-YP2wt-3 cells in the absence and presence of chlorate, respectively, and compared the results with the differences in secretion kinetics between wild-type and mutant YP2 from L-YP2wt-3 and L-YP2mut-4 cells (Fig. 5, A and B). During these studies, we noted that in different sets of experiments, performed several months apart with different batches of L-YP2wt-3 or L-YP2mut-4 cells, the secretion half-times of YP2 varied for the same L cell clone. However, in every single set of experiments, a very similar and highly significant difference in the secretion half-times between wild-type and mutant YP2 was observed, which ranged from 16–18 min (compare Fig. 3, B and B' with Fig. 5 A).

Pulse–chase experiments of L-YP2wt-3 cells in the absence and presence of 5 mM chlorate revealed that the secretion kinetics of the sulfated wild-type YP2 was faster than that of the unsulfated wild-type YP2 produced by chlorate-treated cells, the difference in the secretion half-time being 15 min (Fig. 5 B). This difference was very similar to that seen between sulfated wild-type YP2 and the mutant YP2 (16 min; Fig. 5 A). In the latter set of pulse–chase experiments, the small amount of radiolabeled YP2 still present in the cell at the end of the 180-min chase was not determined, and the total secreted YP2 served as the reference point for the secretion kinetics. This may have resulted in a slight underestimation of the difference in the secretion half-time between wild-type and YP2.) Thus, inhibition of tyrosine sulfation of YP2 by chlorate treatment of cells retarded its transport to the cell surface to the same extent as the mutagenesis of tyrosine 172.

Structural Change from Tyrosine 172 to Phenylalanine 172 Alone Has No Effect on YP2 Transport to the Cell Surface

From the results described so far, one would expect that in the presence of chlorate, the transport of wild-type and mutant YP2 should be the same. Pulse–chase experiments of L-YP2wt-3 and L-YP2mut-4 cells in the presence of 5 mM chlorate showed that the secretion kinetics of the now unsulfated wild-type YP2 and the mutant YP2 were indistinguishable (Fig. 5 C). This was consistent with the assumption that the faster transport of wild-type YP2 relative to mutant YP2 seen in the absence of chlorate (Fig. 5 A) resulted from the sulfation of the wild-type YP2. Moreover, these results indicated that the change in the primary structure of YP2 induced by the mutagenesis of tyrosine 172 to phenylalanine alone had no effect on its transport to the cell surface.

Chlorate Does Not Affect the Transport of Mutant YP2 to the Cell Surface

It was important to show that the effect of chlorate on YP2 transport to the cell surface was specifically caused by the drug's inhibitory action on sulfation and was not the result of an unspecified perturbation of the cells. We therefore investigated the secretion kinetics of an unsulfated secretory pro-

percent of total YP2. The mean of the values obtained from two separate experiments is shown, each experiment consisting of one dish of L-YP2wt-3 cells and one dish of L-YP2mut-4 cells. The bars indicate the variation between the individual values and are only shown when exceeding the size of the square symbols.
Figure 5. Inhibition of sulfation by chlorate retards the intracellular transport of wild-type YP2 to the cell surface as does mutagenesis of tyrosine 172. (A) Kinetics of secretion of wild-type and mutant YP2 in the absence of chlorate. L-YP2wt-3 cells (open squares) and L-YP2mut-4 cells (solid squares) were pulse-labeled for 15 min with [35S]methionine and chased for 180 min, and wild-type and mutant YP2 were purified, as described in the legend to Fig. 3 A. The radioactivity in the YP2 spots was determined. Total secreted YP2 was defined as the radioactive YP2 in the sum of the chase media and the cell surface. To evaluate the significance of the 15–18 min shorter half-time of transport of sulfated YP2 than unsulfated YP2, it was necessary to determine the half-time of transport of wild-type YP2 from the intracellular site of tyrosine sulfation to the cell surface. L-YP2wt-3 cells were pulse labeled for 10 min with [35S]sulfate and chased for various time periods (2, 5, 10, and 20 min). (Labeling for 10 min was found to be required to obtain sufficient sulfate incorporation into YP2 for the reliable quantitation of the early time points.) Analysis of [35S]sulfate-labeled YP2 in the cells and medium showed that after 20 min of chase, almost all YP2 had been secreted (Fig. 7 top). The half-time of secretion of [35S]sulfate-labeled wild-type YP2, which reflected the half-time of transport of wild-type YP2 from the site of min with [3H]tyrosine and chased for 180 min in the continued absence or presence of chlorate, and YP2 was purified, following the protocol described in the legend to Fig. 5 A. The radioactivity in the YP2 spots was determined. Secreted YP2 is expressed as percent of total YP2, calculated as described in the legend to Fig. 5 B. The half-times of secretion (t50) were determined as described in Materials and Methods and Fig. 3 B, and are indicated in parentheses and by the open and solid arrowhead for wild-type YP2 in the absence and presence of chlorate, respectively. The results of a representative experiment are shown. (C) Kinetics of secretion of wild-type and mutant YP2 in the presence of chlorate. L-YP2wt-3 cells (open dotted squares) and L-YP2mut-4 cells (solid dotted squares) were pulse-labeled for 3 h in the presence of 5 mM chlorate. Cells were then pulse-labeled for 15 min with [3H]tyrosine and chased for 180 min in the continued presence of chlorate, and YP2 was purified, following the protocol described in the legend to Fig. 5 A. The radioactivity in the YP2 spots was determined. Secreted YP2 is expressed as percent of total YP2, calculated as described in the legend to Fig. 5 B. The mean of the values obtained from two separate experiments is shown, each experiment consisting of one dish of L-YP2wt-3 cells and one dish of L-YP2mut-4 cells each, and one experiment consisting of one dish of L-YP2mut-4 cells only. The bars indicate the standard deviation and are only shown when exceeding the size of the symbols. The half-times of secretion (t50) were determined from the mean as described in Materials and Methods and Fig. 3 B, and are indicated in parentheses and by the open and solid arrowhead for wild-type YP2 and mutant YP2, respectively.
sulfation within the trans-Golgi to the cell surface, was 10 min (Fig. 7, bottom).

Since YP2 is not N-glycosylated (Friederich et al., 1988) and therefore lacks other trans-Golgi-specific modifications such as galactosylation and sialylation of N-linked oligosaccharides (see Kornfeld and Kornfeld, 1985), the half-times of transport from within the trans-Golgi to the cell surface could not be determined directly for the unsulfated mutant YP2 and the unsulfated wild-type YP2 produced by chlorate-treated cells. In view of the trans-Golgi localization of protein tyrosine sulfation (Baeuerle and Huttner, 1987), these half-times could be deduced from the differences in RER to cell surface transport between sulfated and unsulfated YP2 and from the half-time of transport of [35S]sulfate-labeled wild-type YP2 to the cell surface (Table II). The deduced half-time of transport from within the trans-Golgi to the cell surface was 26–28 min in the case of unsulfated mutant YP2 and 25 min in the case of unsulfated wild-type YP2 from chlorate-treated cells and thus 2.5–2.8-fold greater than the half-time of 10 min determined for sulfated wild-type YP2.

Discussion

Inhibition of Tyrosine Sulfation of Drosophila YP2 Causes Retardation of Its Transport to the Cell Surface

We have shown that inhibition of tyrosine sulfation of a constitutively secreted protein, Drosophila YP2, retards its transport to the cell surface. Two approaches of inhibiting tyrosine sulfation of YP2 were used, which have distinct mechanisms of inhibition: (a) mutagenesis of the single site of YP2 sulfation, tyrosine 172, to the closely related but unsulfatable amino acid phenylalanine; (b) inhibition of synthesis of the cosubstrate of sulfation, PAPS, by chlorate treatment of cells. Both procedures proved highly effective in inhibiting YP2 sulfation: mutagenesis totally blocked tyrosine sulfation of YP2, and chlorate treatment inhibited tyrosine sulfation to 95%. Both procedures led to a virtually identical retardation of YP2 transport to the cell surface: 16–18 min in the case of mutant YP2, 15 min in the case of chlorate treatment.

The advantage of the mutagenesis was that it constituted a highly selective manipulation, limited to one amino acid in the protein under study. On the other hand, we cannot exclude that the difference in transport between wild-type and mutant YP2 was caused by the change in the primary structure per se rather than the lack of the sulfate, which for example resulted in impaired folding of the protein in the RER and retarded exit from this compartment. However, this interpretation of the data is unlikely for the following reason. If the retarded transport of the mutant protein was caused by the tyrosine to phenylalanine change itself and not by the lack of sulfate, transport of the mutant protein should be retarded compared with that of the unsulfated wild-type protein produced in the presence of chlorate. This was not the case. On the contrary, the kinetics of transport of the wild-type and mutant YP2 in the presence of the sulfation inhibitor were identical, showing that the tyrosine to phenylalanine change itself had no effect on transport.

The advantage of the second procedure of inhibiting sulfation, chlorate treatment of cells, was that it did not involve
Table II. Deduced Half-time of Transport of Unsulfated YP2 from within the trans-Golgi to the Cell Surface

| Protein                          | $t_0$ (min) | $\Delta t_0$ to sulfated wild-type YP2 (min) | Deduced $t_0$ to sulfated wild-type YP2 (min) | Fold increase to sulfated wild-type YP2 |
|---------------------------------|-------------|--------------------------------------------|-------------------------------------------|---------------------------------------|
| Unsulfated mutant YP2           | 49 (41/57)  | 16–18                                      | 26–28                                     | 2.6–2.8                               |
| (Fig. 3, B and B', Fig. 5 A)    |             |                                            |                                           |                                       |
| Unsulfated wild type YP2        | 46          | 15                                         | 25                                       | 2.5                                   |
| (chlorate treatment) (Fig. 5 B) |             |                                            |                                           |                                       |

The first column lists the half-times of transport from the RER to the cell surface of unsulfated mutant YP2 (see Fig. 3, B and B' and Fig. 5 A) and unsulfated wild-type YP2 produced in the presence of chlorate (see Fig. 5 B). For unsulfated mutant YP2, an average half-time was calculated from the half-times shown in Fig. 3, B and B' and Fig. 5 A, which are given in parenthesis. The second column shows the differences in the half-times of RER to cell surface transport between unsulfated mutant YP2 and sulfated wild-type YP2 (Fig. 3, B and B', Fig. 5 A), and between unsulfated wild-type YP2 produced in the presence of chlorate and sulfated wild-type YP2 produced in the absence of chlorate (Fig. 5 B), calculated by subtraction. The third column shows the deduced half-time of transport from within the trans-Golgi to the cell surface of unsulfated mutant YP2 and unsulfated wild-type YP2 produced in the presence of chlorate. These deduced half-times were calculated by adding the values shown in the second column to the 10-min half-time determined for sulfated wild-type YP2 (see Fig. 7). The fourth column shows the fold increase in the half-times of trans-Golgi to cell surface transport of unsulfated mutant YP2 and unsulfated wild-type YP2 produced in the presence of chlorate, compared with sulfated wild-type YP2. These values were calculated by dividing the deduced half-times shown in the third column by 10 (minutes), the half-time of sulfated wild-type YP2 (see Fig. 7).

The transport kinetics of a constitutive secretory protein, once this protein is included in transport vesicles destined to the cell surface, are determined solely by the kinetics of vesicle movement and fusion and not by the structure of the protein itself. Second, L cell clones stably expressing wild-type or mutant YP2 were used, so that secretion of YP2 was studied under steady state conditions. This implies that the respective trans-Golgi pool sizes of wild-type and mutant YP2 remained constant during the pulse–chase experiments. This in turn implies that the number of YP2 molecules delivered into the trans-Golgi per unit time (rate of delivery) equals the number of YP2 molecules exiting from this compartment per unit time (rate of exit). Since L-YP2wt-3 and L-YP2mut-4 cells produce very similar amounts of YP2 and since tyrosine sulfation occurs after YP2 has entered the trans-Golgi, the rate of delivery of YP2 into the trans-Golgi should be the same for wild-type YP2, wild-type YP2 in the presence of chlorate, and mutant YP2. Under steady state conditions, the rate of exit of wild-type YP2, wild-type YP2 in the presence of chlorate, and mutant YP2 from the trans-Golgi should therefore also be the same. This leaves us with the most likely explanation that the observed 15–18-min retardation of YP2 transport by inhibition of tyrosine sulfation reflects an increase in the residence time of YP2 in the trans-Golgi. Since the residence time in the trans-Golgi can only account for part of the transport time between the site of sulfation and the cell surface, the increase in residence time by inhibition of sulfation must be even ≥2.5–2.8-fold.

We do not know how the lack of sulfation of tyrosine 172 increases the residence time of YP2 in the trans-Golgi. It is unlikely that mutant YP2 is retarded because it cannot be converted by tyrosylprotein sulfotransferase to the sulfated form, thus remains bound to the enzyme relatively longer than wild-type YP2, and therefore exists from the trans-Golgi more slowly. If this were the case, tyrosylprotein sulfotransferase in L-YP2mut cells should be inhibited by the bound mutant YP2, since the amount of mutant YP2 in the trans-Golgi can be expected to be at least as high as that of the relatively rare enzyme (Niehrs, C., and W. B. Huttner, unpublished data). The observation that the sulfation of fibronectin, a known tyrosine-sulfated secretory protein

any change in the primary structure of the protein under study. However, chlorate inhibited the sulfation of all L-cell proteins (data not shown) and, because of its mechanism of action, probably all intracellular sulfation reactions. In addition, as yet unknown side effects of chlorate, unrelated to the inhibition of PAPS synthesis, could not be excluded. It was therefore important to show that the effect of chlorate on YP2 transport was due to inhibition of tyrosine sulfation of YP2 rather than some other effect unrelated to YP2 sulfation. This was demonstrated by the observation that transport of the unsulfatable mutant YP2 was completely unaffected by chlorate. Moreover, chlorate treatment had no effect on protein synthesis and cell morphology, in agreement with previous findings (Baueuerle and Huttner, 1986), and its inhibitory effect on sulfation was fully reversible.

In view of these results, and considering the distinct sites of action of mutagenesis and chlorate treatment, we conclude that these two manipulations caused the retardation of YP2 transport to the cell surface via the inhibition of sulfation of tyrosine 172.

Retardation of YP2 Transport by Inhibition of Sulfation Results from an Increased Residence Time in the trans-Golgi

Protein tyrosine sulfation has been shown to specifically occur in the trans-Golgi (Baueuerle and Huttner, 1987). Consistent with this finding was the observation that the half-time of secretion of sulfate-labeled YP2 was much shorter than that of amino acid-labeled YP2. Hence, within the various organelles of the secretory pathway, the retardation of YP2 transport by inhibition of its tyrosine sulfation could only have occurred in the trans-Golgi itself or thereafter. Since the transport of sulfated YP2 from the trans-Golgi to the cell surface takes 10 min, the 15–18-min retardation of transport of unsulfated YP2 therefore means that unsulfated YP2 takes 25–28 min to pass through the trans-Golgi and to the cell surface, i.e., 2.5–2.8-fold longer than sulfated YP2 (Table II).

In fact, two lines of considerations suggest that it is the passage of YP2 through the trans-Golgi rather than its transport from this compartment to the cell surface that is retarded upon inhibition of tyrosine sulfation. First, it is likely that protein tyrosine sulfation has been shown to specifically occur in the trans-Golgi (Baueuerle and Huttner, 1987). Consistent with this finding was the observation that the half-time of secretion of sulfate-labeled YP2 was much shorter than that of amino acid-labeled YP2. Hence, within the various organelles of the secretory pathway, the retardation of YP2 transport by inhibition of its tyrosine sulfation could only have occurred in the trans-Golgi itself or thereafter. Since the transport of sulfated YP2 from the trans-Golgi to the cell surface takes 10 min, the 15–18-min retardation of transport of unsulfated YP2 therefore means that unsulfated YP2 takes 25–28 min to pass through the trans-Golgi and to the cell surface, i.e., 2.5–2.8-fold longer than sulfated YP2 (Table II).

In fact, two lines of considerations suggest that it is the passage of YP2 through the trans-Golgi rather than its transport from this compartment to the cell surface that is retarded upon inhibition of tyrosine sulfation. First, it is likely that any change in the primary structure of the protein under study. However, chlorate inhibited the sulfation of all L-cell proteins (data not shown) and, because of its mechanism of action, probably all intracellular sulfation reactions. In addition, as yet unknown side effects of chlorate, unrelated to the inhibition of PAPS synthesis, could not be excluded. It was therefore important to show that the effect of chlorate on YP2 transport was due to inhibition of tyrosine sulfation of YP2 rather than some other effect unrelated to YP2 sulfation. This was demonstrated by the observation that transport of the unsulfatable mutant YP2 was completely unaffected by chlorate. Moreover, chlorate treatment had no effect on protein synthesis and cell morphology, in agreement with previous findings (Baueuerle and Huttner, 1986), and its inhibitory effect on sulfation was fully reversible.

In view of these results, and considering the distinct sites of action of mutagenesis and chlorate treatment, we conclude that these two manipulations caused the retardation of YP2 transport to the cell surface via the inhibition of sulfation of tyrosine 172.

Retardation of YP2 Transport by Inhibition of Sulfation Results from an Increased Residence Time in the trans-Golgi

Protein tyrosine sulfation has been shown to specifically occur in the trans-Golgi (Baueuerle and Huttner, 1987). Consistent with this finding was the observation that the half-time of secretion of sulfate-labeled YP2 was much shorter than that of amino acid-labeled YP2. Hence, within the various organelles of the secretory pathway, the retardation of YP2 transport by inhibition of its tyrosine sulfation could only have occurred in the trans-Golgi itself or thereafter. Since the transport of sulfated YP2 from the trans-Golgi to the cell surface takes 10 min, the 15–18-min retardation of transport of unsulfated YP2 therefore means that unsulfated YP2 takes 25–28 min to pass through the trans-Golgi and to the cell surface, i.e., 2.5–2.8-fold longer than sulfated YP2 (Table II).

In fact, two lines of considerations suggest that it is the passage of YP2 through the trans-Golgi rather than its transport from this compartment to the cell surface that is retarded upon inhibition of tyrosine sulfation. First, it is likely that
(Paul and Hynes, 1984; Liu and Lipmann, 1985), occurred equally well in L-YP2mut cells as in untransfected Ltk−
cells (data not shown; see also Fig. 1 top) suggested that no such inhibition took place. Possible explanations for the shorter residence time of sulfated than unsulfated YP2 in
cells was too low to investigate the molecular mechanism of retardation of unsulfated YP2 in the trans-Golgi by
immunoelectron microscopy or biochemistry in combination with subcellular fractionation. However, although the
molecular mechanism of retardation of unsulfated YP2 in the
trans-Golgi remains to be elucidated, the present results pro-
vide the first evidence for the modulation of intracellular
transport of a secretory protein in the trans-Golgi by a post-
translational modification.

Biological Significance of the Acceleration of YP2 Transport by Tyrosine Sulfation

With the exception of small tyrosine-sulfated peptides such as cholecystokinin (Mutt, 1980), the biological significance of protein tyrosine sulfation is not yet known. This is the first study reporting a role for tyrosine sulfation in a larger poly-
peptide. The retardation of transport of unsulfated YP2, ob-
served here after expression of the protein in mammalian fibroblasts, may be highly significant with respect to the in vivo situation in Drosophila flies. During oogenesis, the fat body and follicle cells of female flies produce large amounts of yolk proteins: a single female fly can produce daily a weight, and up to 20% of the total egg protein is YP2
b) the presence of tyrosine sulfate may induce a structural change in YP2 (for example a local increase in surface hydrophilicity) that facilitates its passage within the trans-Golgi to areas where proteins exit from this compartment; and (c) the presence of tyrosine sulfate may prevent a structural change (for example an aggregation induced by the slightly acidic pH in the trans-Golgi; Anderson and Pathak, 1985) that would slow the passage of YP2 within the trans-Golgi to areas where proteins exit from this compartment. The expression of the transfected YP2 in the L cells was too low to investigate the molecular mechanism of retardation of unsulfated YP2 in the trans-Golgi directly by immunoelectron microscopy or biochemistry in combina-

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