2. MATERIALS AND METHODS

2.1. Materials.

2.1.1 Chemicals and materials.

If not started otherwise, all chemicals were purchased from Sigma (Taufkirchen, Germany) or Merck Biosciences (Bad Soden, Germany). All cell culture media, supplements and materials except of chamber slides (Nunc, Naperville, IL) and S-MEM (Gibco Invitrogen GmbH, Karlsruhe, Germany), were from PAA Laboratories (Cölbe, Germany). Madine-Darby canine kidney cells were a gift of Dr. Swaroop (MDC, Berlin, Germany). [γ-^{32}P]ATP, pGEX-4T-1 vector, Hybond ECL nitrocellulose membrane and Western blotting detection reagents were from Amersham Pharmacia Biotech (Freiburg, Germany). PMA, 1,2-dioctanoyl-sn-glycerol, Gö6976 and GF109203X were from Qbiogene-Alexis GmbH (Grünberg, Germany). Rottlerin was from Merck Biosciences GmbH (Schwalbach, Germany) TRIzol reagent was from Life Technologies GmbH (Technologiepark weg Karlsruhe, Germany). Chelex resin was from Bio-Rad Laboratories GmbH (München, Germany). Disuccinimidyl Suberate (DSS) was from PIERCE (Rockford, IL). Mounting medium was from Shandon (Pittsburgh, PA).

Antibodies and enzymes

Cy3- and HRP-conjugated anti-rabbit and anti-mouse monoclonal antibodies, rabbit polyclonal anti-occludin, anti-claudin-1, anti-pan-cadherin and anti-ZO-2 antibodies as well as mouse anti-ZO-1 antibody were purchased from Zymed Laboratories Inc. (San-Francisco, USA). Alexa-Fluor 488 anti-rabbit antibodies were from Molecular Probes (Eugene, USA). Thermus aquaticus (Taq) Polymerase was from Life Technologies (Eggenstein, Germany). Calf intestinal alkaline phosphatase and Moloney murine leukemia virus (MuMLV) reverse transcriptase were from Promega (Madison, USA). SalI and BamHI restriction endonucleases were from New England Biolabs (Beverly, USA), Protein kinase Cδ was from Merck Biosciences GmbH (Bad Soden, Germany).

2.2. Solutions.

Aqueous solutions were prepared using de-ionised water (electrical resistance, 18.6 MΩ/cm) (Milli-Q, UF Plus, Millipore, Bedford, USA).

2.2.1 Buffer solutions.

Cell culture media.

Complete growth medium for MDCK cells included DMEM supplemented with 2 mM L-glutamine, 100 U penicillin, 100 mg/ml streptomycin, 10% fetal calf serum.

Solutions for pMAL protein expression and purification.

Rich medium	per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose. After autoclave sterilisation, ampicillin was added to 100 µg/ml
Column buffer (CB)	20 mM Tris/HCl pH 7.4, 0.15 M NaCl, 2 mM EDTA, 0.2 mM PMSF, 0.1% TritonX-100, 2 mM DTT
Elution buffer (EB)	20 mM Tris/HCl pH 8.0, 2 mM DTT, 10 mM reduced glutathione

Solutions for SDS-PAGE and Western blot.

30% Acrylamide mix	30% (w/v) Acrilamide, 0.8% (w/v) Bisacrilamide in H_2O
Resolving gel buffer	1.5 M Tris/HCl pH 8.8
Stacking gel buffer	1.0 M Tris/HCl pH 6.8
Running buffer	25 mM Tris, 250 mM glycine, 0.1% SDS
4 x SDS loading buffer	0.2 mM Tris/HCl pH 6.8, 0.4 mM DTT, 4% SDS, 0.4% bromphenol blue, 40% glycerol
Transfer buffer	5.82 g Tris, 2.93 g glycine, 200 ml methanol, 0.375 g SDS, H_2O total 1liter
Wash and incubation buffer	20 mM Tris/HCl pH 7.4, 137 mM NaCl, 0.1% Tween-20
Blocking buffer	5% non-fat milk, 20 mM Tris/HCl pH 7.4, 137 mM NaCl, 0.1% Tween-20

Solutions for cell fractionation.

Buffer for the extraction of TritonX-100 soluble fraction, **1% TX buffer**	1% TX-100, 25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1 µM leupeptin, 0.3 µM aprotinin, 0.1 mM PMSF, 1 µM pepstatin, 0.1 mM PMSF
Buffer for the solubilisation of TritonX-100 insoluble fraction, **1% SDS buffer**	1% SDS, 25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl

Solutions for occludin isolation from rat liver.

Homogenisation buffer	PBS supplemented with 1% TX-100, 4 mM EDTA, 25 mM NaF, 1 µM leupeptin, 0.3 µM aprotinin, 0.1 mM PMSF, 1 µM pepstatin, 0.1 mM PMSF
Buffer for the solubilisation of highly-phosphorylated occludin forms	PBS containing 1% SDS
Dialysis/Immunoprecipitation buffer	25 mM Tris/HCl pH 7.4, 50 mM NaCl, 5% glycerol, 2 mM DTT

Binding/Wash buffer	0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.01 M KCl, pH 7.4 and 0.2% TritonX-100
Elution Buffer	ImmunoPure IgG Elution Buffer, pH 2.8 (PIERCE, Rockford, IL) with 0.1% TritonX-100
Neutralising buffer	1 M Tris/HCl pH 9.5

2.3. Methods.

2.3.1. Cell Culture.

Madine-Darby canine kidney (MDCK) cells were cultured under standard sterile conditions. Cells were placed for cultivation in a cell culture incubator with the following settings: gaseous atmosphere, 5% CO_2, 95% air; temperature, 37°C; humidity, 100%. Cell culture medium was replaced at least every two days. For sub-cultivation, confluent monolayers were washed twice with PBS (without Ca^{2+} and Mg^{2+}). Cells were detached from the cell culture surface by enzymatic digestion with trypsin. After detachment of all cells enzymatic digestion was stopped by the addition of serum-containing medium. The seeding density was approximately $2.5x10^5$ cells/cm^2.

2.3.2 Preparation of low and normal calcium medium.

Low calcium medium was prepared on the basis of calcium-free S-MEM (Gibco). Determinations with a Ca^{2+}-sensitive electrode detects 1-4 µM Ca^{2+} (Balda et al., 1991), therefore, we refer to this medium as low calcium (LC). S-MEM was supplemented with 2 mM L-glutamine, 100 U penicillin, 100 mg/ml streptomycin and 5% fetal calf serum (FCS). To deplete the calcium ions in FCS the latter was incubated with chelating resin Chelex 100 overnight at 4°C. Chelex resin was washed two times with Milli-Q water before use. For 50 ml of FCS 10 ml of wet resin were used. After 5 min centrifugation at 500 x g, FCS was incubated with a new portion of resin for additional 2 hours. For sterilisation purposes and removal of microscopic particles, FCS and LC medium were passed through a 0.22 µm filter (Sterile Filtration Units; Corning-Costar, Cambridge, USA).

Normal calcium medium was prepared from low calcium medium by the addition of $CaCl_2$ to the final concentration 1.8 mM.

2.3.3 Calcium switch procedures.

MDCK cells were grown in DMEM until confluent monolayer was formed (DMEM formulation includes 1.8 mM Ca^{2+}). Cells were washed twice with PBS without Ca^{2+} and then transferred to a low Ca^{2+} medium for 20 hours prior to the addition of normal calcium medium or kinase inhibitors. In separate experiments GF-109203X, rottlerin or Gö6976 at different concentrations were added 10 min before the addition of normal calcium medium with the same inhibitors. Switch from NC to LC was performed in a similar manner, 5 μM Gö6976 being added 10 min before first wash with PBS.

2.3.4 Immunofluorescence microscopy.

MDCK cells were grown on chamber slides, washed with PBS and fixed in 3.7% formaldehyde in PBS for 15 min. After three washes with PBS cells were permeabilised with 0.2% TX-100 in PBS (15 min), soaked in blocking solution (1% bovine serum albumin in PBS) for 60 min and then incubated with 300 μl of primary antibodies (diluted 100 times). Samples were washed three times with 0.2% bovine serum albumin in PBS and then incubated for 60 min with secondary antibodies. After four washes cover slips were mounted over the cells with the aid of mounting medium. Samples were examined using a fluorescence microscope equipped with the AxioVision image acquisition and analysis system (Carl Zeiss, Jena, Germany).

2.3.5 Sample preparation.

MDCK cells were washed twice with PBS and extracted for 30 min with 1% TX buffer (the resulting extract was referred to as the TX-100 soluble fraction). The remaining material was scraped in 1% SDS buffer, boiled for 5 min and centrifuged for 10 min at 14,000 × g (the resulting extract was referred to as the TX-100 insoluble fraction).

2.3.6 One-dimensional SDS-PAGE and Western blot.

SDS-PAGE (8% gel) was performed according to Laemmli (18). For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. The membrane was blocked 1 hour, rinsed with wash buffer and incubated with anti-occludin antibodies for 1 h at room temperature. Membrane was washed 3 times for 10 minutes and incubated with HRP-linked secondary antibody for 40 minutes. After washing, the

immunoreactive bands were detected with ECL kit (Amerscham Biosciences, Freiburg, Germany).

To detect protein phosphorylation, samples were blotted as above and the ^{32}P signal was detected by autoradiography from dried membranes. Proteins transferred on nitrocellulose membrane were visualised by silver staining.

2.3.7 Silver staining of proteins immobilised on nitrocellulose membrane.

Three aqueous solutions were made fresh prior to the staining process: solution A - 275 mg of sodium citrate in 1 ml H_2O, solution B - 117.5 mg $FeSO_4$ x $7H_2O$ in 1 ml H_2O, solution C - 20 mg of $AgNO_3$ in 200 µl H_2O. The solutions were mixed in the next order: 1 ml of solution A, then 800 µl of B and finally 200 µl of C. The volume of resulting staining solution was adjusted with H_2O to 10 ml. The membrane was rinsed briefly with H_2O, shaken in staining solution for a maximum 5 minutes, washed 3 times with 100 ml H_2O for at least 5 minutes and dried 2 hours at room temperature.

2.3.8 RNA isolation.

Total RNA was isolated from murine kidney. 50 mg of tissue was homogenised in 1 ml of TRIzol Reagent (Life Technologies, Eggenstein, Germany). Homogenised samples were incubated at room temperature 5 minutes to permit the complete dissociation of nucleoprotein complexes and mixed with 0.2 ml of chloroform. Following centrifugation at 12000 x g for 15 minutes at 2 °C, RNA from upper aqueous phase was precipitated by 0.5 ml of isopropyl alcohol. Sample was centrifuged at 12000 x g for 10 minutes at 2 °C. RNA pellet was washed with 75% ethanol with subsequent centrifugation at 7500 x g for 5 minutes. RNA pellet was air-dried and dissolved in RNase-free water. The integrity of isolated RNA was checked by electrophoresis through a standard 1% agarose gel. The purity and concentration of RNA was estimated by spectrophotometric technique.

2.3.9 Quantitation of nucleic acids.

Quantitation of nucleic acids was predicted by measuring the light absorbance of the sample at 260 nm. Direct calculation of the concentration was done using the formula:

$$[\text{nucleic acid}]\ \mu g/ml = A_{260} \times \text{dilution} \times \varepsilon$$

where

A_{260} = absorbance at 260 nm

dilution = dilution factor

ε = extinction coefficient, which is approximately 40 for single-stranded DNA/RNA and 50 for double-stranded DNA.

2.3.10 Reverse Transcription-Polymerase chain reaction.

Reverse transcription was performed using 1 µg of isolated murine RNA, 200 U MuMLV reverse transcriptase and 10 µM random hexamer primers (Promega, Madison, USA) at 40°C for 1 hour.

A 777-base pair fragment encoding amino acids 264-521 of murine occludin was amplified using the primers 5'-AGGATCCAAAACCCGAAGAAAGATGGATCGG-3', and 5'-TTGTCGACTAAGGTTTCCGTCTGTCATAGTC-3' (BamHI and SalI sites are underlined). The amplified product was cloned using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA). Several clones were sequenced (Taq DyeDeoxy-Terminator cycle Sequencing Kit, Applied Biosystems, Weiterstadt, Germany); a clone containing no mutations was selected and its BamHI-SalI fragment was subcloned into the pGEX-4T-1 plasmid to produce a plasmid encoding the COOH-terminal domain of occludin fused with glutatione-S-transferase (GST-occludin).

2.3.11 Expression, isolation and purification of GST-fusion proteins.

The fusion protein was overexpressed in E.coli (TOP10F', Invitrogen, USA). An overnight cultures of E.coli containing GST-fusion construct were grown in 400 ml Rich medium at 37°C to $2x10^8$ cells/ml (A_{600}~0.5). The protein expression was induced by adding IPTG to the cell culture to a final concentration of 0.3 mM. Following incubation at 30°C for 2.5 hours, cells were harvested by centrifugation at 4000 x g for 8 minutes. Cells were resuspended in ice-cold column buffer (CB) and sonicated 3 times in short pulses of 15 seconds. After 2 min of centrifugation at 10,000 x g at 4 °C, the cell supernatant was passed through a 0.22 µm filter. Fusion protein was isolated by means of affinity chromatography on 3 ml glutathione-agarose column (Sigma, Taufkirchen, Germany). After extensive washing with 12 column volumes of CB, GST-fusion protein was eluted in EB and applied on HiPrep DEAE anion-exchange column at flow rate 1 ml/min (Amersham Pharmacia Biotech Freiburg, Germany). Proteins were eluted from DEAE column using a linear gradient of NaCl concentration (from 50 to 500 mM NaCl in

40 min at flow rate 2 ml/min). GST-occludin was eluted at about 150 mM NaCl. The purity and integrity of isolated proteins was analysed by SDS-PAGE.

2.3.12 Occludin in vitro phosphorylation.

Purified recombinant occludin fragment (5 µg) was mixed with 16 ng of protein kinase Cδ in 20 mM Tris/HCl buffer pH 7.5 containing 20 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 M NaCl, 40 mM DTT, 0.1 mM ATP and 2.5 µCi of [γ-^{32}P]ATP in a reaction volume of 40 µl. Phosphorylation of GST (the expression product of the pGEX-4T-1 plasmid) was performed as for occludin.

2.3.13 Occludin immunoprecipitation and alkaline phosphatase treatment.

Proteins from Triton-X-100 insoluble fraction of MDCK cells were extracted with 1% SDS buffer and combined with immunoprecipitation buffer IP-buffer (25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% TX-100, 1 µM leupeptin, 0.3 µM aprotinin, 0.1 mM PMSF, 1 µM pepstatin) to adjust a final concentration of SDS to 0.1%. After centrifugation at 4 °C, 13,000 × g for 15 min, the supernatant was pretreated with 15 µl bed volume of protein A-Sepharose. For immunoprecipitation 1 µg of anti-occludin antibodies and 10 µl bed volume of protein A-Sepharose were added to the supernatant and rotated for 1 hr at 4 °C. Beads were washed 5 times with 1 ml of IP-buffer and then resuspended in 100 µl phosphatase buffer (50 mM Tris/HCl, pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine) with or without 20 U of calf intestinal alkaline phosphatase. After 1 hr incubation at 37 °C, SDS-PAGE buffer was added and beads were boiled to elute the immunoprecipitates for subsequent PAGE.

2.3.14 Occludin isolation from rat liver.

Preparation of the fraction enreached in highly phosphorylated occludin forms from rat liver.

For the isolation experiment, three 8-wk-old rats were used. After decapitation, the livers were carefully taken out and frozen in liquid nitrogen. As the first step, the 2.5 g of liver were homogenised in 25 ml of homogenisation buffer, containing 1% Triton X-100, by the use of a loose-fitting homogeniser (Dounce, Cole-Parmer Instrument Co.). The homogenate was centrifuged at 20,000 x g for 10 min at 4°C. The supernatant, which is referred to as Triton-X 100 soluble fraction, was analysed by immunoblot for the presence of occludin. The pellet was

resuspended in 15 ml of 1% SDS buffer to solubilise highly-phosphorylated occludin forms and sonicated four times in short pulses of 15 seconds to destroy the DNA. The resulting suspension was centrifuged for 15 min at 20,000 x g and the supernatant obtained was used for sequential ammonium sulphate protein precipitation. While agitating gently on a magnetic stirrer, saturated solution of ammonium sulphate was slowly added until the concentration of ammonium sulphate reached 7.1%. Precipitation was carried out for at least 30 min at room temperature followed by centrifugation at 20,000 x g for 10 min. The pellet, which was referred to as 7.1% ammonium sulphate precipitate, was frozen. The supernatant was processed for sequential protein precipitation as described above to obtain 10% and 14% precipitates. Each precipitate and supernatant was analysed by immunoblot for the presence of occludin. The precipitate rich in occludin was resuspended in 1 ml of 100 mM NaOH and combined with 1 ml of 2 x concentrated dialysis/immunoprecipitation buffer. This fraction was dialysed using a membrane with a pore size 60,000 Da for 24 hours against 500 ml of dialysis/immunoprecipitation buffer and used for subsequent occludin immunoprecipitaion.

Binding and cross-linking of anti-occludin antibody to protein A.

300 μg of rabbit polyclonal anti-occludin antibodies were incubated with 0.4 ml of the immobilised ProteinA (50% slurry) for 30 min at 4 C to allow the binding of antibody to a gel. The gel was washed 3 times with binding/wash buffer without TritonX-100. The bound antibody was covalently attached to ProteinA gel using 1.6 μg/ml of Disuccinimidyl Suberate (DSS) as a cross-linker. The cross-linking reaction was carried out at room temperature for 30 minutes. The remove uncoupled antibody and excess of DSS the gel was washed five times with elution buffer, followed by 2 washing steps with binding/wash buffer. The prepared immobilised antibodies were used five times for occludin immunoprecipitations.

Occludin immunoprecipitation.

Four 2-ml fractions enriched in phosphorylated occludin, were united pooled to give the final volume 8 ml. This resulting sample was used for occludin immunoprecipitation. Immunoprecipitation was carried out with 300 μg of rabbit anti-occludin antibodies covalently coupled to a gel. After overnight incubation at 10°C, the gel was extensively washed with binding/wash buffer. To avoid the contamination from residual proteins, the flow-through from the wash was controlled by measuring the light absobance at 280 nm, to verify that the gel has

been thoroughly washed. The elution of the immunoprecipitated occludin was performed two times with 380 µl of elution buffer. Resulting two fractions were neutralised by the addition of 20 µl of neutralising buffer and analysed for the presence of occludin by Western blot.

Occludin concentration.

The occludin fractions obtained from five separate immunoprecipitations were united pooled. Occludin from the resulting ~4 ml sample was concentrated by the binding to the 40 µl of diatomaceous earth (PAGEprep^{TM} Support PIERCE.). For this purpose, occludin sample was mixed with an equal volume of DMSO to form a binding solution. DMSO mixture was incubated 1hour with diatomaceous resins with occasional mixing to ensure complete protein absorption. The resins were spin at 5000 rpm for 2 minutes and washed two times with 0.15 ml of 50% DMSO water solution. Occludin was eluted with 50 µl of SDS-PAGE loading buffer (PIERCE). 2 µl of 1M DTT solution was added to the collected eluted sample.

2.3.15 The determination of the molecular weight and sequence of peptides by mass spectrometry.

All mass spectrometry experiments were performed by the Mass Spectrometry Group (FMP).

For MALDI analysis 16 µl of occludin sample isolated from rat liver were resolved by SDS-PAGE. The protein was detected by staining with Colloidal Blue Staining Kit (Novex, San-Diego, CA). Occludin bands were excised from the stained gels, washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, dehydrated in acetonitrile and dried in a vacuum centrifuge. Disulfide bonds were reduced by incubation in 30 µl of 10 mM DTT in 100 mM ammonium bicarbonate for 45 min at 55°C. Alkylation was performed by replacing the DTT solution with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. After 20 min incubation at 25 °C in the dark, the gel pieces were washed with 50-100 µl of 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried in a vacuum centrifuge. The gel pieces were re-swollen in 10 µl of 5 mM ammonium bicarbonate, containing 300 ng endoproteinase Asp-N or trypsin (sequencing grade, Roche Diagnostics, Mannheim, Germany). After 15 min, 5 µl of 5 mM ammonium bicarbonate was added to keep the gel pieces moist during Lys-C cleavage (37°C, overnight). To extract the peptides, 15 µl of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile was added, and the samples were sonicated for 5 min. The separated liquid was dried under vacuum and redissolved in 10 µl of 0.1% (v/v) TFA in

water. The peptides were purified over a C18 reversed-phase minicolumn filled in a micropipette tip, ZipTip C18 (Millipore, Bedford, MA, USA) for mass spectrometry analysis. Purification was performed according to the manufacturer's manual, except that peptides were eluted with 3 µl of 60% (v/v) acetonitrile, 0.3% (v/v) TFA or 5 µl of 60% (v/v) acetonitrile, 0.2% (v/v) formic acid for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nanoelectrospray tandem mass spectrometry (nanoESI-MS/MS) respectively.

MALDI-MS measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, USA). One µl of the analyte solution was mixed with 1 µl of alpha-cyano-4-hydroxy cinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 ml of 0.3% TFA in acetonitrile-water (1:1, v/v). One µl of the resulting mixture was applied to the sample plate. Samples were air-dried at ambient temperature (24°C). Measurements were performed in the reflection mode at an acceleration voltage of 20 kV, 70% grid voltage and a delay time of 150 nsec. Each spectrum obtained was the mean of 150 laser shots.

For nanoESI-MS/MS 5 µl of the peptide mixture were lyophilised and dissolved in 5 µl methanol-1% formic acid (1:1, v/v). The MS/MS measurements were performed with a nanoelectrospray hybrid quadrupole mass spectrometer Q-Tof (Micromass, Manchester, UK). The collision gas was argon at a pressure of 6.0 x 10^{-5} mbar in the collision cell.

2.3.16 Database search.

To confer the identity of isolated protein MS-Fit search program was used (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). MS-Fit is a peptide mass fingerprinting tool that tries to fit a user's mass spectrometry data to a protein sequence in an existing database and thus suggest the identity of the user's protein. Two different databases were searched: Ludwignr. from 08.22.2003 and NCBInr. from 10.21.2003. Parameters used for the MS-Fit searched are:

Digest used:	Asp-N
Maximal missed clevages:	1
Cysteine modification:	acrylamide
Instrument name:	MALDI-TOF
Interpreting peptide masses as	**$[M+H]^+$**
Minimum matches:	5
Peptide masses:	monoisotopic
Mass tolerance:	+/- 0.08 Da
Possible modifications:	acrylamide modified Cys

The search identified *Rattus norvegicus* (rat) occludin as the only protein candidate present in the sample. (accession numbers 13786154 and AB016425 in NCBInr. and Ludwignr., respectively).

To predict potential phosphorylation, ExPASy FindMod program was used (http://www.expasy.org/tools/findmod/). FindMod compares the experimentally measured peptide masses with the theoretical occludin peptides calculated from a specified Swiss-Prot/TrEMBL entry and mass difference is used to predict potential protein post-translational modifications. Specifically, phosphorylation results in monoisotopic mass difference 79.9663 Da (average 79.9799 Da).

Parameters used for the MS-Fit searched are:

Swiss-Prot/TrEMBL ID:	Q9Z303
Enzyme used:	Asp-N
Maximal missed cleavages (#MC):	2
Cysteine modification:	nothing (in reduced form)
Interpreting peptide masses as	$[M+H]^+$
Peptide masses:	monoisotopic
Mass tolerance:	+/- 0.1 Da