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Automated SPR-LC−MS/MS System for Protein Interaction Analysis

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Abstract: We have developed a novel automated system to analyze protein complexes by integrating a surface plasmon resonance (SPR) biosensor with highly sensitive nanoflow liquid chromatography-tandem mass spectrometry (LC−MS/MS). A His-tagged protein, which is also tagged with FLAG and biotinylated sequences, was expressed in mammalian cells. After purification by using the His tag from the cell lysate, the sample protein mixture was applied to an SPR biosensor and the protein complex was captured on the sensor chip. The automated SPR-LC−MS/MS was then performed: (1) two-step on-chip purification of the protein complex by using the FLAG and the biotinylated tags, (2) on-chip protease digestion of the complex, and (3) online nanoflow LC−MS/MS analysis of the resulting peptide fragments for protein identification. All of these processes could be monitored in real-time by the SPR biosensor. We validated the performance of the system using either FK506-binding protein 52 kDa (FKBP52) or ribosomal protein S19 (rpS19) as bait. Thus, the fully automated SPR-LC−MS/MS system appeared to be a powerful tool for functional proteomics studies, particularly for snapshot analysis of functional cellular complexes and machines.

Keywords: surface plasmon resonance • liquid chromatography • mass spectrometry • proteomics • affinity chromatography • protein identification • tandem affinity purification • automated pull-down analysis

Introduction

Recently, a new concept for characterization of femtomolar amounts of protein complexes has emerged.[1−6] This new concept uses a combination of two analytical techniques: surface plasmon resonance (SPR) biomolecular interaction analysis (BIA) and mass spectrometry (MS) to perform SPR-MS. BIA technology is based on the SPR phenomenon and allows highly sensitive detection, real-time monitoring, and characterization of biomolecular interactions. It is widely used for determination of binding and equilibrium constants without destroying the protein sample. Briefly, one partner of an interaction is immobilized on a sensor chip and a solution containing the other partner(s) is then channeled through a flow cell in which one of the flow lines is connected to allow the analyte to pass over the immobilized partner. An interaction alters the index of refraction of the sensor surface, and this change is detected and measured in arbitrary units known as refractive units (RU). Subsequent elution of the ligand likewise affects the refractive index, and analysis of these changes allows calculation of ligand association and dissociation rate constants and the equilibrium dissociation constant. In the SPR-MS analysis, an SPR biosensor is used for ligand fishing. A ligand of interest, such as a protein or a chemical compound, is immobilized on the surface of the sensor chip. Cell extracts or biological fluids are passed over the sensor surface, leading to purification of proteins that specifically interact with the ligand. This ligand-fishing step is followed by enzymatic digestion of the proteins recovered from the sensor chip, which are physically removed from the SPR biosensor port, and the released peptide fragments are identified by MS analysis. The combined SPR-MS approach has been useful for identifying novel interacting proteins for target ligands.[1−6] On the other hand, there are a number of problems with this method: (1) dismantling of the SPR sensor chip and off-line proteolytic digestion is tedious, (2) nonspecific binding of proteins to the sensor surface and to the immobilized ligand can occur, (3) loss of proteins during their recovery on the sensor chip surface can occur, and (4) contamination of nonspecific proteins during manual handling can occur, possibly resulting in misidentification of the interacting proteins.[7]

Here, we present the development of a fully integrated SPR-LC−MS/MS system, in which an SPR biosensor, Biacore 3000, is interfaced with a highly sensitive liquid chromatography (LC)−tandem mass spectrometery (LC−MS/MS) system. The biosensor is used as an affinity chromatography system to isolate proteins interacting with the ligand as well as an apparatus to recover peptides generated from the proteins by

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protease digestion, in which an SPR detector provides quantitative estimates of the amount of bound proteins and recovered peptides. Thus, we used the instrument as a purification machine like columns arranged in sequence with an SPR detector instead of UV. Protein complexes were obtained from cultured cells by employing a novel expression vector that was constructed to isolate proteins bound to a bait protein and then the complexes were subjected to the SPR-LC−MS/MS system. We show that the system is feasible for characterization of protein complexes. Our SPR-LC−MS/MS instrumentation is automated and requires little user interaction after sample injection into the SPR biosensor, which results in decreased presence of contaminants and an overall improvement in reproducibility, thereby facilitating accurate identification of protein complexes.

Experimental Section

Materials. Standard chemical reagents were obtained from WAKO Pure Chemicals (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Sigma-Aldrich Chemicals (Steinheim, Germany). Rabbit anti-FLAG IgG, FLAG peptide, Dulbecco's modified Eagle's medium (DMEM), anti-FLAG M2 affinity gel, and IGEPAL CA630 were purchased from Sigma-Aldrich Chemicals. The amine coupling kit and Sensor Chip CM5 (research grade) were purchased from Biacore (Uppsala, Sweden). The human embryonic kidney cell line 293EBNA, Microto-Midi-Total RNA Purification System, High Fidelity Platinum *Taq* DNA Polymerase, ThermoScript RT-PCR System, oligonucleotides, and TEV protease (recombinant) were obtained from Invitrogen (Grand Island, NY). FITC-conjugated sheep anti-rabbit IgG was purchased from American Qualex Antibodies (Newington, NH). Restriction enzymes were obtained from TAKARA BIO, Inc. (Otsu, Japan). *Achromobacter lyticus* protease I (LEP) was acquired from WAKO Pure Chemicals. Vectashield mounting medium with DAPI was obtained from Vector Laboratories, Inc. (Burlingame, CA). Ni-NTA agarose was obtained from Qiagen GmbH (Hilden, Germany).

Construction of Expression Vectors for SPR-MS. A DNA fragment encoding a 78 amino acid peptide, which consisted of an amino-terminal His_6 tag fused to a sequence derived from the carboxyl terminus (amino acids 524−595) of the *Klebsiella pneumoniae* oxalacetate decarboxylase α subunit that can be biotinylated in mammalian cells,[8] was amplified by PCR using the plasmid pcDNA6/BioEase-DEST (Invitrogen) as a template. The primers used for amplification were the folowing: 5′-ATATAGCTAGCGCCACCATGCACCACCACCACCACCACGGCGCCGGCACCCCGGTG-3′ (forward) and 5′-TATATAAGCTTCGCCAGGGTCATCAGGGTGTC-3′ (reverse). The resulting DNA fragment was ligated into the pcDNA3.1(+) expression vector (Invitrogen) using *Nhe*I and *Hin*dIII and the resulting plasmid was designated as pcDNA3.1(+)-bio. A DNA fragment consisting of a multiple cloning sequence (*Hin*dIII-*Bam*HI-*Eco*RI-*Not*I-*Xho*I) for introduction of a target protein and encoding a TEV protease cleavage site (ENLYFQG)[9] with spacer sequences encoded on the amino and carboxyl termini, and encoding a FLAG sequence (DYKDDDDK) was amplified by PCR using oligonucleotides, 5′-ATATAAAGCTTGGATCCGAATTCGCGGCCGCCCTCGAGGATTATGATATTCCAACTACTG-3′ (forward), 5′-CGGTTTTGAGCTCACCCTGAAAATACAAATTCTCGCTAGCAGTAGTTGGAATATCATAAT-3′ (reverse), 5′-TTCAGGGTGAGCTCAAAACCGCGGCTCTTGCGCAACACGATGAAGCCGACTACAAGGACG-3′ (forward), and 5′-TATATTCTAGATTACTTGTCGTCGTCGTCCTTGTAGTCGGCTTCAT-3′ (reverse). The resulting DNA fragment was introduced between the *Hin*dIII and *Xba*I sites of pcDNA3.1(+)-bio and the resulting vector was designated as pDAP ("Double Affinity Purification" (DAP) tag). DNA fragments encoding human FK506-binding protein 52 kDa (FKBP52)[10] and human ribosomal protein S19 (rpS19) were amplified by PCR using cDNA templates prepared via RT-PCR of total RNA isolated from 293EBNA cells. Primer sets used for cDNA amplification were the following: 5′-ATATACTCGAGATGACAGCCGAGGAGATGAAGGCG-3′ (forward) and 5′-ATATACTCGAGTGCTTCTGTCTCCACCTGAGACTG-3′ (reverse) for FKBP52; 5′-ATATAGGATCCATGCCTGGAGTTACTGTAAAAGAC-3′ (forward) and 5′-ATATACTCGAGATGCTTCTTGTTGGCAGCTGCCAC-3′ (reverse) for rpS19. The resulting DNA fragments corresponding to FKBP52 and rpS19 were introduced into the *Xho*I site (for FKBP52) or between the *Bam*HI and the *Xho*I sites (for rpS19) of the pDAP expression vector and the resulting expression vectors were designated as pDAP-FKBP52 and pDAP-rpS19, respectively.

Cell Culture, Transfection, and Isolation of Protein Complexes. Cell culture and transfection of 293EBNA cells using the expression vectors described above were carried out as previously described.[11] Proteins associated with the bait were isolated by immunoprecipitation and analyzed by 12.5% SDS-PAGE followed by silver staining.[11] For SPR-LC−MS/MS analysis, 293EBNA cells grown on a square culture dish (25 cm × 25 cm) were transfected with the indicated expression vectors. At 48 h post-transfection, the cells were harvested, washed with PBS and then incubated in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% IGEPAL CA630, 1 mM phenylmethanesulfonyl fluoride) on ice for 30 min. The soluble fraction was obtained by centrifugation at 11 000*g* for 30 min at 4 °C and was then incubated with 400 μL of a 50% (v/v) Ni-NTA agarose bead slurry with gentle mixing overnight at 4 °C. After washing the beads twice with PBS, the bound proteins were eluted with 200 μL of PBS containing 1 M imidazole and subjected to SPR-LC−MS/MS analysis.

Immunocytochemistry. 293EBNA cells grown on 8-well glass coverslips (BECTON DICKINSON, Bedford, MA) were transfected with the indicated expression vectors. At 48 h post-transfection, the cells were fixed and then incubated with rabbit anti-FLAG IgG followed by incubation with FITC-conjugated sheep anti-rabbit IgG as described previously.[11] Coverslips were mounted with Vectashield mounting medium with DAPI and analyzed on an Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany).

Purification and On-Chip Digestion of Protein Complexes. Purification and digestion of proteins associated with the bait were accomplished using a Biacore 3000 SPR sensor (Biacore, Uppsala, Sweden). All assays were performed at room temperature. HEPES buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) containing 0.005% (w/v) *n*-octylglycopyranoside (OG) was used as the running buffer. The microfluidic system consisted of four detection surfaces located in four separate flow cells (FC1-FC4) that were accessible either one-by-one or serially in a multichannel mode. Anti-FLAG IgG and streptavidin were immobilized on the surface of the CM5 Research grade Sensor Chip (carboxymethyldextran derivatized surface) in FC1-FC2 and FC3-FC4, respectively, by the standard EDC/NHS [*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide] coupling method.[12] The immobilizing levels were adjusted to 3000−5000 RU for anti-FLAG IgG and to 8000−12000 RU for streptavidin. An aliquot (∼30 μL) of protein complex, preconcentrated using the His_6 tag as described above, was injected into the FC1-FC2 at a flow rate of 1 μL/min, allowing

the first purification step of the complexes on the surface of the sensor chip in the FC1-FC2 via interaction between the FLAG tag and anti-FLAG IgG. Then, the microfluidic system was rinsed sequentially with LEP buffer (Tris-HCl, pH 8.8, 2 M urea, 0.005% OG), 1% (v/v) formic acid, and water, by a MSprep program (Biacore, Uppsala, Sweden). Sequential aliquots of air (5 μL), TEV buffer (20 μL; 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$), air (5 μL), TEV buffer (3 μL), air (5 μL), TEV protease (0.5 units/μL) in TEV buffer (4 μL), air (5 μL), TEV buffer (20 μL), and air (3 μL) were aspirated into the inlet tubing at a flow rate of 5 μL/min and delivered to FC1-FC2, resulting in a surface exposure of the sensor chip to the TEV protease solution. Cleavage between the FLAG sequence and the bait protein was carried out for 30 min by oscillation of the TEV solution at an amplitude of 2 μL and a flow rate of 4 μL/min.[13] The protein complexes, which were released from the surfaces of FC1-FC2, were delivered to FC3-FC4 by an oscillatory flow at an amplitude of 2 μL and a flow rate of 4 μL/min for 60 min, allowing the second purification step of the complexes on the surfaces of the sensor chip in FC3-FC4 via affinity between the biotinylated tag and streptavidin. Then, the anti-FLAG IgG bound to the FLAG tag was removed by injecting 5 μL of 5 mM NaOH/150 mM NaCl, followed by washing with 10 μL of PBS. After this, two-step purification was repeated four times, 5 μL of a 10 μg/mL LEP solution in LEP buffer was delivered to the FC3-FC4 and the protein complexes were digested for 10 h at room temperature. Finally, the resulting peptides were delivered online to the nanoflow LC−MS/MS system and subjected to protein identification analysis. The whole process described above was automated using control software for Biacore 3000 instrument and the computer program is available from the authors by request.

Nanoflow LC−MS/MS Analysis and Protein Identification. The peptide mixtures, which were prepared by the two-step purification of the protein complexes and the following on-chip digestion, were delivered to the nanoflow LC system. A diagram of the flow lines that connect the Biacore 3000 instrument to the nanoflow LC system is shown ((Supplementary Figure 1). Fused-silica capillaries (150 μm i.d. × 375 μm o.d., GL Science, Tokyo, Japan) were used to connect the Biacore 3000 instrument, 6-position switching valves, a syringe pump, a trap column, and the LC system. The peptides were delivered from a recovery port of the Biacore 3000 instrument to the line between two 6-position switching valves (I and II) by an air partition method[3] (Supplementary Figure 1A) under control of the Biacore 3000 software. Then, the flow paths were switched electrically and the peptides were delivered to a trap column (0.5 mm i.d. × 1 mm, LC Assist, Tokyo, Japan) at a flow rate of 300 μL/min by the syringe pump with a stepping motor (KD Scientific, Holliston, MA) equipped with a 1-mL syringe that was filled with 0.1% (v/v) formic acid (Supplementary Figure 1B). Next, the peptides loaded onto the trap column were eluted and reloaded onto a fritless Mightysil-C18 column (3 μm particle, Kanto Chemical, Osaka, Japan).[14] The peptides were eluted with a gradient of acetonitrile 0−40% (v/v) in 0.1% formic acid over 80 min at a flow rate of 50 nL/min by the direct nanoflow LC−MS/MS system,[14] and sprayed directly into the ion source of a quadrupole time-of-flight instrument system (Q-Tof2, Micromass, Manchester, U.K.). MS/MS spectra were acquired by data-dependent collision-induced dissociation. Finally, the resulting fragment mass lists were submitted to automated database searches using the MASCOT software (Matrix Science, Wyndham Place, U.K.) to identify a peptide match. Criteria for match acceptance were as described.[14] The software STEM was used for protein identification.[15]

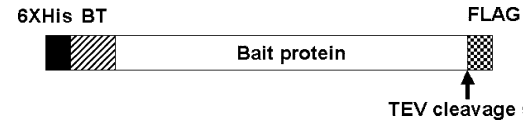

Figure 1. Schematic diagram of the affinity-tagged expression construct of the bait protein used for the SPR-MS experiment. The polyhistidine (His_6, 6XHis) and biotinylated tags (BT) are both located at the amino terminus of the bait protein. The FLAG tag was fused to the carboxyl terminus of the bait protein and the TEV protease cleavage site was introduced between them.

Results

Construction of an Expression Vector for the Bait Protein. We first designed a novel expression vector for the bait protein that contains two sequences encoding affinity purification tags that can be used for the automated tandem purification system (Figure 1). For the two-step purification process, sequences derived from the carboxyl terminus (amino acids 524−595) of the *K. pneumoniae* oxalacetate decarboxylase α subunit, which is biotinylated in mammalian cells,[8] and the FLAG peptide sequence (DYKDDDDK), were fused to the amino and carboxyl termini of the bait protein, respectively, to provide two affinity purification tags. The TEV-protease recognition sequence (ENLYFQG) (Dougherty et al.)[9] was also inserted between the bait protein and FLAG tag (Figure 1). In addition, a His_6 tag was attached to the amino terminus of the biotinylated tag to preconcentrate protein complexes from cell lysates prior to their purification with the Biacore 3000 instrument. When the expression vector is introduced into human cultured cells, the chosen bait protein is expressed and forms complexes with other proteins under physiological conditions. The complexes are preconcentrated from the cell lysates using the His_6 tag to eliminate large amounts of cellular proteins that bind nonspecifically to the flow lines and to the surface of the sensor chip that is covered with a covalently bound matrix (carboxymethylated dextran).

Cellular Localization of the Bait Proteins for Validation Testing. We selected two bait proteins for validation testing of the automated SPR-LC−MS/MS system: FKBP52[10] and rpS19. FKBP52, a member of the immunophilin family, is known to bind to the α and β subunits of the 90 kDa heat shock protein (hsp90), which allows steroid hormone receptor-mediated transcriptional activation of specific genes.[16] RpS19 is a component of the small ribosome subunit, but has yet to be used as an affinity bait for isolation of interacting proteins.

First, we investigated cellular localization of the tagged bait proteins expressed in 293EBNA cells by immunocytochemical analysis (Figure 2). For the control experiment, the expressed polypeptide consisting of the affinity tags without bait protein localized mainly in the cytoplasm and partly in the nucleus (Figure 2A−C). The majority of FKBP52 has been shown to be located in the nucleus; however, substantial amounts were also shown to exist in the cytoplasm.[17] As shown in Figure 2D−F, tagged FKBP52 revealed the same cellular localization as previously described, indicating that the tags do not alter the cellular localization of the bait protein. For rpS19, the tagged protein was detected in the nucleus (Figure 2G−I). In eukaryotic cells, ribosomal proteins can exist in the nucleolus and nucleus as components of synthetic intermediates of ribosomes

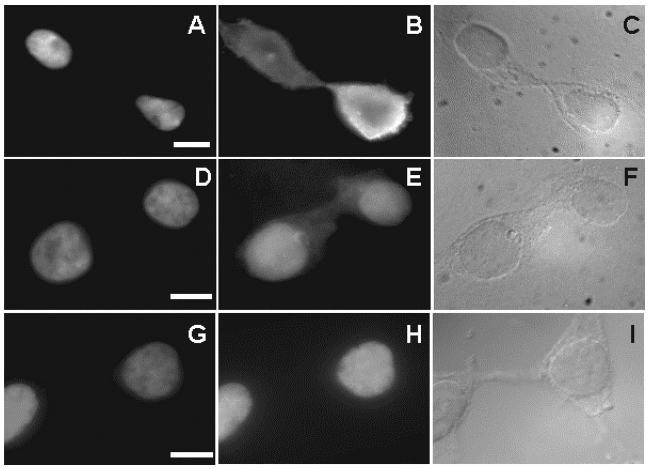

Figure 2. Cellular localization of tagged FKBP52 and rpS19. 293EBNA cells were transfected with pDAP alone (A−C), pDAP-FKBP52 (D−F), and pDAP-rpS19 (G−I). The cells were fixed and stained with DAPI (A, D, and G) or with a primary antibody against FLAG followed by an FITC-conjugated secondary antibody (B, E, and H). Transmitted images are shown in C, F, and I, respectively. Bars, 10 μm.

formed during ribosome biogenesis, whereas mature ribosomes function in the cytoplasm. The nuclear localization of tagged rpS19 suggests that overexpressed rpS19 is retained predominantly in the nucleus. However, it is possible that the antibody used for the immunochemical analysis cannot bind to the FLAG tag of rpS19 that is packed into mature ribosomes because of steric hindrance.

Automated Two-Step Purification and Digestion of the Complexes On-Chip. The essential components of the Biacore 3000 instrument are the sensor chip with a biospecific surface, the optical system responsible for generation and detection of the SPR signal, and a liquid handling system with an integrated microfluidic cartridge (IFC) for controlled transport of samples to the sensor surface. The IFC forms four parallel flow cells (FC1-FC4) on the sensor chip. Interaction between a ligand immobilized to the surface of the sensor chip and an analyte delivered to the surface can be investigated independently by real-time monitoring in each FC. In addition, a combination of either FC1-FC2 or FC3-FC4, for example, can be used as single flow cell units by controlling passage valves that direct the flow path into the IFC. For the first purification step, anti-FLAG was immobilized to the surface of the FC1-FC2 sensor chip; for the second purification step, streptavidin was immobilized to the surface of FC3-FC4.

Before application to the IFC of the Biacore 3000, the protein complexes were preconcentrated from the 293EBNA cell extracts using the His_6 tag, because high concentration of the complexes resulted in more efficient capture of the complexes in the first purification step. When the protein mixtures containing preconcentrated protein complexes were delivered to FC1-FC2, the bait-bound complexes were captured via the affinity between the FLAG tag and the anti-FLAG antibody (Figure 3A). To increase the capture efficiency, the flow rate of the sample delivery was minimized (1 μL/min). Proteins that were nonspecifically bound to the inner surface of flow lines connected to the FC1-FC2 were washed with formic acid, which was delivered by bypassing FC1-FC2.

Next, TEV protease solution was delivered to FC1-FC4 by an air partition method that prevents the enzyme from being

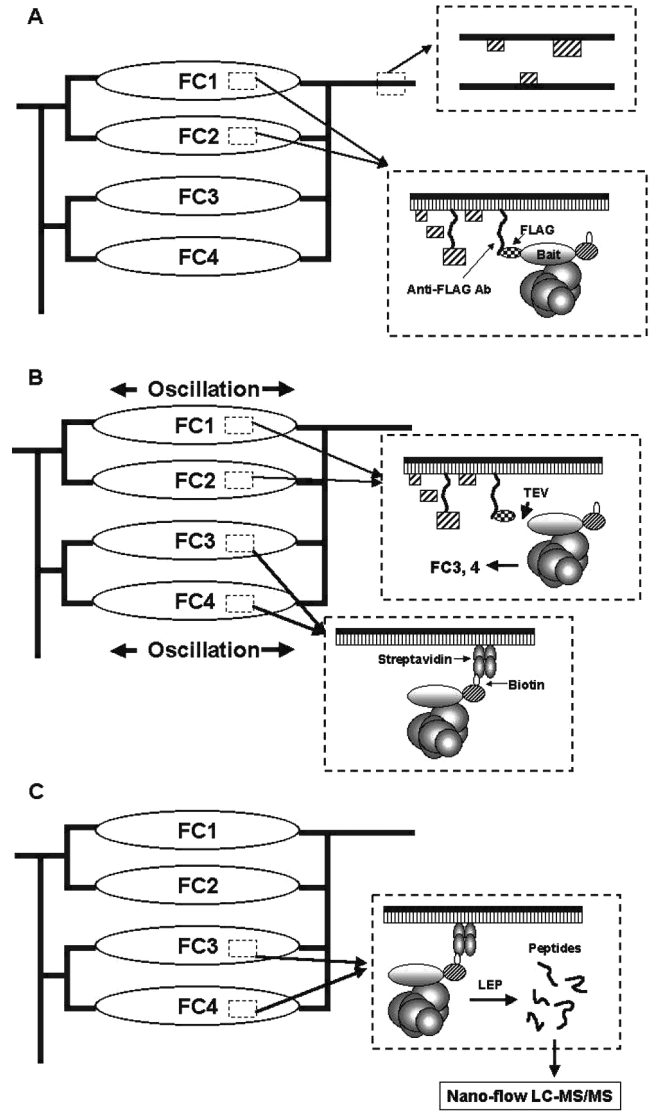

Figure 3. Overview of the two-step purification procedure and the on-chip digestion of the protein complexes using Biacore 3000. (A) Protein complexes associated with the bait protein are captured in FC1-FC2 by the affinity between the FLAG tag and anti-FLAG antibodies, which are immobilized on the surface of the sensor chip. Some proteins (shaded boxes) bind nonspecifically to the flow lines and to the dextran layers on the chip surface. (B) The complexes are released from the chip surface in FC1-FC2 by TEV protease cleavage, delivered to FC3-FC4, and captured by the affinity between biotin and streptavidin. Injection of the TEV protease solution and transfer of the released complexes to FC3-FC4 are performed with an oscillatory flow. (C) The complexes obtained by the two-step purification are subsequently digested by LEP and the generated peptides are delivered to the nanoflow LC−MS/MS system.

diluted[3] and the complexes were removed from the surface of FC1-FC2 by cleavage between the FLAG tag and the bait protein (Figure 3B). Then, the complexes were subjected to the second step purification in FC3-FC4 by the affinity between the biotinylated tag and streptavidin. When the TEV protease solution containing the protein complexes is delivered to FC3-FC4 by a continuous unidirectional flow, it is difficult to recover sufficient amounts of the protein complexes in part because the concentration of the complexes in the TEV protease solution is extremely low but also because the height of the

dextran layer, to which the affinity ligand is immobilized, is only about 1/200 of the depth of the flow cells so that most of the complexes delivered into the flow cell pass above the surface of the sensor chip. To solve these problems, the TEV protease solution containing bait complexes was delivered to the FC3-FC4 with oscillatory flow (Abrantes et al.),[13] which allows efficient mixing of the solution and long contact time between streptavidin and the biotinylated tag, resulting in efficient capture of the complexes in the FC3-FC4 (Figure 3B). The two-step purification was repeated four times to obtain sufficient quantities of the complexes for protein identification by mass spectrometric analysis. Finally, the protein complexes obtained by the two-step purification were digested on FC3-FC4 by the LEP solution, which was delivered to FC3-FC4 by the air partition method and held in the FCs (Figure 3C). We used LEP in place of trypsin, the most common protease in proteomic research. LEP has the advantage that it retains its enzymatic activity even in the presence of protein denaturant at high concentration while trypsin does not. On the other hand, LEP cleaves only carboxyl termini of Lys residues in polypeptides, while trypsin cleaves those of Lys and Arg residues; that is, trypsin can generate larger number of peptides from proteins than LEP. Therefore, trypsin may be suitable for accurate protein identification by MS/MS analysis. In fact, it was estimated that the numbers of peptides generated from FKBP52, for example, by LEP and trypsin, were 44 with average length of 7.1 residues and 64 with length of 10.4, respectively. However, we concluded that LEP could provide enough information, namely, numbers and length of peptides for accurate protein identification in MS/MS analysis, and that the advantageous feature of LEP rather could contribute to maximum recovery of peptides from small amount of the isolated proteins. A representative sensogram of the two-step purification is shown in Figure 4A and B and a representative sensogram of the resulting LEP-treated sample is shown in Figure 4C.

In the representative experiment with FKBP52 as the bait, complexes of about 3725 RU, on average, were captured from each of the initial purification cycles and a total of about 354 RU were collected after four cycles of the second purification step. The amount of peptides recovered by LEP digestion was approximately 148 RU, which corresponds to 148 pg. For the rpS19 experiment, complexes of about 8051 RU, on average, were captured in each initial purification step and a total of 353 RU were collected after four cycles of the second purification step. The amount of peptides recovered was about 193 RU, which corresponds to 193 pg.

Identification of Protein Components in the FKBP52- and rpS19-Associated Complexes. The peptides derived from protein components of the bait-associated complexes were analyzed by a direct nanoflow LC−MS/MS system at the femtomole level (Supplementary Figure 2) and protein identification was performed by a database search using MASCOT software.[14] Analysis of the automated SPR-MS for the FKBP52-associated complexes identified the α and β subunits of hsp90, tubulins, as well as FKBP52 (Table 1, Figure 5). The association between the hsp90 proteins and FKBP52 has been well-established,[10,16,18] whereas tubulins were only recently identified as FKBP52-interacting proteins.[19] The present SPR-MS analysis easily identified those tubulins as interactors of FKBP52. In addition, we detected chaperonin containing TCP1 (Tailless Complex Protein-1 subunits 2, 5, and 7). Although these proteins have not been reported to associate with

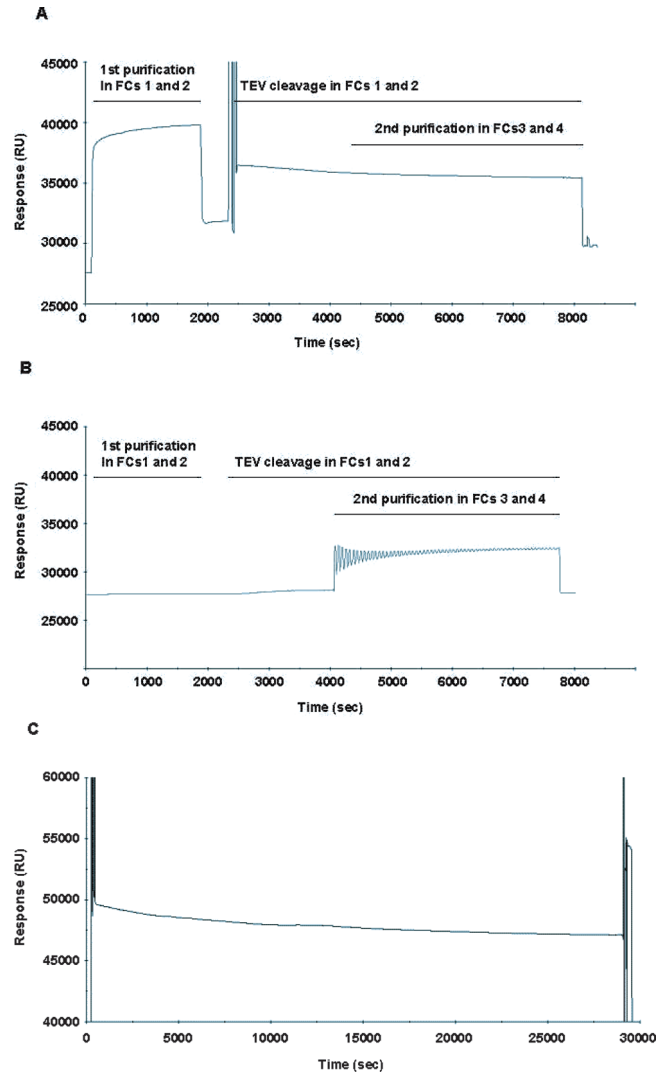

Figure 4. SPR sensograms demonstrating the two-step purification and the on-chip digestion of FKBP52-associated complexes. Representative SPR sensograms from FC1 and FC3 during the first-step purification of the FKBP52-associated complexes, their release from the surface of the sensor chip by TEV cleavage, and their second-step purification are shown in A and B, respectively. (C) Representative SPR sensorgram from FC3 during on-chip digestion of the isolated complexes.

FKBP52, they were probably obtained through an interaction with tubulins.[20] To support these observations, we manually purified the bait-associated complexes that formed in 293EBNA cells transfected with the pDAP-FKBP52 expression vector via the single step purification method using anti-FLAG-IgG-conjugated agarose beads. SDS-PAGE analysis of the FKBP52-associated complexes revealed two predominant protein bands (Figure 6A, lane 2), which were excised and subjected to in-gel digestion and MALDI-TOF/MS analysis: the upper band corresponded to α and β subunits of hsp90 and the lower band corresponded to FKBP52 (Supplementary Table 1). Shotgun analysis of the FKBP52-associated complexes identified seven other proteins, including hsp70 and Cdc37 and hsp90 α and β; however, tubulins and the chaperonin containing TCP1 subunits were not identified (data not shown). The absence of the tubulins and the chaperone containing TCP1 subunits, which were identified by the SPR-MS analysis, could be a result of difference in washing conditions of captured protein complexes

Table 1. Validation of the Automated SPR-MS System[a]

FKBP52-associated complexes
FK506-binding protein 4 (FKBP52)
heat shock 90 kDa protein 1, α
heat shock 90 kDa protein 1, β
tubulin, α, ubiquitous
tubulin, β polypeptide
chaperonin containing TCP1, subunit 2 (β)
chaperonin containing TCP1, subunit 5 (ϵ)
chaperonin containing TCP1, subunit 7 (η)
rpS19-associated complexes
Ribosomal Proteins
L3 L5 L10 L18 L18a L27a P0 S2 S3a S19
Trans-Acting Factors
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21
nucleolin
heterogeneous nuclear ribonucleoprotein U isoform b
Others
U2 small nuclear RNA auxiliary factor 1
β actin
tubulin, α, ubiquitous
tubulin, β polypeptide
heat shock 70 kDa protein 8 isoform 1

[a] Summarized proteins were identified from the automated SPR-MS analysis when FKBP52 and rpS19 were used as bait proteins (see Experimental Section). Peptides detected in one of two independent analyses of the complexes (Supplementary Tables 2−4) are listed. Keratin isoforms and proteins identified from the control experiments were excluded. Protein components of the rpS19-associated complexes are categorized into three functional groups (ribosomal proteins, trans-acting factors, and others).

(see Experimental Section). Cdc37 is known to associate with FKBP52;[21] however, the other proteins have not been reported to interact with FKBP52. The complex was prepared using only the single step purification method, which can isolate weak interactors and other nonspecifically associated contaminants. These results indicate that the SPR-LC−MS/MS analysis is a reliable method for isolation and identification of interaction partners of a bait protein expressed in mammalian cells.

For rpS19, SDS-PAGE analysis showed that single step purification of rpS19-associated complexes contained many protein-staining bands that span a broad range of molecular weights (Figure 6B, lane 2). Analysis of the automated SPR-LC−MS/MS for the rpS19-associated complexes identified 10 ribosomal proteins and three trans-acting factors, which are involved in ribosome biogenesis, and one protein that belongs to another functional category (Table 1). Protein identification was reliable because the main cellular localization of the exogenously expressed rpS19 was the nucleus, including the nucleolus, where ribosome biogenesis occurs. Independent of this analysis, we have shown that the rpS19-associated complex was a preribosomal complex that is formed during ribosome biogenesis in the nucleus.[11,22−24] Although the SPR-LC−MS/MS analysis did not identify the complete range of proteins that were identified by other experimental methods (manuscript in preparation), the SPR-LC−MS/MS system was sufficient for automated protein interaction analysis with high accuracy.

Discussion

Protein interaction analysis requires reliable methods for the purification of protein complexes from crude protein mixtures

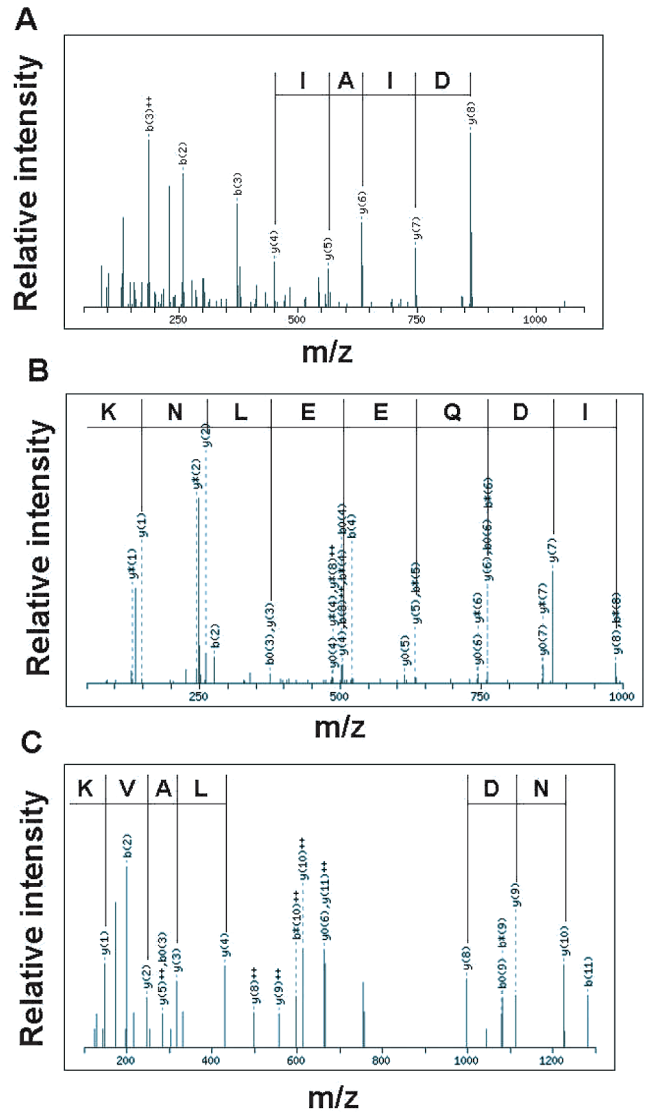

Figure 5. Representative MS/MS spectra used for identification of FKBP52, hsp90 α, and hsp90 β in the SPR-MS analysis of FKBP52-associated proteins. The doubly charged ions at m/z = 560.3615 (A; FKBP52), 576.3413 (B; hsp90 α), and 764.4380 (C; hsp90 β) separated by LC were fragmented, resulting in production of an ion series containing the carboxyl termini (y series). The sets of ion series yielded the amino acid sequences, AWDIAIATMK, YIDQEELNK, and SLTNDWEDHLAVK, which correspond to amino acids 88−97 of FKBP52, 283−291 of hsp90 α, and 306−318 of hsp90 β, respectively.

such as cell lysates. Tandem affinity purification (TAP) technology, where protein complexes containing a bait protein are purified in two steps using a TAP-tag composed of the calmodulin-binding peptide and the IgG binding unit of protein-A of *Staphylococcus aureus* connected by a TEV protease cleavage sequence, has been used extensively for this purpose.[25] In that method, the protein complex bound with a TAP-tagged bait protein was recovered from cell extracts first by affinity selection on an IgG matrix and released by TEV protease, trapped again with calmodulin-coated beads in the presence of calcium ion, and finally recovered by addition of EGTA. This method provided highly purified protein complexes, and has been applied successfully to the large-scale survey of protein complex networks in yeast cells.[26] To purify protein complexes bound to the SPR sensor chip of Biacore 3000, we

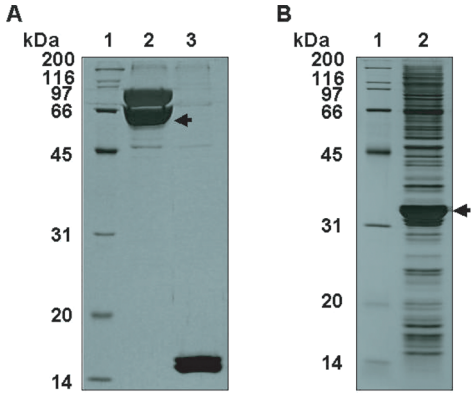

Figure 6. Isolation of FKBP52- and rpS19-associated complexes. FKBP52- and rpS19-associated complexes were immuno-isolated using the FLAG tag from 293EBNA cells transfected with the corresponding expression vectors. The purified protein complexes were subjected to 12.5% SDS-PAGE followed by silver staining. 293EBNA cells transfected with pDAP were used as a control. (A) Lane 1, molecular mass marker; lane 2, FKBP52-associated complexes; lane 3, control experiment. (B) Lane 1, molecular weight marker; lane 2, rpS19-associated complexes. Molecular masses are shown on the left and the bait proteins are indicated by arrows.

initially adopted a single FLAG tag; however, the microfluidic flow lines were heavily contaminated with cellular impurities even after the sample was previously affinity-purified by a His-tag-based pull-down assay. The TAP tag was not necessarily suitable for the automated SPR-LC−MS/MS technology, due to difficulties in expressing the TAP-tagged bait proteins in mammalian cells.[25] Moreover, addition of EGTA during the second step of purification could lead to removal of proteins from the complex that require metal ions such as Ca^{2+} and Mg^{2+} for binding. Thus, we developed a novel tandem affinity expression tag which allowed purification of the tagged protein complex on a sensor chip for Biacore instruments. Our DAP tag has a relatively small size compared to the original TAP tag and did not affect the subcellular localization of FKBP52 (Figure 2). In addition, the automated SPR-LC−MS/MS led to identification of a number of known binding partners of FKBP52, suggesting that the DAP tag did not impair protein function and that the method allowed analysis of physiologically significant protein interactions. We also demonstrated that the analysis of protein complexes associated with one of the ribosomal proteins, rpS19, resulted in the identification of many nucleolar components, which are typical of a preribosomal particle formed during ribosome biogenesis.

However, the current SPR-LC−MS/MS technology requires further improvement. First, the sample binding capacity of the SPR sensor chip needs to be increased for the analysis of less abundant protein complexes in the cell. This could be achieved by immobilizing the tagged ligand at higher densities to the surface of the sensor chip (e.g., setting longer coupling times with higher ligand concentrations). It may also be effective to use tags that bind to their respective ligands with a lower dissociation constant (K_d). While streptavidin has one of the highest affinities for it is ligand (K_d of $\sim 10^{-15}$ M^{-1}) among known biomolecular interactions, the anti-FLAG M2 antibody has an affinity with a K_d of $\sim 10^{-6}$ M^{-1}. Therefore, the FLAG tag may need to be replaced by alternative tags with stronger binding affinity to prevent the loss of protein complexes during the on-chip purification. Second, the protein complexes captured on the sensor chip need to be stabilized for comprehensive protein identification. Although the automated SPR-LC−MS/MS allowed the identification of many rpS19-associated components as described above, our extensive snapshot analyses of mammalian ribosome biogenesis proved that the rpS19-associated preribosome particle contained several more protein components (manuscript in preparation). This suggested that a portion of the components were dissociated from the complex during the purification process. This might be the most difficult problem of the affinity-based protein interaction analysis to overcome; however, we hypothesize that an additional process, such as chemical cross-linking of the protein complex captured on the sensor chip, will prevent the loss of components and increase the protein coverage and recovery.

Recent comprehensive analyses of protein interactions have provided vast amounts of information on the protein networks of the cell and have demonstrated how proteins are assembled together into complexes to carry out cellular functions. In the past, most protein complexes were viewed as static; however, a new concept is emerging in which protein complexes are considered to regulate cellular functions by dynamic changes with various interacting components. This implies that the protein complexes acquire a specific function essential to cells only by changing a portion of their components, and therefore, the components of a particular complex may vary depending on the physiological conditions of the cell. In this context, extensive snapshot analyses that can describe the dynamic aspects of cellular protein complexes and machines have become a major focus of functional proteomics. We expect that the automated SPR-LC−MS/MS system described here will serve as a tool for collecting protein complex snapshots and thereby facilitate our understanding of cellular functions under physiological as well as pathological conditions.

Conclusions

We described here an integrated SPR-LC−MS/MS system for fully automated analysis of protein complexes and interactions. Although the technique still needs improvement with respect to protein coverage and recovery, the advantages of its real-time selection of protein interactions and simultaneous identification of protein complexes will open new opportunities for the snapshot analysis of protein complexes, allowing our understanding of many cellular processes controlled by networked proteins.

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Supporting Information Available: Schematic diagram of the interface between Biacore 3000 and nanoflow LC (Supplementary Figure 1). In the SPR-LC−MS/MS system, Biacore 3000 and nanoflow LC are interfaced by two six-port valves (I and II), which direct the flow path of the system. The detailed procedure for the delivery of the peptide mixtures from Biacore to the trap column with the interface is described in the Experimental Section. Ion chromatograms for nanoflow LC−MS/MS analysis (Supplementary Figure 2). The peptides generated from the two-step purification and on-chip digestion of the FKBP52-associated protein complexes with Biacore 3000 were automatically analyzed by nanoflow LC−MSMS system.

Four precursor peptide ions were selected for parallel data-dependent collision-induced dissociation. A chromatogram of the total ion count (TIC) and the MS/MS chromatograms (MS/MS1−4) of base peak intensity (BPI) are shown. Supplementary Table 1 indicates the FKBP52-associated proteins by MALDI-TOF/MS. Peptides detected in each of the two independent analyses of the FKBP52- and rpS19-associated complexes are listed in Supplementary Tables 2−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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