Chip-Based Analysis of Protein−Protein Interactions by Fluorescence Detection and On-Chip Immunoprecipitation Combined with μLC−MS/MS Analysis

ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 2003

Impact Factor: 5.64 · DOI: 10.1021/ac034258u · Source: PubMed

10 AUTHORS, INCLUDING:

Maria Mariano
Illumina
2 PUBLICATIONS **52** CITATIONS
SEE PROFILE

Peter Wagner
Zyomyx
34 PUBLICATIONS **2,656** CITATIONS
SEE PROFILE

Anal. Chem. **2003,** *75,* 6163−6170

Chip-Based Analysis of Protein−Protein Interactions by Fluorescence Detection and On-Chip Immunoprecipitation Combined with μLC−MS/MS Analysis

Jens R. Sydor,[†,‡] Mark Scalf,[†] Steve Sideris,[†] Guo Dong Mao,[§] Yash Pandey,[†] Ming Tan,[†] Maria Mariano,[†] Michael F. Moran,[§] Steffen Nock,[†] and Peter Wagner*[,†]

Zyomyx, Inc., 26101 Research Road, Hayward, California 94545, and MDS Proteomics Inc., 251 Attwell Drive, Toronto, Ontario, Canada M9W 7H4

A new chip-based method to identify protein−protein interactions was developed using the guanine nucleotide exchange factor GRF2 and two interacting proteins, Ras and calmodulin, as model proteins. A generic immobilization strategy for FLAG-tagged bait proteins on a protein-repellent streptavidin chip surface was implemented by presentation of an oriented anti-FLAG antibody. A flow cell device, integrating different chip surfaces, was developed, and the interaction of immobilized GRF2 with the two analytes was verified by fluorescence assays. On-chip tryptic digest assays were then performed on the capture surface and analyzed by μLC−MS/MS. The interaction of GRF2 with calmodulin and Ras was demonstrated, and the lower limit of detection was determined. We also implemented an on-chip immunoprecipitation assay to identify GRF2-binding partners from complex protein mixtures. Cells overexpressing FLAG-GRF2 were lysed and then incubated with the anti-FLAG chip. In addition to detecting GRF2, we also identified calmodulin, demonstrating that this technique can successfully identify endogenous levels of proteins, bound to recombinant bait proteins. This chip-based method has the advantage that no subsequent gel separations of protein complexes prior to LC−MS analysis are required and is therefore amenable to miniaturized high-throughput determination of protein−protein interactions.

The elucidation of protein−protein interactions is one of the most challenging but important tasks in proteomics research. A variety of techniques have been developed for the investigation of these interactions. The two-hybrid system and protein complementation assays are commonly used to detect binary protein interactions[1−6] whereas coimmunoprecipitation assays can be applied to identify protein complexes.[7,8] Immunoprecipitation assays in combination with mass spectrometry have recently been demonstrated to be a very powerful technique for identifying components of protein complexes.[9,10] In these studies, bait proteins, containing a common tag sequence, are overexpressed in cells. After immunoprecipitation or affinity purification of the bait protein complexes by a strategy using the common tag, proteins are separated by SDS−PAGE, excised, and digested by endoproteinases, most commonly trypsin. The resulting peptide fragments are then separated and sequenced by μLC−MS/MS to identify the individual components. However, this approach requires a significant amount of material and labor due to the large number of manipulations involved. To save precious samples and reduce manual interventions, it is desirable to combine miniaturized devices with μLC−MS/MS for the isolation, digestion, and identification of protein complexes.

* To whom correspondence should be addressed. Phone: +1-510-266-7503. Fax: +1-510-266-7792. E-mail: peter.wagner@zyomyx.com.

[†] Zyomyx, Inc.

[‡] Current address: Infinity Pharmaceuticals Inc., 780 Memorial Drive, Cambridge, MA 02139.

[§] MDS Proteomics Inc.

(1) Fields, S.; Song, O. *Nature* **1989**, *340*, 245−246.

(2) Uetz, P.; Giot, L.; Cagney, G.; Mansfield, T. A.; Judson, R. S.; Knight, J. R.; Lockshon, D.; Narayan, V.; Srinivasan, M.; Pochart, P.; Qureshi-Emili, A.; Li, Y.; Godwin, B.; Conover, D.; Kalbfleisch, T.; Vijayadamodar, G.; Yang, M.; Johnston, M.; Fields, S.; Rothberg, J. M. *Nature* **2000**, *403*, 623−627.

(3) Ito, T.; Chiba, T.; Ozawa, R.; Yoshida, M.; Hattori, M.; Sasaki, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4569−4574.

(4) Pelletier, J. N.; Campbell-Valois, F.-X.; Michnick, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12141−12146.

(5) Wehrman, T.; Kleaveland, B.; Her, J.-H.; Balint, R. F.; Blau, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3469−3474.

(6) Remy, I.; Galarneau, A.; Michnick, S. W. *Methods Mol. Biol.* **2002**, *185*, 447−459.

(7) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Press: Cold Spring Harbor, NY, 1988.

(8) Figeys, D.; McBroom, L. D.; Moran, M. F. *Methods* **2001**, *24*, 230−239.

(9) Ho, Y.; Gruhler, A.; Heilbut, A.; Bader, G. D.; Moore, L.; Adams, S.-L.; Millar, A.; Taylor, P.; Bennett, K.; Boutilier, K.; Yang, L.; Wolting, C.; Donaldson, I.; Schandorff, S.; Shewnarane, J.; Vo, M.; Taggart, J.; Goudreault, M.; Muskat, B.; Alfarano, C.; Dewar, D.; Lin, Z.; Michalickova, K.; Willems, A. R.; Sassi, H.; Nielsen, P. A.; Rasmussen, K. J.; Andersen, J. R.; Johansen, L. E.; Hansen, L. H.; Jespersen, H.; Podtelejnikov, A.; Nielsen, E.; Crawford, J.; Poulsen, V.; Sorensen, B. D.; Matthiesen, J.; Hendrickson, R. C.; Gleeson, F.; Pawson, T.; Moran, M. F.; Durocher, D.; Mann, M.; Hogue, C. W. V.; Figeys, D.; Tyers, M. *Nature* **2002**, *415*, 180−183.

(10) Gavin, A.-C.; Bösche, M.; Krause, R.; Grandi, P.; Marzioch, M.; Bauer, A.; Schultz, J.; Rick, J. M.; Michon, A.-M.; Cruciat, C.-M.; Remor, M.; Höfert, C.; Schelder, M.; Brajenovic, M.; Ruffner, H.; Merino, A.; Klein, K.; Hudak, M.; Dickson, D.; Rudi, T.; Gnau, V.; Bauch, A.; Bastuck, S.; Huhse, B.; Leutwein, C.; Heurtier, M.-A.; Copley, R. R.; Edelmann, A.; Querfurth, E.; Rybin, V.; Drewes, G.; Raida, M.; Bouwmeester, T.; Bork, P.; Seraphin, B.; Kuster, B.; Neubauer, G.; Superti-Furga, G. *Nature* **2002**, *415*(6868), 141−147.

10.1021/ac034258u CCC: $25.00
Published on Web 10/10/2003

Several approaches have been explored to perform tryptic digestion of proteins within microfluidic devices. One of the most advanced integrated systems has been reported by Wang et al., where trypsin-immobilized beads are packed into a reservoir of a glass chip for rapid digestion of proteins.[11] The resulting peptides were separated by on-chip CE and analyzed by ESI-MS from the chip. Other examples of on-chip processing include sample digestion by trypsin adsorbed onto a porous membrane in the flow channel of a microfluidic device[12] or by utilizing a sample reservoir on the chip to incubate the trypsin and protein samples, followed by separation on a glass chip and analysis by ESI-TOF-MS.[13] Another integrated system uses matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) detection in conjunction with a porous surface digestion chip.[14] A piezoelectric dispenser was used to deposit digested samples and matrix into nanovials on a MALDI target plate. All these approaches, however, lack the ability to integrate the isolation of protein complexes with a subsequent tryptic digest followed by MS analysis.

We addressed this issue by developing a novel chip assay format, in which bait proteins are immobilized on a protein-repellent chip surface. Upon incubation with the biological sample, interacting proteins are captured, which are then analyzed by on-chip tryptic digestion and subsequent μLC−MS/MS analysis. We used these assay formats to investigate the interaction of the guanine nucleotide release factor GRF2 with its interaction partners Ras and calmodulin.[15−17] FLAG-tagged GRF2 was affinity-immobilized on different chip surfaces, and the interaction with Ras and calmodulin was verified by fluorescence assays and by on-chip tryptic digest followed by μLC−MS/MS analysis. In addition, we were able to coimmunoprecipitate and identify FLAG-GRF2 together with one of its interaction partners, calmodulin, directly from mammalian cell lysates.

EXPERIMENTAL SECTION

Materials. Restriction enzymes for generating the FLAG-tagged GRF2 construct were from New England Biolabs (Beverly, MA). Anti-FLAG M2 agarose, HEPES, sodium chloride, ethylenediaminetetraaceticacid (EDTA), ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), monobasic sodium phosphate, and Tween-20 were all purchased from Sigma Chemical Co. (St. Louis, MO). AlexaFluor$_{594}$-labeled calmodulin was purchased from Molecular Probes (Eugene, OR). Pierce Chemical Co. (Rockford, IL) was the supplier of streptavidin. Nonidet P-40 (NP-40) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Roche Diagnostics (Indianapolis, IN). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and L-glutamine were purchased from Invitrogen Canada (Burlington ON, Canada). Anti-FLAG M2 agarose, FLAG peptide, anti-FLAG antibody, aprotinin, leupeptin, AEBSF, and other chemicals were all purchased from Sigma Chemical Co.

Expression and Purification of FLAG-Tagged GRF2 and TAMRA-Labeled Ras. The full-length murine Ras-GRF2 was cloned as described previously.[15] Human embryonic kidney 293 cells were grown in DMEM containing 10% FBS, 0.29 g of L-glutamine/L, and 100 μM nonessential amino acids. The 293 cells were transfected with pcDNA3-FLAG-GRF2 by calcium phosphate precipitation as described previously.[18] Stable transformants were selected in G418-containing (400 μg/mL) medium, and the expression of FLAG-GRF2 was detected by western immunoblot analysis using anti-FLAG antibody.

To purify FLAG-tagged GRF2, stably transfected 293 cells were washed with ice-cold Tris saline and lysed with 20 mM HEPES (pH 7.4) containing 100 mM NaCl, 0.5% NP-40, 0.1% CHAPS, 1 mM EDTA, 3 mM EGTA, 0.2 mM AEBSF, 1.0 μg/μL leupeptin, and 1.5 μg/μL aprotinin. Lysates were clarified by centrifugation at 27000*g* for 30 min and incubated with anti-FLAG M_2 agarose (1 mg of lysate protein: 4 μL of M_2 agarose) for 4 h at 4 °C with agitation. The slurry was then loaded into a column and washed with 20 volumes of lysis buffer and 10 volumes of washing buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, and 0.1% CHAPS). FLAG-GRF2 was eluted from the column with washing buffer containing FLAG-peptide (400 μg/mL) and analyzed by SDS−PAGE. FLAG-GRF2-containing fractions were collected and dialyzed against 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, 0.1% CHAPS, and 10% glycerol, frozen in liquid nitrogen, and stored in −80 °C.

Full-length human H-Ras was expressed as an intein fusion protein and labeled with a TAMRA-containing peptide as previously described.[19]

Preparation of Site-Specifically Biotinylated Anti-FLAG Antibody and Fab Fragment. The site-specific biotinylation of the anti-FLAG antibody by derivatization of oxidized sugar moieties on the Fc region of the antibody and the site-specific biotinylation of the Fab fragment by a reaction of the single thiol group in the hinge region was performed as described elsewhere.[20]

Preparation of Protein-Repellent Surfaces and Assembly of Flow Cell Device. The protein-repellent surfaces were prepared on Au or TiO_2 chips. Au surfaces were prepared with a biotinylated self-assembled monolayer formed on a gold-coated glass surface by immersion of 15 mm × 25 mm (flow cell device) or a 10 mm × 10 mm chips (planar chips) in an ethanolic solution of an unsymmetrical alkane disulfide.[20] The ω functionalities of the oligo(ethyleneglycol)-containing alkane disulfides are *N*-hydroxysuccinimide and methoxy groups. This monolayer was then reacted with tri(ethylene glycol) amino biotin to give a biotinylated surface. The biotin groups on the surface allowed for the binding of streptavidin (SA).

(11) Wang, C.; Oleschuk, R.; Ouchen, F.; Li, J.; Thibault, P.; Harrison, D. J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1377−83.

(12) Gao, J.; Xu, J.; Locascio, L. E.; Lee, C. S. *Anal. Chem.* **2001**, *73*, 2648−2655.

(13) Lazar, I. M.; Ramsey, R. S.; Ramsey, J. M. *Anal. Chem.* **2001**, *73*, 1733−1739.

(14) Ekström, S.; Önnerfjord, P.; Nilsson, J.; Bengtsson, M.; Laurell, T.; Marko-Varga, G. *Anal. Chem.* **2000**, *72*, 286−293.

(15) Fam, N. P.; Fan, W.-T.; Wang, Z.; Zhang, L.-J.; Chen, H.; Moran, M. F. *Mol. Cell. Biol.* **1997**, *17*, 1396−1406.

(16) Fan, W.-T.; Koch, C. A.; de Hoog, C. L.; Fam, N. P.; Moran, M. F. *Curr. Biol.* **1998**, *8*, 935−938.

(17) De Hoog, C. L.; Fan, W.-T.; Goldstein, M. D.; Moran, M. F.; Koch C. A. *Mol. Cell. Biol.* **2000**, *20*, 2727−2733.

(18) Stone, J. C.; Moran, M. F.; Pawson, T. *Methods Enzymol.* **1991**, *200*, 673−692.

(19) Sydor, J. R.; Mariano, M.; Sideris, S.; Nock, S. *Bioconjugate Chem.* **2002**, *13*, 707−712.

(20) Peluso, P.; Wilson, D. S.; Do, D.; Tran, H.; Venkatasubbaiah, M.; Quincy, D.; Heidecker, B.; Poindexter, K.; Tolani, N.; Phelan, M.; Witte, K.; Jung, L. S.;Wagner, P.; Nock S. *Anal. Biochem.* **2003**, *312*, 113−124.

TiO_2 chips were prepared by incubating 15 × 25 mm silicon chips or 10 × 10 mm silicon chips (tryptic digest assays) containing a 20-nm layer of TiO_2 with 1 mg/mL PLL-*g*-PEG-B30% for 1 h at room temperature as described previously.[21] The chips were washed with 50 mL of PBS and then incubated with premixed 0.4 μM streptavidin and 0.8 μM anti-FLAG Fab in PBS for 1 h at room temperature.

A flow cell device was positioned over the 25 mm × 15 mm chips to create four individual flow cells for the fluorescence or tryptic digest assays. For this purpose, an EPDM O-ring was used to create the channels with a surface area of ~0.35 cm^2 between the chip and a PMMA coverslip.

BIAcore Studies. All surface plasmon resonance assays were performed on the monolayer-coated Au surface in a BIAcore 3000 at 25 °C in PBS with 0.05% Tween-20. Streptavidin was loaded onto the surface at a flow rate of 30 μL/min at 0.1 mg/mL until the surface was completely saturated. After SA deposition, the different biotinylated anti-FLAG antibodies were loaded at 50 μg/mL concentration with a flow rate of 30 μL/min until saturation was observed. For FLAG-GRF2 binding, flow rates of 30−50 μL/min were used. The surface was subsequently washed to determine the stability of the anti-FLAG antibody/FLAG-GRF2 complex.

Chip-Based Fluorescence Assays in the Flow Cell Device. For fluorescence assays using the flow cell device, TiO_2 surfaces were favored over the Au surfaces due to the fluorescence-quenching properties of Au. Nucleotide-bound Ras assays were performed in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, 0.1% CHAPS, 10% glycerol, 1 mM $MgCl_2$, 10 μM GDP. Nucleotide-free Ras assays were performed in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, 0.1% CHAPS, 10% glycerol, 10 mM EDTA. A buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween-20, and 100 μM $CaCl_2$ was used for the calmodulin assays.

The flow cell assays were performed on 15 mm × 25 mm TiO_2-coated silicon chips that were placed into the flow cell device after the streptavidin/antibody complex had been immobilized. After assembly of the device, 100 μL of 10 mM biotin was flowed through each flow cell at 50 μL/min to block any remaining streptavidin sites. Flow cells were washed with 500 μL of buffer at 100 μL/min to remove excess biotin. FLAG-tagged GRF2 was immobilized on the anti-FLAG chip surface by flowing 100 μL of 10 μg/mL FLAG-GRF2 through the appropriate channels at 10 μL/min. After a 5-min buffer wash (500 μL of buffer at 100 μL/min), 500 nM TAMRA-labeled Ras or AlexaFluor594-labeled calmodulin flowed through the appropriate channels. Final buffer washes of 200 μL at 100 μL/min for Ras and 200 μL at 200 μL/min for calmodulin were performed through each flow cell to eliminate any unbound protein. Chips were subsequently scanned using a confocal fluorescence scanner (ScanArray 5000; GSI Lumonics, Billerica, MA) with a 543-nm excitation laser and a 578-nm emission filter.

On-Chip Immunoprecipitation and Tryptic Digest Assays. For the on-chip tryptic digest assays on planar chips (without flow cell device in a humidity chamber), 100 μL of 10 μg/mL FLAG-GRF2 in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% NP-40, 0.1% CHAPS, and 10% glycerol was incubated on the chips for 1 h. Chips were rinsed with 10 mL of PBS to remove unbound GRF2. A total of 100 μL of 500 nM Ras, 500 nM calmodulin, or mammalian cell lysate was added to the chips and incubated at room temperature for 1 h. The chips were subsequently washed with 25 mL of the appropriate assay buffer and 25 mL of 100 mM NH_4HCO_3, pH 7.8. They were then incubated with 100 μL of 100 nM trypsin in 100 mM NH_4HCO_3, pH 7.8, at 37 °C for 2 h in a humid environment. Digested samples were recovered and analyzed by μLC−MS/MS.

Tryptic digest flow cell assays were performed on a 15 mm × 25 mm Au or TiO_2-coated silicon in the flow cell device in a way similar to the fluorescence assays. After assembly of the device, 100 μL of 10 mM biotin flowed through each flow cell at 50 μL/min to block any remaining streptavidin sites. Flow cells were washed with 500 μL of buffer at 100 μL/min to remove excess biotin. A total of 100 μL of 10 μg/mL FLAG-GRF2 subsequently flowed through the appropriate flow cells at 10 μL/min, followed by an immediate wash with 500 μL of buffer at 100 μL/min. A total of 100 μL of 500 nM calmodulin, nucleotide-bound Ras, or nucleotide-free Ras then flowed through the appropriate flow cells at a rate of 10 μL/min. The final buffer wash was performed using 150 μL of 100 mM NH_4HCO_3, pH 7.8 at a flow rate of 100 μL/min. Trypsin solution (~50 μL) was then introduced into each channel. The flow rate for trypsin was 100 μL/min at a concentration of 100 nM. The entire flow cell device was then incubated at 37 °C for 2 h to allow for trypsin cleavage of bound proteins. Drying out of the chip surface during all steps was carefully avoided. Samples were recovered from the flow cell device and were analyzed by μLC−MS/MS.

Chromatography and Mass Spectrometry. The μLC−MS/MS system consists of an Agilent 1100 capillary HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Finnigan LCQ deca (Thermo Finnigan) ion trap mass spectrometer. A home-built nanospray source was used in place of the standard ESI source provided with the instrument. The nanospray source consists of a modified optics stage used to position a 365 μm o.d. × 75 μm i.d. × 15 μm tip diameter Picofrit column (New Objective, Cambridge, MA) packed with 5 cm of BetaSil C18 beads (5-μm diameter) (Keystone Scientfic, Bellefonte, PA) in front of the entrance to the heated desolvation capillary of the mass spectrometer. High voltage (1.8 kV) was applied through a stainless steel union (Western Analytical Products, Murietta, CA) connecting the HPLC with the nanospray source.

The elution profile ramped from 0 to 70% acetonitrile in 0.25% acetic acid over 72 min, at flow rates ranging from 1 μL/min (loading of sample) down to 300 nL/min. The ion trap mass spectrometer was set up to run in "triple-play" mode. A full-mass scan was performed between 400 and 2000 m/z, followed by a full-mass "zoom" scan focused in a 10 amu window around the parent ion in order to obtain charge-state information by determining the isotopic distribution of the parent ion, followed by full MS/MS scans of the parent ion at 40% relative collision energy. Dynamic exclusion was enabled with a repeat count of 3, an exclusion duration of 3 min, and a repeat duration of 4 min.

The aquired MS/MS spectra were searched against a non-redundant human protein database using the SEQUEST program (Thermo Finnigan). Proteins were considered positively identified

(21) Ruiz-Taylor, L. A.; Martin, T. L.; Zaugg, F. G.; Witte, K.; Indermuhle, P.; Nock, S.; Wagner, P. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 852−857.

if two or more unique peptides received Xcorr scores of 2.5 or greater.

RESULTS AND DISCUSSION

Surface Immobilization Strategy for the FLAG-Tagged Bait Proteins. We developed a generic immobilization strategy for bait proteins on biochip surfaces using GRF2, modified to contain an amino-terminal, seven amino acid FLAG epitope sequence, as a model. The strategy relies on the immunoprecipitation of FLAG-tagged proteins and their interaction partners from complex mixtures onto an anti-FLAG tag antibody-derivatized surface. The advantage of this approach is that it can be used to capture any recombinant FLAG-tagged protein directly from cell lysates together with interaction partners, as well as for an immobilization of tagged proteins themselves without any need for prior purification or modification.

It has been shown that oriented presentation of antibodies on planar surfaces leads to greater antigen-binding capacity than does random orientation.[20] Therefore, we investigated the performance of three different anti-FLAG antibody preparations: site-specific biotinylation of the antibody by derivatization of oxidized sugar moieties on the Fc region of the antibody, site-specific biotinylation of the Fab fragment by a reaction of the single thiol group in the hinge region, and randomly biotinylated antibody.

Immobilization of the Fab or Ab on the biochip surface and the interaction with GRF2 were investigated by surface plasmon resonance using a BIAcore instrument. The results are consistent with the published data, showing that a higher surface coverage can be achieved using a site-specifically biotinylated antibody or antibody fragment versus a random biotinylated antibody.[20] The site-specifically biotinylated anti-FLAG Fab binds to the streptavidin surface with a higher surface density compared to the carbohydrate-biotinylated anti-FLAG Ab. However, the binding capacity for FLAG-GRF2 is similar for both surfaces due to the fact that the antibody has two binding sites whereas the Fab fragment is monovalent (Figure 1). Due to the similar binding capacity for FLAG-GRF2, site-specifically biotinylated anti-FLAG Fab and Ab were both used for the subsequent experiments and are referred to as anti-FLAG surface.

Development of a Flow Cell Device Integrating Chip Surfaces for Different Assay Setups. Different properties, which are necessary for the investigation of protein interactions, had to be incorporated into a device integrating chip surfaces for the capture and digestion of protein complexes with subsequent analysis by fluorescence and μLC−MS/MS. The surface area in each flow cell must be large enough to capture sufficient amounts of protein material for the MS analysis. For this purpose, an EPDM O-ring was used to create four channels with a surface area of ~0.35 cm^2 each. Based on the results of the BIAcore experiments, at least 1 pmol/cm^2 analyte binds to the surface. Therefore, the amount of analyte available for tryptic digestion and μLC−MS/MS analysis is ~350 fmol, which should be sufficient for our μLC−MS/MS setup.

The flow cell must allow for rapid, dead volume-free washing to ensure that the generally weak interactions in a protein complex are retained. We therefore built a device that allows for the performance of protein immobilization, capture of the complex, digestion, and elution in one compartment with minimal wash volume (Figure 2).

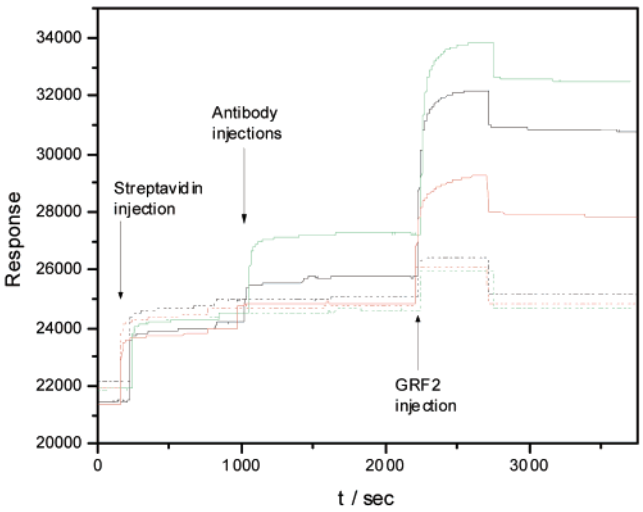

Figure 1. BIAcore sensorgram for the immobilization of biotinylated anti-FLAG antibodies and FLAG-GRF2. Green solid line: immobilization of site-specifically biotinylated anti-FLAG Fab followed by FLAG-GRF2 injection; green dotted line, control flow cell without anti-FLAG Fab; black solid line, immobilization of site-specifically biotinylated anti-FLAG antibody followed by FLAG-GRF2 injection; black dotted line, control flow cell without site-specifically biotinylated anti-FLAG antibody; red solid line, immobilization of random biotinylated anti-FLAG antibody followed by FLAG-GRF2 injection; red dotted line, control flow cell without random biotinylated anti-FLAG antibody.

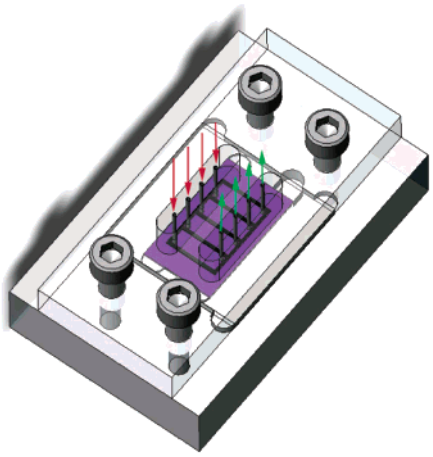

Figure 2. Schematic description of the flow cell device. Red arrows, flow in; green arrows, flow out. The device creates four channels that cover a chip surface area of ~0.35 cm^2.

Development of Fluorescence Assays for the Interaction of GRF2 with the Analytes Ras and Calmodulin in the Flow Cell Device. To test the performance of our flow cell device, we designed a fluorescence-based interaction assay using GRF2 as a bait protein and fluorescently labeled calmodulin and Ras as interaction partners. GRF2 is a multidomain protein known to interact directly with calmodulin and Ras through its IQ motif and CDC25-related domain, respectively.[22] The interaction of GRF2 with calmodulin is calcium-dependent,[15] and GRF2 exhibits a greater affinity for nucleotide-free Ras compared with GDP or GTP liganded forms.[16]

(22) De Hoog, C. L.; Koehler, J. A.; Goldstein, M. D.; Taylor, P.; Figeys, D.; Moran, M. F. *Mol. Cell. Biol.* **2001**, *21*, 2107−2117.

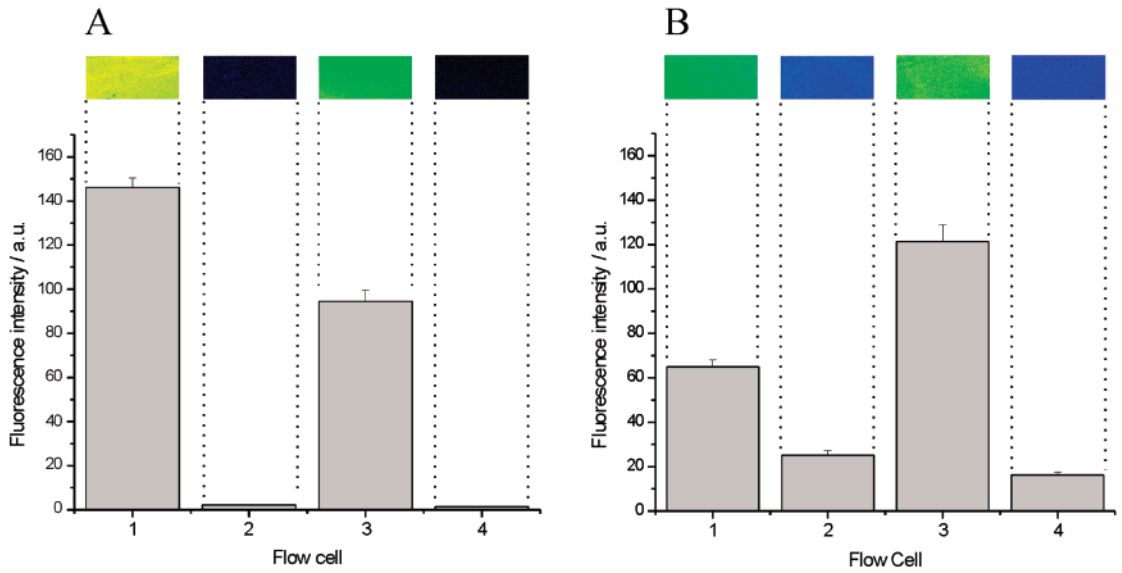

Figure 3. Biochip fluorescence assay for the determination of the interaction of surface-immobilized FLAG-GRF2 with fluorescently labeled Ras and calmodulin. Fluorescence assays were performed on chips incorporated into the flow cell device. Eight spots on the chip in each flow cell, with a diameter of 200 μm, were screened and averaged. (A) GRF2-calmodulin assay. Flow cell 1: immobilized FLAG-GRF2 incubated with 500 nM AlexaFluor594-labeled CaM and washed with 200 μL at 200 μL/min. Flow cell 2: same conditions as flow cell 1, but without immobilized FLAG-GRF2. Flow cell 3: immobilized FLAG-GRF2 incubated with 500 nM AlexaFluor594-labeled CaM and washed with 400 μL at 200 μL/min. Flow cell 4: same conditions as flow cell 3 without immobilized FLAG-GRF2. (B) GRF2−Ras assay. Flow cell 1: immobilized FLAG-GRF2 incubated with 500 nM TAMRA-labeled RasGDP and washed with 200 μL at 100 μL/min. Flow cell 2: same conditions as flow cell 1 without immobilized FLAG-GRF2. Flow cell 3: immobilized GRF2 incubated with 500 nM TAMRA-labeled nucleotide-free Ras and washed with 200 μL at 100 μL/min. Flow cell 4: same conditions as flow cell 3, but without immobilized FLAG-GRF2.

The device was assembled after immobilizing the streptavidin−Ab complex, and the subsequent incubations were performed in the flow cells. The TiO_2 surface was used for these fluorescence assays in order to prevent fluorescence quenching, which might occur on the Au surface. After immobilization of FLAG-tagged GRF2 on the anti-FLAG surface, TAMRA-labeled Ras and Alexa-$Fluor_{594}$-labeled calmodulin were used for the fluorescence assays.

The assays clearly showed an interaction on the chip between the immobilized GRF2 and both fluorescently labeled analytes under the defined conditions (Figure 3). Additionally, the fluorescence assays represent a validation of the device setup and the conditions that were used for the interaction assays, i.e., washing conditions, as shown for the GRF2−CaM assay (Figure 3A).

Interestingly, the results show a difference in the interaction of GRF2 with nucleotide-free Ras versus RasGDP (Figure 3B). The difference between the fluorescence intensity in the flow cells with nucleotide-free Ras (nfRas) and RasGDP is seen consistently and correlates with results from published immunoprecipitation and in vitro binding experiments.[15,16] The preference of GRF2 for nucleotide-free Ras was also confirmed by the results of the mass spectrometry experiments, shown below.

On-Chip Immunoprecipitation and Tryptic Digest Combined with μLC−MS/MS Analysis of GRF2 Protein Complexes. Purified FLAG-GRF2 bait protein was exposed to the TiO_2/PLL-*g*-PEG-B30%/streptavidin/biotinylated anti-FLAG antibody surface, followed by washing of the chip surface, on-chip tryptic digestion, and μLC−MS/MS analysis. These experiments served both to evaluate the efficiency of on-chip tryptic digestion and as a control to identify proteins that remain bound to the purified GRF2. Interestingly, we were able to identify calmodulin associated with the purified GRF2 in these experiments. Figure 4 shows (A) the TIC for the elution of the peptides, (B) an example MS spectrum of a calmodulin peptide eluting at 48.8 min, and (C) the MS/MS spectrum of the calmodulin peptide from (B). The aquired MS/MS spectra were searched against a nonredundant human protein database using the SEQUEST program.

Repetitive experiments consistently identified GRF2 and calmodulin with a high confidence. This observation is consistent with previous work indicating a relatively stable and EGTA-sensitive association between GRF2 and calmodulin.[15] The only other protein to be identified consistently with a high confidence when searching against the human database was immunoglobulin. The immunoglobulin match is consistent with the anti-FLAG antibody bound to the surface, which would be expected to undergo some digestion by trypsin. Searching against the entire nonredundant protein database shows only a single peptide matching to streptavidin. The lack of streptavidin digestion suggests that the packing of antibodies on the surface is tight enough to sterically hinder the trypsin from penetrating further to the surface or that the streptavidin is resistant to trypsinolysis under these conditions. The increased intensity at the end of the TIC is caused by the elution of the detergents. The presence of detergents might suppress some hydrophobic peptide ions that elute at higher acetonitrile concentrations. However, most of the peptides that were fragmented for protein identification eluted before $t = 60$ min.

To remove calmodulin that remained bound to the purified GRF2, a washing step using EGTA to chelate Ca^{2+}, necessary for calmodulin binding, was implemented for subsequent assays. After washing with an EGTA buffer prior to tryptic digestion, μLC−

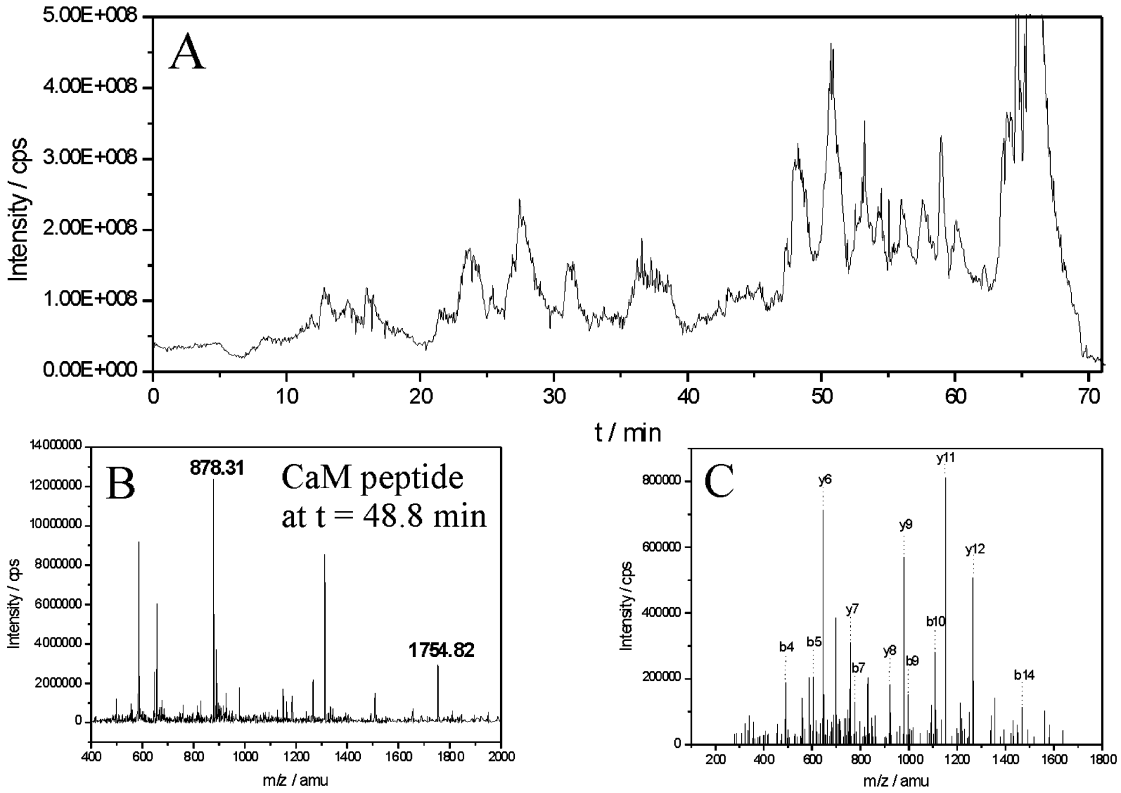

Figure 4. μLC−MS/MS analysis of the on-chip tryptic digest assay of FLAG-GRF2 immobilized on an anti-FLAG surface. (A) TIC; (B) MS of a selected CaM peptide; (C) MS/MS of the CaM peptide and assignment of selected b and y ions used for the identification of CaM by SEQUEST.

MS/MS results showed either only one or no calmodulin peptide. Most of the bound calmodulin was therefore washed away from the GRF2.

In the next set of experiments, FLAG-GRF2 was immobilized on the surface, washed with EGTA buffer, and incubated with purified samples containing a 500 nM concentration of nucleotide-free Ras, RasGDP, or CaM before on-chip tryptic digest μLC−MS/MS analysis. These experiments served to positively identify these three analytes as GRF2-binding partners. Figure 5 shows (A) the TICs of the chip assay with 500 nM nucleotide-free Ras as the analyte together with (B) an example MS spectrum and (C) the aquired MS/MS of the corresponding peptide. The TIC of the sample from the surface with FLAG-GRF2 clearly shows an increased total ion count as compared to the sample from the surface without FLAG-GRF2. Nucleotide-free Ras, RasGDP, and CaM were successfully identified as GRF2 binders in these experiments.

The analytes were then titrated to determine the lower limits of detection for the chip assays. Although the EGTA wash did not seem to release all prebound calmodulin, the data indicate that we can still detect calmodulin at a concentration of 1 nM. Nucleotide-free Ras could be detected at a concentration of 10 nM. We did not perform these experiments with RasGDP as the nucleotide-free form interacts more strongly with GRF2 and consistently showed a higher sequence coverage in these assays. This observation is in agreement with the fluorescence assays, which demonstrated a stronger interaction between GRF2 and nucleotide-free Ras, compared to RasGDP. The same samples from anti-FLAG chip surfaces without FLAG-GRF2 consistently gave no peptide identification for the analytes, demonstrating both the specific capture of the analytes by FLAG-GRF2 and the protein-repellent properties of the surface.

In addition to the performance of the protein interaction assays on planar 10 × 10 mm surfaces in a humidity chamber, we wanted to reproduce these assays in the flow cell device integrating the chips for the GRF2 interaction assays, including protein complex formation, washing, and on-chip tryptic digestion. The flow cell device setup had already been proven useful for the fluorescence assays in which we clearly detected the interaction of GRF2 with Ras and calmodulin. We were able to confirm these results in the flow cell setup with the tryptic digest assay. For example, the interaction between nucleotide-free Ras as an analyte and GRF2 was detected in the flow cell assay setup by identifying GRF2 with 9% sequence coverage and Ras with 11% coverage. In the control flow cell without immobilized GRF2, analyte peptides were not detected. The sequence coverages in these assays were slightly lower than in the assays on planar chips in the humidity chamber, which could be attributed to a slightly reduced recovery of the assay sample after release from the flow cell.

The approach of on-chip tryptic digestion combined with μLC−MS/MS would clearly be a technique amenable to high-throughput screening, if protein complexes could be captured from cell lysates without prior purification. This on-chip immunoprecipitation scheme requires affinity chip surfaces with protein-repellent properties to avoid a high background in the subsequent μLC−MS/MS analysis. To test the applicability of our biotin derivatized poly(L-lysine)-grafted poly(ethylene glycol) copolymer surface with immobilized streptavidin for this approach, a capture of the FLAG-tagged bait together with interacting proteins from complex mixtures was performed. For this, the planar chip

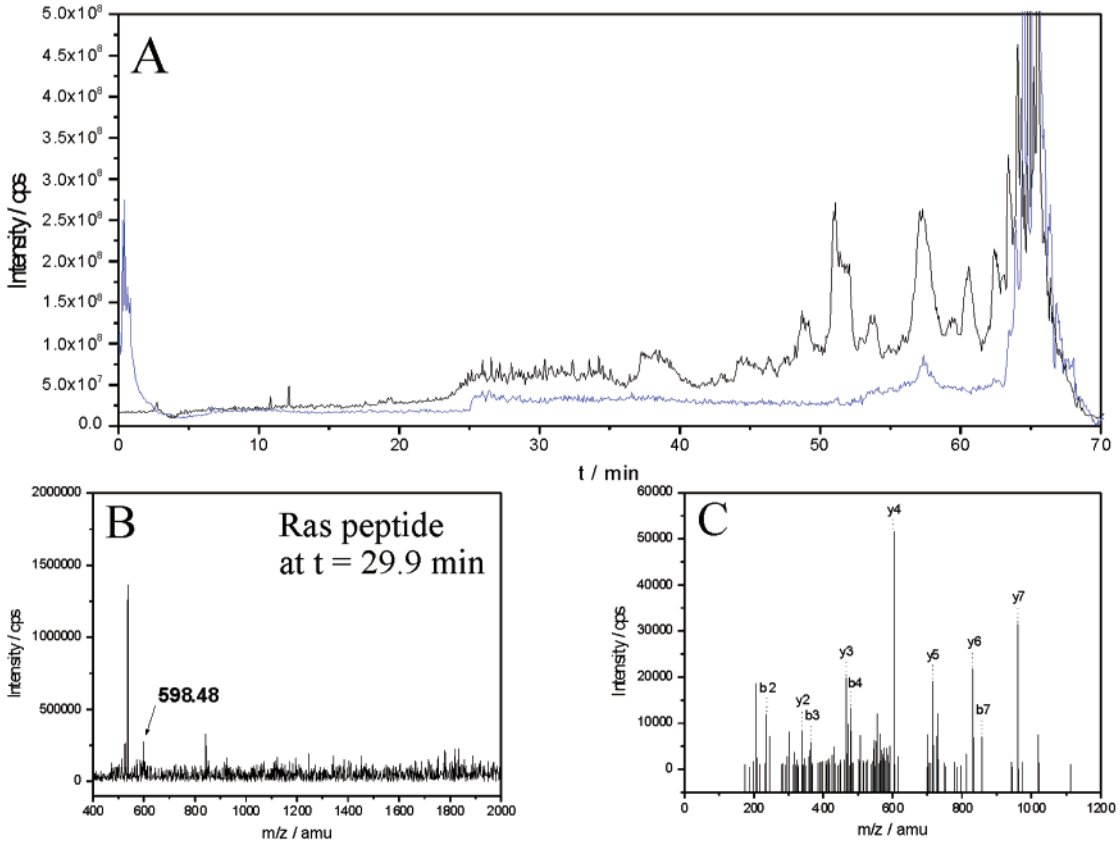

Figure 5. μLC−MS/MS analysis of the on-chip tryptic digest assay of the interaction between immobilized FLAG-GRF2 and nucleotide-free Ras. (A) Black line, TIC of the sample from the surface containing FLAG-GRF2; blue line, TIC of the control sample from the surface without FLAG-GRF2; (B) MS of a selected Ras peptide; (C) MS/MS of the Ras peptide and assignment of selected b and y ions used for the identification of Ras by SEQUEST.

surfaces, containing the biotinylated anti-FLAG antibody, were incubated with cell lysates from 293T cells overexpressing FLAG-GRF2. As a control, the same antibody surface was incubated with cell lysates from nontransfected cells. Figure 6 shows (A) both TICs of the on-chip tryptic digest μLC−MS/MS experiments and (B/C) selected MS as well as (D/E) MS/MS data for the identification of GRF2 and CaM from the experiment with overexpressed GRF2 in the cell lysate.

The tryptic digest sample from the chip that was incubated with the lysate containing overexpressed FLAG-GRF2 clearly shows a higher ion count in the μLC−MS/MS. In this experiment, GRF2 was identified with a sequence coverage of 11% and a comparable number of peptide hits in relation to the experiments with preimmobilized FLAG-GRF2. The identification of the tagged bait protein will serve as a confirmation of both its expression and location when this method is adapted to a higher throughput process, involving a number of different bait proteins on a single chip. In addition to the capture of GRF2 by an anti-FLAG antibody on a chip surface, calmodulin was also identified as an interaction partner for GRF2 from a complex biological mixture. This observation is in accordance with published experiments in which calmodulin was identified as a GRF2 binder in coimmunoprecipitations.[15] This experiment shows the potential of our chip platform for direct immunoprecipitation assays. Bait proteins can be overexpressed in their native cellular environment, consequently engage in their normal protein−protein interactions, and then become directly immobilized on a chip after cell lysis for on-chip tryptic digest μLC−MS/MS analysis of their interacting proteins.

The results presented in this paper demonstrate the feasibility of using a chip format to capture and analyze protein complexes from biological mixtures such as tissue and whole cell extracts. The applied strategy of capturing overexpressed proteins with affinity tags from cell extracts has been proven successful to elucidate native protein−protein interactions in a variety of studies from high-throughput interaction screening to the identification of interaction partners for specific proteins of interest in important interaction pathways.[8−10,23,24] Such protein complexes to a great extent represent the metabolic and signal transduction pathways in cells.

Our method has the inherent advantage of confirming the correct identity of the bait protein, in addition to its interacting proteins. Recently, Ho et al. delineated the interactions of more than a quarter of the budding yeast genome by using a similar μLC−MS/MS platform as described herein, but in conjunction with a conventional and therefore laborious protein capture method requiring the dispensing and collection of bead-associated immune complexes.[9] Our results suggest that, by adapting such large-scale interaction mapping endeavors to a chip-based format, much smaller amounts of starting material from cultured human cells may be employed and that a higher throughput may be achieved as a consequence of the automation of the chip-based

(23) Figeys, D. *Curr. Opin. Biotechnol.* **2003**, *14*, 119−125.
(24) Cheng, E. H.-Y.; Sheiko, T. V.; Fisher, J. K.; Craigen, W. J.; Korsmeyer, S. J. *Science* **2003**, *301*, 513−517.

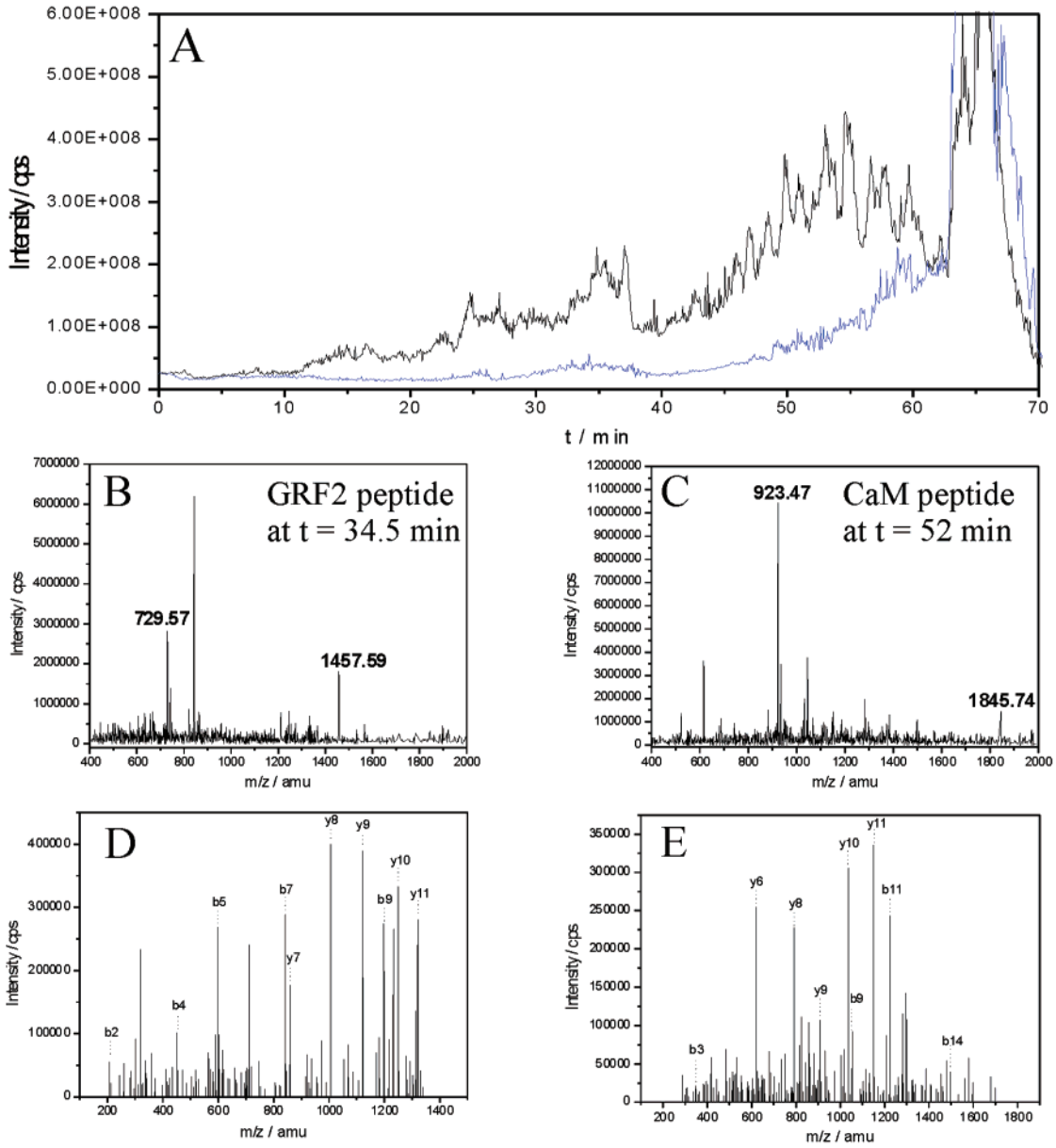

Figure 6. μLC−MS/MS analysis of the on-chip immunoprecipitation and tryptic digest assay of FLAG-GRF2 from crude cellular extracts. (A) Black line, TIC of the sample from the surface incubated with 293T cell lysate containing overexpressed FLAG-GRF2; blue line, TIC of the control sample from the surface incubated with nontransfected 293T cell lysate; (B) MS of a selected GRF2 peptide; (C) MS of a selected CaM peptide; (D) MS/MS of the GRF2 peptide and assignment of selected b and y ions and; (E) MS/MS of the CaM peptide and assignment of selected b and y ions used for the identification of by SEQUEST.

protein complex capture process. This will provide a means to investigate at a functional level numerous cellular states, which is required to generate information on the dynamic nature of proteomes affected by disease or therapeutic treatments. The ability to analyze protein interactions in whole proteomes, or subsets of interest, such as drug target families or pathways of interest, in a chip format may have great utility in drug discovery.

ABBREVIATIONS

EPDM, ethylene−propylene rubber; ESI, electrospray ionization; GDP, guanosine 5′-diphosphate; GRF2, guanine nucleotide release factor 2; GTP, guanosine 5′-triphosphate; IQ motif, calmodulin-binding motif; μLC−MS/MS, micro liquid chromatography combined with tandem mass spectrometry; PLL-*g*-PEG-B30%, poly(L-lysine)-grafted-poly(ethylene glycol) with 30% biotin content; PMMA, poly(methyl methacrylate); TIC, total ion chromatogram.

ACKNOWLEDGMENT

We thank Daniel Figeys, Shane Climie, Leroi Desouza. and Paul Peluso for technical advice.

AC034258U