Detection and Identification of Protein Interactions of S100 Proteins by ProteinChip Technology

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Detection and Identification of Protein Interactions of S100 Proteins by ProteinChip Technology

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The aim of this work was to establish an approach for identification of protein interactions. This assay used an anti-S100A8 antibody coupled on beads and incubated with cell extract. The bead eluates were analyzed using ProteinChip technology and subsequently subjected to an appropriate digestion. Molecular masses of digestion fragments were determined by SELDI−MS, and database analysis revealed S100A10 as interacting protein. This result was confirmed by co-immunoprecipitation and immunocapturing. Using S100A10 as new bait, a specific interaction with S100A7 was detectable.

Keywords: S100 proteins • protein−protein interaction assay • surface-enhanced laser desorption/ionization−mass spectrometry (SELDI−MS) • immunoprecipitation

Introduction

Functional characterization of the proteome interaction studies are of particular interest, because it is known that most of the proteins are usually interconnected. Interacting proteins are often involved in the same biochemical pathway. The identification of an interacting partner of a protein of unknown function can help to clarify its function. Defining the interaction partners of known proteins is invaluable to detailing its mechanism of action and regulation. The idea of temporal and spatial localization of different proteins at specific sites in the cell suggests that interactions of different components involved in the same cellular processes are required. The clarification of the association of a molecular machine or regulatory factor may help to understand cellular regulatory mechanisms.[1] Recently, many different techniques have been used to define protein−protein interactions.[2] In vitro techniques are recognized as often generating false positives.[3] To avoid this problem, a multitude of in vivo methods have been developed including yeast two hybrid assays.[4] However, yeast two hybrid screens can also generate a fairly high number of both false positives and negatives.[5] Recently, a new technique named SELDI−MS (surface-enhanced laser desorption/ionization mass spectrometry) was developed which comprises ProteinChip arrays and mass spectrometry.[6,7] The ProteinChip array carries spots with different chromatographic surfaces to retain proteins regarding their physicochemical properties. The spots can easily be purified from contaminants such as buffer salts or detergents by washing, thus eliminating the need for prepurification techniques as required with other MS techniques. For this reason, crude biological extracts, such as cell lysis extract, serum ,or other biological fluids can be spotted directly on the ProteinChip arrays for mass spectrometric analysis. One application of SELDI−MS is the analysis of protein patterns of different biological samples mainly derived from cancer and noncancer patients.[8−12] In a former protein profiling study of head and neck squamous cell carcinoma (HNSCC) carried out by SELDI−MS, a decreased expression of S100A8/A9 in microdissected tumor tissue was detected.[13] These proteins are members of the S100 Ca^{2+} binding family and have received increased attention because of their involvement in several human diseases such as rheumatoid arthritis, acute inflammatory lesions, cardiomyopathy, Alzheimer's disease, and cancer.[14,15] The aim of the present study was the proof of principle of a procedure composed of immunoprecipitation techniques and SELDI technology to detect and identify protein−protein interactions from crude cell extracts. On this account, we started to analyze the interaction partners of S100A8/A9. Hereby, we were able to detect specific interactions between different S100 proteins using specific immunoaffinity beads. These interactions were visible by SELDI−MS and the interaction partners were identified as S100A10 (calpactin light chain; p11) and S100A7 (psoriasin), respectively, and further confirmed using co-immunoprecipitation and immunocapturing.

Materials and Methods

Cell Culture. HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were cultured in DMEM supplemented with 10% fetal bovine serum.[16] Cells were grown to 80% confluence and were passaged at a split ratio of 1:4. At 95% confluence, cells were harvested and lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM $MgCl_2$, 3 mM 2-β-mercaptoethanol, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF. After centrifugation (15 min; 15 000 rpm) the supernatant was used immediately.

Protein−Protein Interaction Assay. A twenty-microliter portion of Interaction Discovery Mapping (IDM) beads (Ciphergen Biosystems Ltd., Fremont, Ca) was incubated with 4 μL protein A (Sigma) overnight at 4 °C. A pellet was generated by centrifugation, and the supernatant was discarded. The pellet was washed twice with a buffer containing 50 mM sodium acetate pH 5.0. Afterward, the beads were incubated in a buffer containing 0.5 M Tris/HCl pH 9.0, 0.1% Triton X-100

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for 2 h at room temperature for blocking residual reactive groups. The beads were washed three times with 1× PBS. Thereafter, 40 μL of specific antibody (0.2 μg/μL) against human S100A8 (S13.67; BMA Biomedicals; Augst, Switzerland), human S100A9 (S36.48; BMA Biomedicals; Augst, Switzerland), and human S100A10 (H-21; Swant; Bellinzona, Switzerland), respectively, or normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) as negative control, in 50 mM sodium acetate pH 5.0 were applied to the beads and allowed to bind at room temperature for 1 h in an end-over-end mixer. Unbound antibody was removed by washing in 50 mM sodium acetate. Unspecific binding sites were blocked using a buffer containing 2 mg/mL bovine serum albumin in 0.5 M Tris/HCl pH 9.0, 0.1% Triton X-100 for 2 h at room temperature in an end-over-end mixer. Afterward, the beads were washed in 1× PBS, 0.1% Triton X-100 and in 1× PBS and incubated with 100 μL of crude HaCaT cell extract overnight at 4 °C in an over-end-over mixer. The unbound proteins were washed away by sequential washes in PBS, 0.5 mM sodium chloride, 0.05% Triton X-100, 1 M urea in PBS, PBS, and aqua bidest. Bound proteins were eluated from the IDM beads by 10 μL 50% acetonitrile/0.5% trifluoroacetic acid and gently vortexed for 5 min. Five μL of the eluated samples were applied to the activated, hydrophobic surface of an H50 ProteinChip Array (Ciphergen Biosystem Inc.; Fremont) and dried on air. After washing with 3 μL aqua bidest 0.5 μL sinapinic acid (saturated solution in 0.5% TFA/50% Acetonitrile) was applied twice and the array was analyzed in a PBS II reader (Ciphergen; Fremont) according to an automated data collection protocol.

Tryptic Digestion of IDM Eluates. For the analysis of fragment masses proteins eluated from IDM beads were tryptic digested. Hereby, the volume of the samples was reduced to nearly 5 μL, mixed with 5 μL of a buffer containing 6 M urea, 3 mM 2-β-mercaptoethanol and denaturated for 20 min at 95 °C. Afterward, sample volume was increased until samples contained a maximum of 1 M urea, 15 μL of a tryptic solution (4 ng/μL; in 25 mM ammonium bicarbonate) was added and incubated for 24 h at 35 °C. Samples were sonicated for 5 min, 5 μL of them were applied directly to the activated reverse phase surface of an NP20 array (Ciphergen; Fremont) and dried on air. After addition of the matrix (CHCA), peptide fragment masses were analyzed using the PBS II instrument. A standard protein mix (all-in-1 peptide standard mix; Ciphergen, Fremont), including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin beta-chain (3495.94 Da) was used for calibration. Proteins were identified using the fragment masses generated through trypsin digestion by searching in a publicly available database (*http://129.85.19.192/profound_bin/WebProFound.exe)*.

Immunocapturing Assays. For immunocapturing of specific target proteins 2 μL protein A in 2 μL of 50 mM $NaHCO_3$ pH 9.2 was directly coupled to RS100 ProteinChip arrays (Ciphergen; Fremont) overnight at 4 °C in a humidity chamber. After incubation the supernatant was removed and 2 μL BSA (2μg/μL) (Sigma) in 2 μL 1× PBS pH 7.2 was spotted for 2 h at room temperature to block any remaining active sites. Afterward, BSA was removed, spots were washed with 1× PBS pH 7.2 and 2 μL of specific antibody (0.2 μg/μL) against human S100A10 (H-21; Swant; Bellinzona, Switzerland) or human S100A7 (ab13680; Abcam; Cambridge, UK), respectively, or, as negative control, normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) in 2 μL of 50 mM $NaHCO_3$ pH 9.2 were applied to the arrays for 2 h at room temperature in a humidity chamber. Unbound antibody was removed and spots were washed with 5 μL 1× PBS for 2 min. Afterward, the ProteinChip array was fixed in a Bioprocessor (Ciphergen, Fremont) and 50 μL of crude cell extract diluted in 50 μL of a 0.1% Triton X-100 in 1× PBS pH 7.2 were applied to each spot and incubated for 2 h at room temperature on a shacking platform. The unbound proteins were washed away by sequential washes in 1× PBS, 0.5 mM sodium chloride, 0.05% Triton X-100, 1 M urea in 1× PBS, 1× PBS, and aqua bidest. The array surface was allowed to dry for 5 min, 0.5 saturated sinapinic acid was added twice and the ProteinChip was analyzed in a PBS II reader according to an automated data collection protocol.

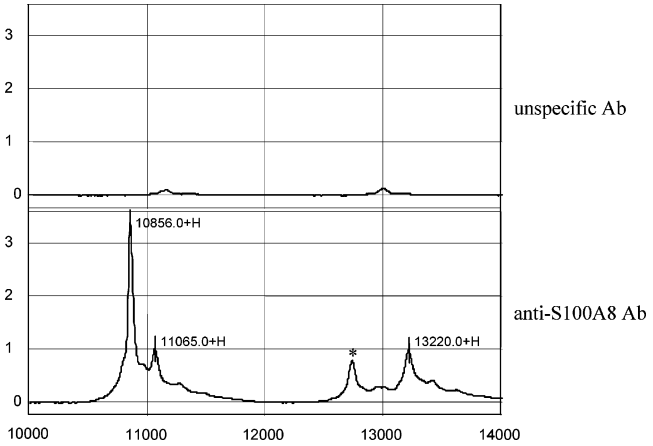

Figure 1. Protein−protein interaction assay using specific anti-S100A8 antibody. An anti-S100A8 antibody was bound on IDM beads, incubated with crude HaCaT cell extract and binding partners were analyzed by SELDI−MS. Among signals of 10.87 kDa and 13.22 kDa that represent S100A8 and S100A9, respectively, a signal of nearly 11.06 kDa was detected. In a negative control using a nonspecific antibody no specific signal was detectable. (Signal labeled by asterisk is still to identify).

Co-immunoprecipitation. Antibodies against human S100A7 (ab13680; Abcam; Cambridge, UK), human S100A8 (S13.67; BMA Biomedicals; Augst, Switzerland), human S100A9 (S36.48; BMA Biomedicals; Augst, Switzerland), and human S100A10 (H-21; Swant; Bellinzona, Switzerland), or, as negative control, normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) were bound on protein A-agarose beads. Crude extract (250 μL) from human HaCaT cells was incubated with the antibody loaded beads for 1 h at 4 °C. Then the resins were washed three times with Co−IP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Bound proteins were subjected to 10% SDS-PAGE and detected by immunoblotting with anti-S100A7, anti-S100A8, anti-S100A9 (S36.48; BMA Biomedicals; Augst, Switzerland), or anti-S10010 antibody, respectively.

Results

Detection of S100A8 Protein Interaction Partners. We first assessed possible interaction partners of S100A8/A9 by SELDI−MS. Hereby, among other peaks we found a specific signal at nearly 11.07 kDa using anti-S100A8 antibody-coupled IDM beads compared to approaches using nonspecific antibody (Figure 1). Beside this mentioned signal, we detected a signal at nearly 13.22 kDa, too. This molecular mass correlates well to the molecular mass of S100A9 that is known as a binding partner of S100A8.

Protein Identification by Tryptic Digestion and Database Search. Proteins eluated from both anti-S100A8 antibody

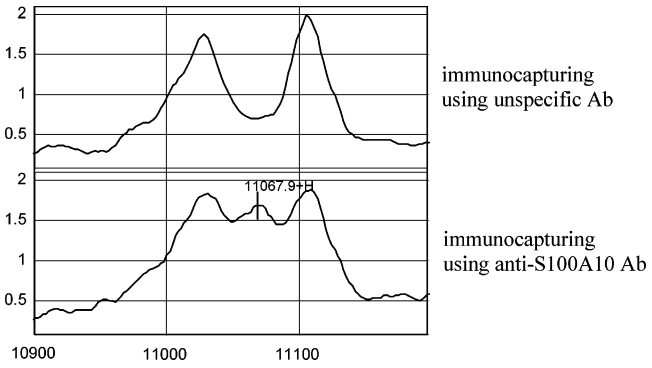

Figure 2. Normalized ProteinChip Arrays spectra of the immunocapturing assay of crude cell extract. S100A10 was captured from HaCat cell extracts by the corresponding monoclonal antibody bound on RS100 ProteinChip arrays. The peak at 11.07 kDa representing S100A10 was clearly detectable. In control assays without the specific antibody no S100A10 signal was captured.

coupled or nonspecific antibody coupled IDM beads were tryptic digested for analysis of fragment masses. The digested solutions were spotted on an NP20 array and the size of the obtained fragments was determined by the PBS II instrument. In parallel, a theoretical tryptic digestion of known proteins of the eluates (e.g., the bait, known interaction partners of the bait, antibody subunits etc.) was carried out using a public database (Expasy findpept tool; http://us.expasy.org/tolls/findpept.html). Afterward, peptide fragments derived from known proteins were eliminated. Database searches (Profound; http://129.85.19.192/profound_bin/WebProFound.exe) using retained peptide fragments revealed S100A10 as a possible candidate. The calculated mass of human S100A10 is 11.072 kDa (www.expasy.ch), and thus slightly higher than the average mass of about 11.065 kDa found in these experiments (Figure 1).

Immunocapturing of S100A10. For confirmation of the identity of the interaction partner of S100A8, an immunoassay was performed using a monoclonal antibody against S100A10. Hereby, a specific antibody bound on RS100 ProteinChip arrays captured S100A10 from HaCaT cell extract. The spectra of the analysis showed a peak corresponding to S100A10. In a control assay using a nonspecific antibody no proteins specific for S100A10 were captured (Figure 2).

Co-immunoprecipitation of S100A8/A9 by an anti-S100A10 Antibody. In a co-immunoprecipitation assay, we were able to precipitate both S100A8 and S100A9 using protein A-agarose beads with a specific antibody against S100A10 from a crude extract of HaCat cells (Figure 3, lane 1 and 3, respectively). In an approach using a nonspecific antibody bound on protein A-agarose neither S100A8 nor S100A9 was detectable (Figure 3, lane 2 and 4, respectively). As a control the specific antibody against S100A10 precipitated its antigene (Figure 3, lane 5).

Detection and Identification of a S100A10 Protein Interaction Partner. In the next step, we assessed S100A10 for possible protein interaction partners. Hereby, we found an interaction partner possessing a molecular mass of nearly 11.29 kDa using anti-S100A10 antibody coupled IDM beads compared to nonspecific antibody coupled IDM beads by SELDI−MS (Figure 4A). Database search (profound) of the tryptic digestion fragments of the eluted proteins revealed S100A7 as a good candidate. For confirmation of this result, an immunocapturing assay using an anti-S100A7 antibody was carried out. Hereby, a signal of nearly 11.37 kDa was detectable. This molecular mass is slightly higher (0.39%) than the calculated mass (expasy). In the control assay, using a nonspecific antibody no signal compared to the molecular mass of S100A7 was detectable (Figure 4B). In a co-immunoprecipitation approach, both an anti-S100A10 and an anti-S100A7 antibody were able to precipitate S100A7 (Figure 4C, lane 1 and 3, respectively). When we used antibodies that recognize S100A8 or S100A9, no clear S100A7 signal was detectable (Figure 4C, lane 2 or data not shown).

Discussion

In the present study, we performed an approach to investigate protein−protein interactions by ProteinChip technology. By the appearance of both false positive and negative results in other in vivo interaction assays we analyzed the endogenous interacting partners in crude cell extract by mass spectrometry.[17] The assay comprises the detection of interaction partners by SELDI−MS and the identification using immunocapturing experiments and coprecipitation, respectively. In our knowledge, it is one of the first studies using SELDI−MS to assess protein interactions.[18,19] As a model system we used members of the S100 Ca^{2+}-binding family. A multitude of protein interactions is known to be performed by S100 proteins.[20] S100 proteins are involved in several human diseases such as

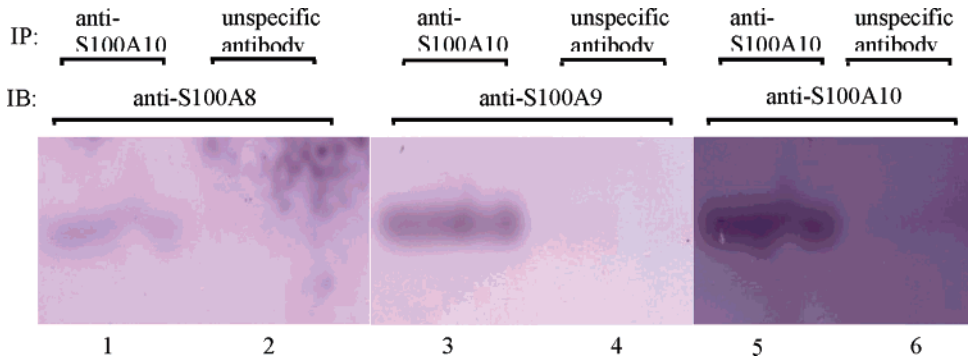

Figure 3. Co-immunoprecipitation of S100A8/A9 and S100A10. Specific anti-S100A10 antibody or, as negative control, nonspecific antibody was coupled to protein A-agarose beads and incubated with HaCaT cell extracts. Beads were washed with Co−IP buffer and bound proteins were subsequently subjected to SDS-PAGE and analyzed by immunoblotting using specific antibodies against S100A8 (lane 1), S100A9 (lane 3), and S100A10 (lane 5), respectively. Co-immunoprecipitation assays using a nonspecific antibody showed no signal (lanes 2, 4, 6).

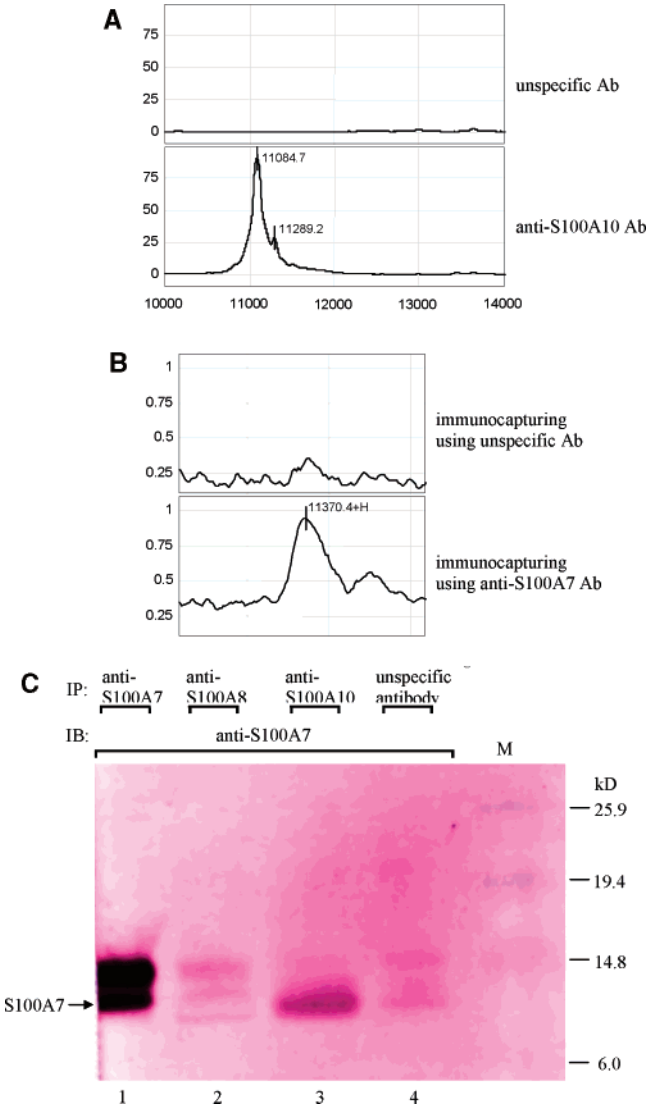

Figure 4. Detection and identification of S100A7 as specific S100A10 interaction partner. (A) Anti-S100A10 antibody was coupled on IDM beads and incubated with HaCaT cell extracts. Bound proteins were analyzed by SELDI−MS. Besides a signal representing S100A10, a signal at nearly 11.29 kDa was detectable that is absent in the assay using nonspecific antibody. (B) In an immunocapturing assay a specific anti-S100A7 antibody was bound on an RS100 ProteinChip array and captured a signal corresponding to S100A7 from crude HaCaT cell extracts. In a control assay without a specific antibody, this signal was not detectable. (C) In a co-immunoprecipitation assay using an S100A10 antibody, S100A7 was detectable subsequently in an immunoblot (lane 3). A similar signal was also detectable using an S100A7 antibody for precipitation (lane 1). No S100A7 signal was detectable using an anti-S100A8 antibody or a nonspecific antibody, respectively (lanes 2 or 4).

rheumatoid arthritis, acute inflammatory lesions, cardiomyopathy, Alzheimer's disease, and cancer.[14,15] Recently, we showed in a study that S100A8/A9 are less expressed in head and neck squamous cell carcinoma compared to normal tissue.[13] S100A8 and S100A9 achieve an interaction together and under physiological conditions, a heterodimer is often existent.[21] Among different protein binding partners S100A8 forms specific interactions to for example cytochrome b558, p67 (phox), and Rac-2 and initiates so the NADPH oxidase activation.[22,23] As we demonstrated, the interaction between S100A8/A9 and S100A10 is detectable under in vivo conditions by mass spectrometry and immunological techniques. It is not clear whether the single components of the S100A8/A9 complex alone are able to bind S100A10 or if this interaction requires a full complex of both. In our knowledge, this study shows for the first time an interaction between S100A8/A9 and S100A10. Further studies will be needed to reveal a potential biological context of this protein interaction. S100A10 forms an interaction with annexin II that results in the formation of a heterotetramer.[24] This interaction can be inhibited by a protein kinase C dependent phosphorylation of a specific serine residue in the annexin II.[25] The S100A10/annexin II complex has been implicated in the structural organization and dynamics of endosomal membranes and cell membrane cytoarchitecture, and is as well involved in the formation of adherent junctions.[26−28] Surprisingly, we only detected the mentioned interaction between S100A10 and annexin II when we used an anti-S100A10 antibody. In the protein−protein interaction assays carried out with the anti-S100A8 antibody, we detected S100A10 but never annexin II (supplementary Figure 1). Until now, we have no explanation for this fact. It might be possible that S100A8 as well as annexin II share a similar binding site to S100A10, because there are no data available about the biological function of S100A10 beyond the complex with annexin II. The interaction between S100A10 and S100A7 detected by several proteomic approaches is described in the present study for the first time. Due to our data, we do assume that S100A8/A9 is not involved in this mentioned interaction as a bridging protein, because no S100A7 signal was detectable using specific anti-S100A8 or anti-S100A9 antibodies in co-immunoprecipitation. The S100A7 Ca^{2+}-binding protein is highly upregulated in psoriatic epidermis as well as in primary human keratinocytes undergoing abnormal differentiation.[29] It is primarily expressed in breast cancer cells and at very low level in normal breast epithelial cells.[30] Also an overexpression in gastric cancer is described.[31] S100A7 interacts with Jab 1 in ductal carcinoma in situ (DCIS) of the breast and because of that it may influence the progression of breast carcinoma.[32] Besides this interaction, an interaction with RanBPM may contribute to breast tumor progression, as well as interactions in keratinocytes with both E-FABP and epidermal fatty acid binding protein are described.[33−35] It seems that several members of the S100 Ca^{2+}-binding protein family form interactions with each other that could be involved in different physiological processes. To assess the biological role of these protein interactions, additional studies are needed. The identification of further interaction partners that is still in process may clarify possible biological functions. In this study, we show a possible proteomic approach composing ProteinChip technology and immunological techniques to detect and identify protein−protein interactions.

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Supporting Information Available: Supplementary Figure 1. This material is available free of charge at http://pubs.acs.org.

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