Interactome Analysis Reveals that C1QBP (complement component 1, q subcomponent binding protein) Is Associated with Cancer Cell Chemotaxis and Metastasis*§

Xiaofang Zhang‡§¶, Fei Zhang‡¶, Lin Guo§, Yanping Wang‡, Peng Zhang§, Ruirui Wang‡, Ning Zhang‡¶, and Ruibing Chen‡¶

The complement component 1, q subcomponent binding protein (C1QBP/p32/HABP1) is a ubiquitously expressed and multicompartmental cellular protein involved in various biological processes. In order to further understand its biological functions, we conducted proteomics analysis of its interactome in this study. An improved sample preparation and mass spectrometric identification strategy was developed combining high-speed centrifugation, formaldehyde labeling, and two-dimensional reverse-phase liquid chromatography. Using this approach, we identified 187 interacting proteins and constructed a highly connected interacting network for C1QBP. Moreover, we explored the interaction between C1QBP and protein kinase C ζ, a key regulator of cell polarity and migration. The results indicated that C1QBP regulated the activity of protein kinase C ζ and modulated EGF-induced cancer cell chemotaxis. In addition, C1QBP was required for breast cancer metastasis in a severe combined immunodeficiency mouse model. Furthermore, C1QBP was observed to be overexpressed in breast cancer tissues, and its expression level was closely linked with distant metastasis and TNM stages. In summary, C1QBP was identified as a novel regulator of cancer metastasis that may serve as a therapeutic target for breast cancer treatment. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.029413, 3199–3209, 2013.

The complement component 1, q subcomponent binding protein (C1QBP/p32/HABP1)¹ is an acidic protein that binds

¹ The abbreviations used are: C1QBP, complement component 1, q subcomponent binding protein; coIP, co-immunoprecipitation; EGF, epidermal growth factor; GSK3β, glycogen synthase kinase 3 β; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MS, mass spectrometry; PRKCZ, protein kinase C ζ; RP-RP-LC, double reverse-phase liquid chromatography; SCID, severe combined immunodeficiency; UPLC, ultrahigh-performance liquid chromatography.
C1QBP Regulates Cancer Cell Chemotaxis and Metastasis

ing proteins via relative quantification (16–18). Formaldehyde isotopic labeling is a fast and simple reaction that has been extensively applied for quantitative proteomics (19, 20). However, a test study of its application in coIP-MS analysis has yet to be performed. The other source of false positives that has often been overlooked is the interference from contaminating DNA in the cell lysate. It has been reported that ethidium bromide can be used to inhibit DNA–protein interactions and eliminate DNA-dependent interaction in coIP assays (21). Another option is to add nucleases to degrade nucleic acids in the cell lysates (22). In this study, we discovered that DNA contamination can be removed efficiently with high-speed centrifugation, and this simple sample preparation step can also reduce the presence of background binding proteins.

In this study, we established an improved sample preparation and mass spectrometric identification strategy to study protein–protein interaction combining high-speed centrifugation, formaldehyde labeling, and two-dimensional reverse-phase liquid chromatography (RP-LC). Using this approach, we studied the protein complexes associated with C1QBP. The proteomics data show that C1QBP binds to protein kinase C ζ (PRKCZ), a pivotal modulator of tumor cell chemotaxis. Mechanistic studies revealed that C1QBP mediates breast cancer cell chemotaxis and metastasis by interacting with PRKCZ on the cell membrane. Furthermore, a high level of C1QBP was detected in breast cancer tissues via immunohistochemical staining, and pathological analysis suggested a close link between C1QBP expression and distant metastasis.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and Animals—Dithiothreitol (DTT), iodoacetamide, urea, formaldehyde, deuterated formaldehyde, sodium cyanoborohydride, monoclonal antibody against γ-tubulin, monoclonal antibody against Flag, and anti-Flag antibody-conjugated agarose beads were purchased from Sigma; antibodies against C1QBP, PRKCZ, actin, and phosphor-cofilin (Ser3) were obtained from Santa Cruz Biotecology (Santa Cruz, CA); and antibodies against phosphor-PRKCZ (Thr410), GSK3β, phosphor-GSK3β (Ser9), and cofilin were bought from Cell Signaling Technology (Danvers, MA). LC–MS-grade water and acetonitrile were purchased from Merck (Whitehouse Station, NJ). Micro-Boyden chambers were purchased from Neuro Probe (Gaithersburg, MD). Fibronectin and recombinant human EGF were purchased from R&D Systems (Minneapolis, MN). Mitochondrial oxidative phosphorylation complex I activity assay kits were purchased from MitoSciences Co. Ltd. (Eugene, OR). SCID mice were purchased from Wei Tong Li Hua Experimental Animal Co. Ltd. (Beijing, China).

Cell Culture, Plasmid, siRNA, and Transfection—All the cell lines used in this study were obtained from American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% glutamine Pen-Strep solution at 37 °C and 5% CO2. C1QBP-Flag was amplified via PCR and cloned into vector pcDNA 3.1. The C1QBP-Flag plasmids were then transfected into HEK293T cells using Lipofectamine 2000. The expression of C1QBP-Flag fusion protein was confirmed by Western blotting with both anti-C1QBP and anti-Flag antibodies. For the C1QBP knockdown, two StealthTM siRNAs against human C1QBP (#1: 5’-GAAAGCCCTTGTGT-TGAGACTGTCAT-3’; #2: 5’-ACTGCCGATCTGAAATGGAAGATA-3’) and a scrambled siRNA were synthesized by Invitrogen; for stable clones, a shRNA expression plasmid containing #2 target sequence and a vector containing a scrambled sequence were obtained from Genechem Co. (Montreal, Quebec). For PRKCZ silencing, siRNA (5’-GAGGAATGAGACATGTTG-3’) was purchased from Genescript (Piscataway, NJ).

Co-immunoprecipitation—Two 10-mm dishes of cells cultured to 80% to 90% confluence were lysed with Triton X-100 buffer (40 mM Tris, 120 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM Na3VO4). The same amount of cells transfected with empty plasmid was used as the control. The cell lysates were then centrifuged at 15,000 × g at 4 °C for 15 min, and the pellets were removed. For further sample cleanup, the lysates were either centrifuged at 50,000 × g for 1 h or incubated with Benzonase™ at 4 °C for 4 h. The total protein concentrations of the cell lysates were measured with a BCA assay, and the same amount of proteins was used for following procedure: The cell extracts were first precleared with protein G agarose beads. Next, C1QBP and its interacting proteins were pulled down with anti-Flag antibody-conjugated agarose beads, and mass spectrometric analysis or Western blotting analysis was conducted on the samples.

Proteolysis and Formaldehyde Labeling—C1QBP and its interacting proteins were eluted from the agarose beads with 6 M urea. The samples were reduced via incubation with 10 mM DTT at 37 °C for 1 h. The reduced proteins were alkylated for 1 h in darkness with 40 mM iodoacetamide. The alkylation reaction was quenched by DTT added to a final concentration of 50 mM. The urea in the solution was exchanged with 25 mM sodium bicarbonate buffer by means of centrifugation using 3-kDa ultrafiltration devices (Millipore, Billerica, MA). The samples were incubated with trypsin at 37 °C overnight to allow the digestion to complete.

The isotopic labeling experiment was performed as described previously (19). Briefly, sodium cyanoborohydride was added to the protein digest to a final concentration of 50 mM. Samples were labeled with 0.2 mM formaldehyde or 0.2 mM deuterated formaldehyde. The mixed peptides were vortexed and incubated at 37 °C for 1 h. To quench the reaction, 2 μl NH3·H2O was added and the mixture was immediately dripped in a SpeedVac. The samples were reconstituted in water before HPLC separation.

High-pH RP-HPLC Separation—Samples were injected onto an HPLC system (Waters, Milford, MA) coupled with a high-pH stable C18 column (Phenomenex Gemini C18, 150 × 2.1 mm, 3 μm) at a flow rate of 150 μl/min. The peptides were eluted with a 40-min gradient of 5%–45% buffer B (buffer A: 50 mM ammonium formate, pH 10; buffer B: acetonitrile). Fractions were collected every 3 min for 60 min. Collected fractions were dried in a SpeedVac and reconstituted in 20 μl of 0.1% formic acid. 5 μl of each of the 11 fractions containing peptides were subjected to nano-ultrahigh-performance liquid chromatography (UPLC)-MS/MS.

LC-MS/MS and Data Analysis—A nanoUPLC system (Waters) was used to separate the tryptic peptides. Samples were loaded on a trap column and flushed with mobile phase A (0.1% formic acid in H2O) at 5 μl/min for 3 min before being delivered onto a nanoUPLC column (C18, 150 × 0.075 mm, 1.7 μm). The peptides were eluted using a 7%–45% B gradient (0.1% formic acid in acetonitrile) over 90 min into a nano-electrospray ionization LTO Orbitrap mass spectrometer (Thermo Electron Corp.). The mass spectrometer was operated in data-dependent mode in which an initial Fourier transform scan re-
corded the mass range of m/z 350–2000 and then the eight most abundant ions were automatically selected for collision-activated dissociation. The spray voltage was set at 2.0 kV. The normalized collision energy was set at 35% for MS/MS. Raw data were searched against the International Protein Index human protein database (release version 3.83) containing 93,289 sequence entries using the SEQUEST algorithm embedded in the Protein Discoverer 1.3 Software (Thermo Electron Corp.). The following parameters were applied during the database search: 10 ppm precursor mass error tolerance; 1 Da fragment mass error tolerance; static modifications of carbamidomethylation for all cysteine residues, dimethylation for the formaldehyde labeling (Δ28 Da), or deuterated-formaldehyde labeling (Δ32 Da) on lysines and the N terminus; flexible modification of oxidation modifications for methionine residues; and one missed cleavage site of trypsin allowed. A reversed database was searched to evaluate the false discovery rate, and a rate of <0.05 was used as a filtering criterion for all identified peptides. In addition, only proteins identified with two or more tryptic peptides were considered, and proteins identified with the same set of peptides were grouped and treated as one.

Quantitation analysis was conducted using Protein Discoverer 1.3 software (Thermo Electron Corp.). Ratios between control and C1QBP-Flag samples were calculated based on the extracted ion chromatography areas (n = 3). A ratio of >10 was used as a cutoff threshold. Gene ontology annotation information was also acquired through Protein Discoverer 1.3. Pathway analysis was performed using DAVID. Known protein–protein interaction information was obtained by searching protein–protein interaction databases using VisAnt. The interaction data obtained through database searching and mass spectrometric analysis were then integrated in Excel and imported into Cytoscape v2.8.3 for network visualization.

**Immunofluorescence and Confocal Microscopy**—Cells were plated in 12-well plates containing sterile glass coverslips, allowed to grow for 24 h, and stained in serum-free medium for at least 3 h. After stimulation with 10 ng/ml EGF for 10 min at 37 °C, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature, permeabilized in 0.2% triton/PBS for 10 min, and blocked in 3% BSA in PBS for 1 h at room temperature. The cells were incubated with primary antibodies at 4 °C overnight and then stained with Alexa Fluor 488 and 546-conjugated secondary antibodies for 1 h at room temperature. 1 ng/ml DAPI was used for nuclear staining. Coverslips were counterstained with DAPI, mounted with ProLong® Gold antifade reagents, and visualized with confocal laser scanning microscopy (Leica TCS SP5). For quantitative measurement, the level of DNA was extracted with methanol at 4 °C for 1 h, and the F-actin content was measured using a microplate fluorescence reader with an excitation wavelength of 578 nm and an emission wavelength of 600 nm. Results were expressed as the relative F-actin content, where (F-actin) / (fluorescence) = (fluorescence) / (fluorescence).

**In Vivo Metastasis Assay**—For experimental metastasis studies, 1 × 10⁶ control cells and shC1QBP cells (equal number of clone 1 and clone 6 cells) in 100 μl of PBS were injected into the tail veins of SCID mice (n = 6). The number of metastatic nodes in the lung was macroscopically counted after 7 weeks. The lungs were fixed with formalin and embedded in paraffin. Serial sections were analyzed with H&E staining to verify lung micrometastasis. The animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

**Immunohistochemical Staining**—The 145 formalin-fixed, paraffin-embedded invasive breast cancer tissues and 17 hyperplasia lobular tissues were obtained from the Department of Breast Cancer, Tianjin Medical University Cancer Institute and Hospital. The use of tissue sections in this experiment was approved by the Ethics Committee of Tianjin Medical University. Immunohistochemical staining was performed as described previously (24, 25). Briefly, the sections were deparaffinized in xylene and rehydrated in graded series of ethanol solution. After antigen retrieval with sodium citrate buffer, the sections were incubated with mouse anti-human C1QBP monoclonal antibody at 4 °C overnight and then stained with HRP-conjugated secondary antibody. The expression was revealed by the addition of 3,3-diaminobenzidine buffer. PBS was used in place of the primary antibodies in all negative controls. The high or low expression of C1QBP in breast cancer tissues was scored semi-quantitatively by two experienced pathologists. The statistical analysis was performed using SPSS 17.0 software. The relationships between C1QBP expression and clinical parameters were analyzed via chi-squared test, and a p value less than 0.05 was considered statistically significant.

**RESULTS**

**Improvement of Sample Preparation for colP-MS**—Typically, in the sample-preparation step of colP assays, nuclei and cell debris were precipitated from the cell lysates via centrifugation (~ 15,000 × g at under 4 °C for 15 min). After the first step of centrifugation, a wide range of DNA fragments were observed from the supernatant (Fig. 1A). The presence of DNA fragments may cause false positives in interacting protein identification and decrease the sensitivity of detection. As shown in Fig. 1B, extending the duration of the centrifugation to 1 h greatly reduced the level of DNA contamination in the supernatant; however, a certain amount of DNA fragments remained. The amount of remaining DNA fragments decreased with increasing centrifugation speed, and no DNA...
fragments were observed after centrifugation at 50,000 \times g. We also compared the use of a nuclease with centrifugation—in this case, Benzonase™ was added. As shown in Fig. 1C, after incubation with Benzonase™ at 4 °C for 4 h, the amount of DNA fragments decreased, but not as efficiently as with 50,000 \times g centrifugation. Incubation of the cell lysates with Benzonase™ at 37 °C could increase the efficiency of nucleic acid removal (data not shown). However, it might cause protein degradation and affect the results of interacting protein identification.

High-speed centrifugation of cell lysates before coIP also reduced the presence of background binding proteins. As shown in Fig. 1D, the concentration of the extracted cellular protein was not affected by the addition of Benzonase™, and it was slightly reduced after the cell lysates were centrifuged at 50,000 \times g. Using nanoUPLC-MS/MS, we detected 230 proteins from the control group, 267 proteins after incubation with Benzonase™, and 143 proteins after high-speed centrifugation (n = 3; Fig. 1E). The most abundant background proteins were cytoskeleton proteins (i.e. actin, tubulin, and myosin). In addition, a large number of ribosomal protein isoforms were detected in the control and Benzonase™ groups, which could contribute to the sample background and interfere with the detection of actual interacting proteins. Most of these ribosomal proteins were removed by high-speed centrifugation (Fig. 1F).

Establishment of the Interactome of C1QBP—As discussed above, when high-speed centrifugation was used, the presence of DNA fragments and background interfering proteins was greatly reduced for the subsequent coIP and mass spectrometric analysis. In addition, to increase the coverage of interacting protein identification, two-dimensional RP-RP-LC-MS/MS was applied. The first offline HPLC separation was performed on a C18 column at pH 10. 11 fractions were subjected to the secondary online C18 nanoUPLC separation. A comparison of one-dimensional and two-dimensional separation is shown in supplemental Fig. S2. As a result, a ~4-fold increase in the total number of identified protein groups was achieved with two-dimensional LC-MS/MS relative to one-dimensional LC-MS/MS. However, to further distinguish nonspecific binding proteins with genuine interacting partners, an accurate quantitation strategy should be established.

In this study, the application of formaldehyde labeling was investigated. Fig. 2 shows an overview of the workflow employed in this study. Examples of the quantification results are shown in Fig. 3. Samples from cells transfected with empty Flag plasmid were labeled with formaldehyde, and samples from C1QBP-Flag cells were labeled with deuterated formaldehyde. A 4-Da mass change was observed for each label. Only the heavy labeled peaks were observed for the target protein C1QBP. Peak pairs with a 1:1 ratio were observed for the background proteins (i.e. actin). Interacting proteins were detected with either heavy labeled peaks or a much higher intensity in the heavy labeled state. Using a ratio of 10 as cutoff threshold, 187 proteins were determined as C1QBP interacting proteins (supplemental Table S1).

Most of the identified C1QBP interacting proteins were localized in the cytoplasm and nucleus (Fig. 4A). The other cellular components included organelle lumen, cytosol, cell membrane, cytoskeleton, and mitochondrion. Molecular function analysis revealed that most of the identified proteins were related to protein binding (24.92%), catalytic activity (16.58%),

C1QBP Regulates Cancer Cell Chemotaxis and Metastasis
and nucleotide binding (15.45%) (Fig. 4B). Several well-known C1QBP interacting proteins were identified in this study, such as large proline-rich protein BAT2 (26), coiled-coil-helix-coiled-coil-helix domain-containing protein 2, mitochondrial (27), and lethal giant larvae homolog 1 (28). However, most of the identified proteins were novel interacting proteins that had not been previously reported. The interacting proteins were analyzed using DAVID for related signaling pathways (Table I). The results showed that C1QBP was highly involved with gene transcription and gene expression, in agreement with earlier reports (29, 30). A KEGG database search also indicated that C1QBP was associated with multiple signaling molecules in the Tight Junction pathway, which plays an important role in the regulation of cell-cell interaction.

For network construction, the identification data were integrated with interaction information obtained through protein–protein interaction database searches and imported to Cytoscape for visualization. As shown in Fig. 4C, a highly connected network composed of 90 proteins and 174 connections was mapped (only proteins with a degree of connection of ≥2 are shown). Some of these proteins may function as “hubs” in the network (i.e. ATF2, HDGF, and MEPCE). Some of them formed highly connected subclusters that indicated the presence of a large complex consisting of multiple proteins (i.e. GTF3C). Taken as a whole, the interactome analysis provided a more complete picture of the biological functions of C1QBP, suggesting its potential roles in various biological processes.

**FIG. 2.** The sample preparation and mass spectrometric identification workflow employed in this study. Cell lysates were first cleaned up via high-speed centrifugation and then incubated with Flag antibody-conjugated beads. Two-dimensional RP-RP-LC-MS/MS coupled with formaldehyde labeling was applied to identify the interacting proteins.

**FIG. 3.** Relative quantification for identifying C1QBP interacting proteins using formaldehyde labeling. Peaks from the control (derivatized with formaldehyde) are labeled with circles, and peaks from C1QBP-Flag cells (derivatized with deuterated formaldehyde) are labeled with diamonds. Examples of tryptic peptide peak pairs from four proteins are shown: (A) C1QBP, (B) HDGF, (C) actin, and (D) SERBP1.
C1QBP Interacted with PRKCZ and Regulated Its Activity—In the proteomics analysis, PRKCZ was identified as a C1QBP interacting protein. PRKCZ plays a pivotal role in chemotaxis signaling networks (12, 31). Both EGF and chemokine ligand 12 elicit plasma membrane translocation of PRKCZ and its activation, which in turn regulates cell polarity.

Table I

| Databases   | Related pathways                                      | Number of genes | p value      | Gene symbols                      |
|-------------|-------------------------------------------------------|-----------------|--------------|-----------------------------------|
| Biocarta    | RNA polymerase III transcription                       | 5               | 4.0E-6       | SSB, GTF3C1, GTF3C3, GTF3C4, GTF3C5 |
|             | PRC2 complex sets long-term gene silencing            | 4               | 1.1E-3       | EED, EZH2, RING1, SUZ12           |
| KEGG        | Spliceosome                                           | 5               | 1.3E-2       | U2AF1, U2AF2, HNRNPC, SNRNP27     |
|             | Tight junction                                         | 5               | 1.6E-2       | CSA, LLGL1, PRKCZ, PRKCI, TJ1     |
| Reactome pathway | Transcription                                      | 11              | 3.1E-7       | RNMT, U2AF1, U2AF2, CCNT1, CDK9, GTF3C1, GTF3C2, GTF3C3, GTF3C4, GTF3C5, PABPN1 |
|             | Influenza infection                                   | 6               | 1.9E-2       | U2AF1, U2AF2, YBX1, CCNT1, CDK9, HNRNPC, PABPN1, RPL8 |
|             | Gene expression                                       | 9               | 1.9E-2       | RNMT, U2AF1, U2AF2, YBX1, CCNT1, CDK9, HNRNPC, PABPN1, RPL8 |
|             | Cell cycle, mitotic                                   | 7               | 1.9E-2       | MADIL1, MLF1IP, MCM3, NUMA1, PTTG1, PSMD1, RCC2 |

**Fig. 4.** Interacting proteins of C1QBP. A, diagram showing the cellular components of the identified C1QBP interacting proteins. B, diagram showing the related molecular functions of the identified C1QBP interacting proteins. The annotation information was acquired through analysis using Protein Discovery 1.3. C, network of C1QBP interacting proteins. Proteins and their interactions are shown as nodes and edges. The node size reflects the interaction degree. The network was constructed by integrating the proteomics analysis data and VisAnt interaction database search results and then visualized using Cytoscape.

**TABLE I**

| Databases | Related pathways | Number of genes | p value  | Gene symbols                      |
|-----------|------------------|-----------------|----------|-----------------------------------|
| Biocarta  | RNA polymerase III transcription                       | 5               | 4.0E-6   | SSB, GTF3C1, GTF3C3, GTF3C4, GTF3C5 |
|           |                  |                 | 1.1E-3   | EED, EZH2, RING1, SUZ12           |
| KEGG      | Spliceosome      | 5               | 1.3E-2   | U2AF1, U2AF2, HNRNPC, SNRNP27     |
|           | Tight junction   | 5               | 1.6E-2   | CSA, LLGL1, PRKCZ, PRKCI, TJ1     |
| Reactome  | Transcription    | 11              | 3.1E-7   | RNMT, U2AF1, U2AF2, CCNT1, CDK9, GTF3C1, GTF3C2, GTF3C3, GTF3C4, GTF3C5, PABPN1 |
|           | Influenza infection | 6              | 1.9E-2   | U2AF1, U2AF2, YBX1, CCNT1, CDK9, HNRNPC, PABPN1, RPL8 |
|           | Gene expression  | 9               | 1.9E-2   | RNMT, U2AF1, U2AF2, YBX1, CCNT1, CDK9, HNRNPC, PABPN1, RPL8 |
|           | Cell cycle, mitotic | 7              | 1.9E-2   | MADIL1, MLF1IP, MCM3, NUMA1, PTTG1, PSMD1, RCC2 |

C1QBP Regulates Cancer Cell Chemotaxis and Metastasis
through par3/GSK3β and mediates actin polymerization through coflin (12, 24, 32). As shown in Fig. 5A, the interaction between PRKCZ and C1QBP was further confirmed by Western blotting, and their interaction was enhanced upon EGF stimulation. To further explore the nature of their interaction, coIP assays were performed on different constructed fragments of C1QBP and PRKCZ. C1QBP co-immunoprecipitated with the regulatory domain of PRKCZ (Fig. 5B). In addition, coIP assays showed that both of the C1QBP fragments containing the 74–174 amino acid sequence interacted with PRKCZ, whereas its C-terminus fragment, 175–282aa, could not co-immunoprecipitate with PRKCZ (Fig. 5C). These results indicate that the N terminus of the mature form of C1QBP interacts with the regulatory domain of PRKCZ. Furthermore, the subcellular distribution of C1QBP and PRKCZ was studied via confocal fluorescent microscopy. C1QBP was mainly distributed in the mitochondria, and treatment with EGF induced an enrichment of C1QBP on the plasma membrane, leading to co-localization with PRKCZ (Fig. 5D).
To investigate the effect of C1QBP on the biological activity of PRKCZ, the expression of C1QBP was depleted in MDA-MB-231 cells and the phosphorylation and membrane translocation of PRKCZ, hallmarks of its activation during EGF-induced chemotaxis, were examined. As shown in Fig. 4E, EGF-induced phosphorylation of PRKCZ in the activation loop (Thr410) was impaired in C1QBP-depleted cells, and a confocal fluorescent microscopy study showed that ablation of C1QBP also inhibited EGF-induced membrane translocation of PRKCZ (Fig. 5F). However, in PRKCZ knockdown cells, EGF-induced C1QBP translocation to the plasma membrane was intact, suggesting that PRKCZ had no effect on the activation of C1QBP, and C1QBP may function upstream in the signaling pathway (Fig. 5G). Taken together, the results demonstrated that EGF induced an interaction between C1QBP and PRKCZ, and that C1QBP has a great effect on the biological activity of PRKCZ.

C1QBP Regulates Cancer Cell Chemotaxis through Interaction with PRKCZ—To investigate the potential roles of C1QBP involved with cancer cell chemotaxis, we first analyzed the expression level of C1QBP in human breast epithelial cell line and various breast cancer cell lines, including MCF-7, T47D, BCap37, ZR-75-30, BT549, SK-BR-3, and MDA-MB-231 (Fig. 6A). Its expression in human breast epithelial cell line was modulated by regulating cell polarity and EGF-induced actin polymerization.

A, Western blotting analysis of the expression of C1QBP in seven different breast cancer cell lines and breast epithelial cell line HBL-100. B, Western blotting analysis indicated that the expression of C1QBP was dramatically reduced in two stable clones (clone 1 and clone 6). C, chemotaxis assay of controls and two C1QBP knockdown stable clone cells. The quantitative data are expressed as means ± S.E. from three independent experiments. Statistical analysis was performed via two-way analysis of variance (**p < 0.0001). D, C1QBP deletion impaired cell migration in the wound-healing assay. The migrating distances of the control and two C1QBP knockdown cells at different time points are plotted. The quantitative data are expressed as means ± S.E. from three independent experiments. E, down-regulation of C1QBP reduced the EGF-induced phosphorylation of GSK3β in MDA-MB-231 cells. F, down-regulation of C1QBP impaired wound-healing-induced Golgi and γ-tubulin reorientation in MDA-MB-231 cells (left); the percentage of leading edge cells with Golgi reorientation was counted and summarized (right). Quantitative data are expressed as means ± S.E. from three independent experiments. Statistical analysis was performed via one-way analysis of variance (*p < 0.05). G, down-regulation of C1QBP impaired the phosphorylation of cofilin in MDA-MB-231 cells. H, fluorescence microscopy analysis indicated EGF-induced actin polymerization was impaired in C1QBP knockdown cells. Scale bar = 25 μm.
The epithelial cell line HBL-100 was lower than that in the seven types of cancer cells. Highly migratory cancer cells (i.e., MDA-MB-231, ZR-75-30, and BT-549) appeared to express higher levels of C1QBP than low-migratory cells (i.e., T47D). The migration ability of four breast cancer cell lines was examined with a wound-healing assay, and the results are shown in supplemental Fig. S2.

Secondly, two stable C1QBP knockdown clones (clones 1 and 6) were generated (Fig. 6B). Down-regulation of C1QBP levels inhibited MDA-MB-231 cell migration in both EGF-induced chemotaxis and wound-healing assays, suggesting that C1QBP plays an important role in cell migration (Figs. 6C and 5D). A similar phenomenon was also observed in two other breast cancer cell lines, BT549 and ZR-75-30 (supplemental Fig. S2). Although C1QBP is well known for its function in oxidative phosphorylation, a major pathway for energy metabolism (9), the data indicate that oxidative phosphorylation was not directly required for its regulatory role in chemotaxis (supplemental Fig. S3).

PRKCZ regulates cancer cell chemotaxis by organizing cell polarity and inducing actin polymerization (12, 33). If C1QBP regulates cell migration through PRKCZ, both responses should be detected in C1QBP-depleted cells. Indeed, silencing of C1QBP inhibited EGF-induced phosphorylation of GSK3β, which is a key modulator of cell polarity (Fig. 6E) (31). In addition, as shown in Fig. 6F, migrating cells lost their polarity, as indicated by disorientation of the Golgi in the wound-healing assay (p < 0.05). Furthermore, EGF-induced coflin phosphorylation was inhibited in the C1QBP knockdown cells, as indicated by Western blotting analysis (Fig. 6G), which in turn caused impairment in actin polymerization, as demonstrated by confocal microscopy (Fig. 6H) and quantitative measurements (supplemental Fig. S4). These results indicate that C1QBP mediates chemotaxis through the regulation of cell polarity and cytoskeleton rearrangement.

C1QBP Was Required for Breast Cancer Cell Metastasis in Vivo—To test the biological relevance of C1QBP in cancer metastasis, an experimental metastasis model using SCID mice was employed. The control cells and a mixture of two stable C1QBP knockdown clones (clone 1 and clone 6) were injected into the tail veins of SCID mice. Seven weeks later, it was found that tumor foci had formed in the lungs of the mice that were injected with control cells. In contrast, few foci were detected in the lungs of mice injected with stable C1QBP depleted clones (Figs. 7A and 6B). The results suggest that C1QBP knockdown cells failed to form tumors in SCID mouse lungs and that C1QBP was required for breast cancer metastasis.

Furthermore, the expression of C1QBP and its association with cancer metastasis was investigated using clinical samples. Paraffin-embedded tissues from 145 cases of invasive ductal breast carcinoma and 17 cases of lobular hyperplasia were examined via immunohistochemical staining using an anti-C1QBP antibody. Among the 145 specimens of invasive ductal carcinoma, 107 samples showed positive staining, whereas only 6 out of 17 cases of lobular hyperplasia were positive (Fig. 7C and supplemental Table S2). The results suggest that a high level of C1QBP was expressed in breast cancer tumor tissues. Further clinical pathological analysis revealed that the expression of C1QBP was closely associ-
C1QBP Regulates Cancer Cell Chemotaxis and Metastasis

| Parameters/markers | Total | C1QBP expression | p value |
|--------------------|-------|------------------|---------|
| Age (years)        |       |                  |         |
| <50                | 67    | 21               | 0.256   |
| ≥50                | 78    | 17               |         |
| Tumor size (cm)    |       |                  |         |
| <2                 | 22    | 9                | 0.114   |
| ≥2                 | 123   | 29               |         |
| TNM stage          |       |                  |         |
| I                  | 12    | 6                | 0.012   |
| II                 | 75    | 24               |         |
| III                | 29    | 6                |         |
| IV                 | 29    | 2                |         |
| Lymph node status\textsuperscript{a} |     |                  |         |
| Negative           | 64    | 18               | 0.705   |
| Positive           | 81    | 20               |         |
| Distant metastasis\textsuperscript{b} |     |                  |         |
| Negative           | 116   | 36               | 0.008   |
| Positive           | 29    | 2                |         |

\textsuperscript{a} The lymph node status with cancer cells is positive. The lymph node status with no detectable cancer cells is negative.

\textsuperscript{b} Distant metastasis includes patients with lung and liver metastasis. Positive: lung and liver with cancer cells; negative: lung and liver with no detectable cancer cells.

Table II: Correlation between C1QBP expression and clinic pathologic parameters of patients with invasive ductal carcinoma

In summary, we established an efficient coIP-MS strategy for studying protein–protein interactions and established the interactome network for C1QBP. In-depth biological studies revealed a novel signaling pathway mediated by C1QBP that was essential for cancer cell chemotaxis and metastasis. This study provides evidence of a promising biomarker and drug target for cancer therapeutics.

Acknowledgments—We thank Dr. Yi Yang for assistance with the confocal microscope and Dr. Lisha Qi for immunohistochemical evaluation. We also thank Erin Gempel from the University of Wisconsin at Madison for her help with manuscript editing.

REFERENCES

1. Ghebrehiwet, B., Lim, B. L., Pearschule, E. I., Willis, A. C., and Reid, K. B. (1994) Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. J. Exp. Med. 179, 1809–1821

2. Muta, T., Kang, D., Kitajima, S., Fujiwara, T., and Hamasaki, N. (1997) p32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phos-
C1QBp Regulates Cancer Cell Chemotaxis and Metastasis

10. Kim, K. B., Yi, J. S., Nguyen, N., Lee, J. H., Kwon, Y. C., Ahn, B. Y., Cho, K. H., Kim, Y. K., Yoo, H. J., Lee, J. S., and Ko, Y. G. (2011) Cell-surface C1qR regulates cancer cell migration and metastasis. J. Biol. Chem. 286, 23093–23101.

11. He, J., Gu, D., Wu, X., Reynolds, K., Duan, X., Yao, C., Wang, J., Chen, C. S., Chen, J., Wildman, R. P., Klag, M. J., and Whelan, P. K. (2005) Major causes of death among men and women in China. N. Engl. J. Med. 353, 1124–1134.

12. Sun, R., Gao, P., Chen, L., Ma, D., Wang, J., Oppenheim, J. I., and Zhang, N. (2005) Protein kinase C zeta is required for epidermal growth factor-induced chemotaxis of human breast cancer cells. Cancer Res. 65, 1433–1441.

13. Roussos, E. T., Condeelis, J. S., and Patsialou, A. (2011) Chemotaxis in cancer. Nat. Rev. Cancer 11, 573–587.

14. Muller, A., Horney, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50–56.

15. Ghosh, I., Chowdhury, A. R., Rajeswari, M. R., and Datta, K. (2004) Differential expression of hyaluronic acid binding protein 1 (HABP1)/C1QBp during progression of epithelial carcinoma. Mol. Cell. Biochem. 267, 133–139.

16. Chen, R., Wang, Y., Liu, Y., Zhang, Q., Zhang, X., Zhang, F., Shieh, C. H., Yang, D., and Zhang, N. (2013) Quantitative study of the intercorrelate of PKCzeta involved in the EGF-induced tumor cell chemotaxis. J. Proteome Res. 12, 1478–1486.

17. Mann, M. (2006) Functional and quantitative proteomics using SILAC. Nat. Rev. Mol. Cell. Biol. 7, 952–958.

18. Wang, T., Gu, S., Ronni, T., Du, Y. C., and Chen, X. (2005) In vivo dual-tagging proteomic approach in studying signaling pathways in immune response. J. Proteome Res. 4, 941–949.

19. Chen, R., Hui, L., Cape, S. E., Wang, J., and Li, L. (2010) Comparative proteomic analysis of food intake via a multi-faceted mass spectrometric approach. ACS Chem. Neurosci. 1, 204–214.

20. Hsu, J. L., Huang, S. Y., Chow, N. H., and Chen, S. H. (2003) Stable-isotope dimethyl labeling for quantitative proteomics. Anal. Chem. 75, 6843–6852.

21. Lai, J. S., and Herr, W. (1992) Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. U.S.A. 89, 6958–6962.

22. Nguyen, T. N., and Goodrich, J. A. (2006) Protein-protein interaction assays: eliminating false positive interactions. Nat. Methods 3, 135–139.

23. Tsboi, S. (2006) A complex of Wiskott-Aldrich syndrome protein with mammalian verprolins plays an important role in monocyte chemotaxis. J. Immunol. 176, 6576–6585.

24. Zhang, F., Zhang, X., Li, M., Chen, P., Zhang, B., Guo, H., Cao, W., Wei, X., Cao, X., Hao, X., and Zhang, N. (2010) mTOR complex component Rictor interacts with PKCzeta and regulates cancer cell metastasis. Cancer Res. 70, 9360–9370.

25. Nesrine, M., Sellami, R., Doghri, R., Rifi, H., Raies, H., and Mezlini, A. (2012) Testicular synovial sarcoma: a case report. Cancer Biol. Med. 9, 274–276.

26. Lehner, B., Semple, J. I., Brown, S. E., Counsell, D., Campbell, R. D., and Sanderson, C. M. (2004) Analysis of a high-throughput yeast two-hybrid system and its use to predict the function of intracellular proteins encoded within the human MHC class III region. Genomics 83, 153–167.

27. Seo, M., Lee, W. H., and Huh, K. (2012) Identification of novel cell migration-promoting genes by a functional genetic screen. FASEB J. 26, 464–478.

28. Bialucha, C. U., Ferber, E. C., Pichaud, F., Peak-Chew, S. Y., and Fujita, Y. (2007) p32 is a novel mammalian Lgl binding protein that enhances the activity of protein kinase Czeta and regulates cell polarity. J. Cell Biol. 178, 579–581.

29. Petersen-Mahrt, S. K., Estner, C., Ohrlmalm, C., Matthews, D. A., Russell, W. C., and Akusjarvi, G. (1999) The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation. EMBO J. 18, 1014–1024.

30. Chattopadhay, C., Hawke, D., Kobayashi, R., and Maity, S. N. (2004) Human p32 interacts with B subunit of the CCAAT-binding factor, CBF/ NF-Y, and inhibits CBF-mediated transcription activation in vitro. Nucleic Acids Res. 32, 3632–3641.

31. Etienne-Manneville, S., and Hall, A. (2003) Cdc42 regulates GSK-3beta and adenomatous polyps in cell control cell polarity. Nature 421, 753–756.

32. Etienne-Manneville, S., and Hall, A. (2001) Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106, 489–498.

33. Etienne-Manneville, S., Manneville, J. B., Nicholls, S., Ferenzi, M. A., and Hall, A. (2006) Cdc42 and Par6-PKCzeta regulate the spatially localized association of Dlg1 and APC to control cell polarization. J. Cell Biol. 170, 895–901.

34. Amamoto, R., Yagi, M., Song, Y., Oda, Y., Tsuneyoshi, M., Naito, S., Yokomizo, A., Kuroiwa, K., Tokunaga, S., Kato, S., Hiura, H., Samori, T., Kang, D., and Uchiumi, T. (2011) Mitochondrial p32/C1QBp is highly expressed in prostate cancer and is associated with shorter prostate-specific antigen relapse time after radical prostatectomy. Cancer Sci. 102, 639–647.