Published in final edited form as:

Proteomics. 2009 December ; 9(24): 5414–5424. doi:10.1002/pmic.200800864.

Biotin tagging coupled with amino acid-coded mass tagging (BioCAT) for efficient and precise screening of interaction proteome in mammalian cells

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Abstract

In mammalian cells, when tandem affinity purification (TAP) approach is employed, the existence of untagged endogenous target protein and repetitive washing steps together result in overall low yield of purified/stable complexes and the loss of weakly and transiently interacting partners of biological significance. To avoid the trade-offs involving in methodological sensitivity, precision, and throughput here we introduce an integrated method, biotin tagging coupled with amino acid-coded mass tagging (BioCAT) for highly sensitive and accurate screening of mammalian protein-protein interactions (PPIs). Without the need of establishing a stable cell line, using a short peptide tag which could be specifically biotinylated *in vivo*, the biotin-tagged target/bait protein was then isolated along with its associates efficiently by streptavidin magnetic microbeads in a single step. In a pulled-down complex amino acid-coded mass tagging (AACT) serves as ‘in-spectra’ quantitative markers to distinguish those bait-specific interactors from non-specific background proteins under stringent criteria. Applying this BioCAT approach, we first biotin-tagged *in vivo* a multi-functional protein family member, 14-3-3ε, which was expressed at close to endogenous level. Starting with approximately 20 millions of 293T cells which were significantly less than what needed for a TAP run, 266 specific interactors of 14-3-3ε were identified in high confidence.

Keywords

Affinity purification; Amino acid-coded mass tagging; Biotin tagging; Protein-protein interaction; 14-3-3ε

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Supporting Information Available: Supplementary Figure 1 and Tables 1 and 2. This material is available free of charge via the Internet.

1 Introduction

Cellular functions, either under normal or pathological conditions, or different stresses, are the results of the coordinated action of multiple proteins interacting in macromolecular complexes or assemblies [1-4]. Therefore, precise determination of specific composition of protein complexes, especially using scalable and high-throughput methods, represents a systematic approach toward revealing particular cellular biological functions [2-6].

Immunoprecipitations (IP) via available antibodies against target proteins are commonly used to selectively enrich and pull-down target protein and its interacting partners for concurrent MS analysis of their identities [3, 4, 7]. However, in an IP complex pull-down experiment the antibody against the target protein may interfere with the binding sites on the target protein for recruiting its interactors. Tagging the target gene with known epitope sequence(s) followed by IP experiments using epitope-specific antibody becomes a popular way to pull down target protein-specific protein complexes. This type of approaches allows for ‘opening’ these binding sites at the target protein for its partners so that the naturally occurring endogenous interactions can be preserved. As a commonly used approach of the epitope tagging strategy, tandem affinity purification (TAP) was developed to isolate target protein-specific interacting complexes in high purity [5, 7, 8]. The rationale behind TAP tag method is that the contaminating proteins bound non-specifically to the bait protein can be removed by multiple steps of epitope-based affinity purifications [7, 8]. In addition to the tedious step of establishing a cell line that stably expresses TAP-tagged target/bait protein, the repetitive washing not only demands for considerable amounts of the cells starting with for purification to homogeneity (typically around 10^9 cells), but also, as a trade-off for high complex purity, can lead to the loss of weak or transient interactions of biological relevance.

In MS analysis ‘in-spectra’ quantitative markers can be introduced through metabolic incorporation of stable isotope-enriched amino acids using our amino acid-coded mass tagging (AACT) approach [9, 10], or stable isotope labeling by amino acids in cell culture (SILAC as named by another group) [11], to improve the sensitivity, accuracy, and throughput of measuring protein abundance changes [9, 11, 12]. We have previously developed a ‘dual-tagging’ (both epitope tag and stable isotope tag) quantitative proteomic method [13-15] that integrates the capabilities of natural complex formation, epitope affinity isolation, and ‘in-spectra’ quantitative markers to systematically distinguish those interacting proteins from a large non-specific binding background. The sensitive detection of signal proteins can then be achieved with a single isolation step which can minimize the loss of weakly or transiently interacting partners. However, unlike the recombination mechanism capable of completely ‘knock-outing’ the untagged endogenous version of target protein in yeast cells [16], in mammalian cells the residual expression of the endogenous untagged target protein can lead to the competition with its epitope-tagged counterpart for recruitment of its interacting partners, which could lower the yield of pulling down the target-specific complex [7, 8]. It is a reasonable assumption that an epitope tag with stronger affinity could allow for higher recovery of tagged bait and hence its interacting partners in the pulled down complex. Because biotin-avidin binding is the strongest non-covalent interaction known in nature, a high-affinity isolation of protein complex could be achieved through biotinylating a target protein by co-expressing bacterial BirA biotin ligase and then purifying the biotin-

tagged protein using streptavidin beads [17]. To further improve the specificity, accuracy, and sensitivity of our dual-tagging strategy, here we introduce a convenient and efficient method for screening interaction proteome or interactome in mammalian cells by using *in vivo* biotin tagging coupled with amino acid-coded mass tagging or we named as BioCAT. We showed that a target protein with a short artificial tag of 15 amino acids [18, 19] could be specifically biotinylated either *in vitro* or *in vivo*. Through transient co-transfection with BirA biotin ligase the biotin-tagged target protein was biotinylated *in vivo*. Also the biotin-tagged target protein was expressed at lower or close to the level of endogenous untagged counterpart which allows us to profile target protein-specific interactions at physiologically relevant conditions.

In our model system of biological interest, the 14-3-3 proteins, that are small, acidic, and abundant proteins with propensity to form both homo- and heterodimers, belong to a family of conserved regulatory molecules expressed in all eukaryotic cells. In mammals seven isoforms including 14-3-3 β , γ , ϵ , η , σ , θ (or τ) and ζ are known to possibly interact with more than 300 target proteins [20-22]. These isoforms have high homology with approximately 50% amino acid identity [22]. There is no apparent single conserved function for all members of 14-3-3 family instead one can hypothesize that each type of 14-3-3 isomers can be differentially involved in regulating various cellular processes through interacting with different groups of functionally characteristic proteins [20-23]. As the first step to reveal the multi-functional nature of the least characterized 14-3-3 isomer, 14-3-3 ϵ , we illustrate the unique strength of this BioCAT approach for screening 14-3-3 ϵ -specific interactome. Note that compared to a TAP-based method of QTAX [16] which was demonstrated only for yeast cells, our method bypasses tedious procedure of establishing stable cell line, meanwhile our parallel uses of a single high affinity tag instead of TAP tag for high yield pull-down and amino acid tags for increasing signal specificity will ensure the efficiency and accuracy of distinguishing specific protein interactions in mammalian cells.

2 Methods

2.1 Plasmid/Vector Generation

The DNA sequence coding for the 15-aa acceptor peptide (AP) tag (GLNDIFEAQKIEWHE, refs. 18, 19) is amplified by PCR by using two partly complementary primers. The sequences of the primers used in our experiments were given as follows: for the AP-tag at N-terminus, the sense primer 5'-CACCATGTCCGGCCTGAACGACATCTTCGAGGC TCAGAAAATCG-3', antisense 5'-TTCGTGCCATTTCGATTTTCTGAGCCTCG-3'; for the AP-tag at C-terminus, sense 5'-CACCTCCGGCCTGAACGACATCTT CGAGGCTCAGAAAATCG-3', antisense 5'-TCATTCGTGCCATTTCGATTTTCTGAGCCTCG-3. The coding region of the *Escherichia coli* birA biotin-protein ligase gene was amplified from genomic DNA by PCR using the primers with sense 5'-CACCATGAAGGATAACACCGTGCCACTG-3' and antisense 5'-GGTAGAAGAGGTACACTACGC-3. These PCR products were then introduced directly into the expression vector pcDNA3.1 using the directional TOPO Cloning technology (Invitrogen). The constructs were then named as pAP-C, pAP-N and pBirA, respectively. The cDNAs coding for VAV1, a signaling mediator in many types of immune cells, and

14-3-3 ϵ were amplified by RT-PCR from NK92MI cell and 293T cell, respectively, and then introduced into the corresponding restriction enzyme sites of pAP-C or pAP-N (Hind III/BamH I or EcoR V/Xba I) to generate new expression vectors, where the AP tag is genetically encoded at the C terminus or N terminus of the protein of interest. The vectors were then named as pVAV1-AP for carrying the VAV1 gene with the AP tag at the C-terminus; p1433-AP for carrying the 14-3-3 ϵ gene with the AP tag at the C-terminus; and pAP-1433 for carrying the 14-3-3 ϵ gene with the AP tag at the N-terminus. The primers used for these constructs were as follows: pVAV1-AP, sense 5'-GCTTATCAAGCTTAGGCGGTAGCCATGGAG-3', antisense 5'-GCACCGGATCCCAGCAGTATTCAGAATAATC-3'; pAP-1433, sense 5'-CGTTGATATCGATGATCGAGAGGATCTGGTG-3', antisense 5'-CCAGTCTAGATCACTGATTTTCGTCTTCCACG-3'; p1433-AP, sense 5'-CGACGACAAGCTTATGGATGATCGAGAGGATCTGG-3', antisense 5'-CTATGGGATCCTGATTTTCGTCTTCCACGTC-3. All vectors were verified by sequencing.

2.2 Reagents

Recombinant biotin ligase BirA and AP-tagged standard protein, MBP-AviTag either fully biotinylated or un-biotinylated was obtained from Avidity (Denver, CO). Anti-14-3-3 ϵ antibody was obtained from Chemicon (Temecula, CA). Anti-14-3-3 ζ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NonO antibody was obtained from Protein Tech Group (Chicago, IL). Leucine-d₃ was purchased from Cambridge Isotope Laboratories (Andover, MA).

2.3 Cell Culture and Transfection

The 293 and 293T cells were cultured in DMEM (Invitrogen, Calsbad, CA) supplemented with 10% FBS (Invitrogen), 1% penicillin, and 100 μ g/mL streptomycin. The NK92MI cells were cultured in α -MEM (Invitrogen) supplemented with 12.5% fetal calf serum, 12.5% horse serum, 0.2 mM inositol, 0.1 mM -mercaptoethanol and 0.02 mM folic acid. The hepatocellular carcinoma cell line 7703 was cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS. The 293T cells for AACT were maintained in DMEM with leucine replaced with leucine-d₃ until the leucine-d₃ was fully incorporated into cellular proteins [10, 11]. Transient transfection of 293T cells was performed in 6-well plate by using FuGENE HD transfection reagent according to the manufacture's instruction (Roche Diagnostics, Indianapolis, IN). For comparative analysis we used same total amount of plasmids when transfecting the cells either with one type of plasmids or co-transfecting with both types of plasmids.

2.4 Immunoblot Analysis

The proteins were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore, Bedford, MA). For analysis of biotinylation, filters were blocked for 1 h in 5% BSA/1 $\times$ TBS/0.1% Tween-20 and incubated for 1 h at room temperature with streptavidin-horseradish peroxidase conjugate (HRP). For the analysis of protein expression, filters were incubated with the specified primary antibody followed by incubation with a

secondary antibody conjugated with HRP. The ECL substrate was then added and the blot was developed.

For the purpose of validating newly identified 14-3-3ε interacting partners, the 293T cells were transiently transfected alone with the expression plasmid pBirA or co-transfected with both plasmids as indicated. Forty hours after the transfection, the biotin-tagged AP-1433 and its interactors were pulled down together by streptavidin microbeads and then separated by SDS-PAGE for Western blotting against, *e.g.*, anti-streptavidin or anti-14-3-3 or anti-NonO antibodies.

2.5 *In vitro* Biotinylation

The cells were lysed in the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). The cellular soluble extracts (200 μl) were then transferred into a Microcon centrifugal filter (YM-3, 3000 molecular weight cutoff, Millipore) to desalt. The Microcon tube was spun at 14,000 × *g* for 1 h, after which about 25 μl remained. The sample in the Microcon was diluted in 100 μl of dilution buffer (10 mM Tris-HCl, pH 8.0) and respun for 40 min, and the cycle repeated twice, with a final volume of about 50 μl. For biotinylation, recombinant biotin ligase BirA was added to the desalted protein solution with ATP and biotin for 1 hr at 30°C, according to the manufacture's protocol.

2.6 One-step Purification

The biotin-tagged proteins were purified by using streptavidin magnetic microbeads through magnetic sorting according to the manufacturer's instructions. For purification of the standard protein, 0.22 or 0.67 μg of MBP-AviTag protein was diluted in 200 μl of binding buffer (10 mM Tris-HCl, pH 8.0, 0.5% BSA) and then incubated with 100 μl of streptavidin magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). For purification of the tagged proteins that have been biotinylated *in vivo* by co-expressed BirA ligase, the cellular soluble extracts (200 μl) were directly incubated with 100 μl of streptavidin magnetic microbeads in the lysis buffer. The biotin-avidin binding was usually completed in seconds as suggested by the manufacturer. After rinsing the μ Column in the magnetic field of the μMACS separator with the appropriate equilibration buffer (Miltenyi Biotec) and the binding buffer, the binding reaction (following 1 min incubation at room temperature) was applied onto the top of the column matrix. The column was then rinsed with the lysis buffer, which was used for cell lysis as described above, to remove non-specifically binding molecules. In the last step, elution of the biotinylated protein was performed by applying 100 μL of 1× SDS protein loading buffer after removing the column out of the magnetic field. For purification of the tagged protein that was biotinylated *in vitro*, before binding with streptavidin microbeads, the protein was first removed free biotin by using Microcon centrifugal filter (3000 molecular weight cutoff) as described above. The purified proteins in 1× SDS protein loading buffer were boiled for 5 min before resolved by SDS-PAGE. The gel was stained by Coomassie brilliant blue or silver using standard protocols. We used Bandscan 5.0 software for performing the densitometry analysis.

2.7 LC-MS/MS Analysis

The procedure for in-gel digestion and peptide extraction was similar to that previously described [13, 14]. Nanocapillary liquid chromatography electrospray ionization tandem mass spectrometry (nanoLC-MS/MS) was performed on a hybrid linear quadrupole ion trap/Orbitrap (LTQ Orbitrap) mass spectrometer (Thermo Electron, San Jose, CA). An Agilent 1100 series nanoflow HPLC is coupled on-line to a nanospray ionization source (Proxeon Biosystems) holding a column packed with 3- μ m-diameter ReproSil Pur C18 beads into a 15-cm-long, 75- μ m-inner diameter fused silica emitter. The peptide mixture eluted from each gel band was separated by using a gradient, Buffer A consists of 0.5% acetic acid, and buffer B consists of 0.5% acetic acid and 80% acetonitrile (ACN). The gradient elution was run from 6% to 30% ACN over 60 min, then 30% to 80% of Buffer B in the next 10 min, held at 80% B for 5 min, and then dropped to 6% B for another 15 min to recondition the column. The LTQ-Orbitrap was set to acquire a full-range scan at 60,000 resolution from 350 to 1500 Th in the Orbitrap and to simultaneously fragment the top five abundant peptide ions in each cycle in the LTQ. Because Orbitrap data were to be used for quantitative analysis, blank gradients where buffer B was injected were interspersed between two analytical gradients to eliminate sample carryovers.

2.8 Protein Identification

MS/MS data was searched against the IPI human protein database (version 3.13) using the SEQUEST algorithm (Thermo Electron) incorporated into the BioWorks software (version 3.2). Searches were performed with modifications on methionine (oxidation), or leucine with masses 15.9949 or 3.018 Da respectively. The peptide mass tolerance and fragment mass tolerance were at 50 ppm and 1 Da respectively. SEQUEST results were filtered by Xcorr in coordinating with the charge state of peptides. Xcorr values of 1.9 for singly charged ions, 2.7 for doubly charged ions, and 3.75 for triply charged ions were considered as the positive matches. We set Delta Cn 0.1 and the probability limit 0.001. MS/MS files were searched with a tryptic enzyme constrain against a forward and decoy human IPI protein database using the SEQUEST algorithm to yield an estimated 1% false-positive rate. Each protein identification requires the matches of at least two peptides.

2.9 Protein Quantitation

The light-to-heavy isotope ratio (L/H) of each leucine-containing peptide was measured by selecting ion chromatogram extraction using the PepQuan ICAT software in BioWorks 3.2. For the default parameter, we selected leucine as the 'modified' amino acid residue with a mass shift of 3.0188Da between its light and heavy isotope version within the mass tolerance of 0.03. The identified peptides with a minimum score cutoff of 30 were used to obtain the L/H ratio for the corresponding proteins. For a protein L/H ratio obtained from multiple leucine-containing peptides the standard deviation (sd) was used to calculate the accuracy of L/H ratio. The proteins with their L/H sd larger than 20% were checked on their MS signals manually to compare the ratio of peak intensity with the area of XIC. Two biological replicates of the samples were performed and compared. Only consistent results of protein identification and quantitation from both runs were considered as positive and were included in our report.

2.10 Functional analysis by bioinformatics

Functions of those identified specific interactors were described using gene ontology (GO) terms. Gene annotations were performed using the Functional Annotation Tool at DAVID site (<http://david.abcc.ncifcrf.gov>, May 2008 version). For each GO term (GOTERM_BP_3), we assembled the group of genes annotated with the term when the number of the genes was more than 8.

The analysis of the associations of the identified specific interactors was performed by STRING, which is a database of known and predicted protein-protein interactions (PPIs) (<http://string.embl.de/>, May 2008 version).

3 Results

3.1 Efficiency and Specificity of Biotinylation of Target/Bait Protein in Mammalian Cells

An artificial peptide tag of 23 amino acids (aa) has been shown to be biotinylated *in vitro* by bacterial BirA biotin ligase [19] or *in vivo* in mammalian cells by co-expressed BirA ligase [17]. A shorter 15 aa acceptor peptide (AP) tag could be site-specifically biotinylated by BirA ligase at the lysine residue within the AP sequence *in vitro* [19]. It has been reported that the surface proteins tagged with an AP tag could be biotinylated *in vitro* by adding BirA ligase to a medium culturing mammalian cells [18]. We selected the shorter AP tag which may reduce the effect on target protein-specific interactions to examine the efficiency and specificity of *in vivo* biotinylation in mammalian cells. We then transfected 293T cells with both plasmids carrying the genes coded for AP-tagged protein VAV1 (VAV1-AP) and for BirA ligase. Our results indicated that the AP tag is unlikely to be recognized and biotinylated by endogenous biotin ligases in mammalian cells, such as holocarboxylase synthetase, *etc* [24]. AP-VAV1 was biotinylated in the cells co-expressing BirA, but not in those with the absence of BirA (Figure 1A), indicating the AP tag-dependent specificity for biotinylation. In addition, we also observed that AP-VAV1 could be biotinylated *in vitro* by recombinant BirA (Figure 1A).

We then placed the AP tag at either the N or the C terminus of 14-3-3 ϵ protein, an important multi-functional protein involving in widespread biological processes [20-23]. We observed that both terminus versions of AP-tagged 14-3-3 ϵ protein were specifically biotinylated either *in vivo* (Figure 1B, lanes 3 and 4) or *in vitro* (Figure 1B, lanes 7 and 8). In addition, very little nonspecific biotinylation background was observed in the 293T cells expressing only BirA (Figure 1B, lanes 2, 3 and 4), or only AP-tagged 14-3-3 ϵ protein (Figure 1B, lanes 5 and 6). Using anti-14-3-3 ϵ antibody, both the slower-migrating tagged protein and the endogenous 14-3-3 ϵ were detected in the cellular extracts from transfected cells (Figure 1C). Note that unlike those cells transfected with AP-tagged 14-3-3 ϵ alone, in the case of the co-transfection with BirA-expressing vector, the expression level of AP tagged 14-3-3 ϵ either at N- or C-terminal was found close to that of the untagged endogenous 14-3-3 ϵ to ensure the analysis of the endogenous protein components. For the purpose of comparison, the half amount of 14-3-3 ϵ vector was used for the co-transfection compared to when transfected with AP-tagged 14-3-3 ϵ alone. Taken together, these results indicate that AP-

tagged protein could be specifically and efficiently biotinylated by BirA ligase both *in vivo* and *in vitro*.

3.2 Efficient Purification of Biotin-Tagged Target Protein and Its Associates in Complex Using Streptavidin Magnetic Microbeads

Efficient pull-down of the tagged bait protein and its specific associates is critical for protein identification by MS which is abundance-based. Because of the strong affinity between biotin and streptavidin, biotin-tagged protein could be isolated and purified by using streptavidin beads [17, 25]. Here we explored the possibility for convenient and efficient purification of biotinylated AP-tagged bait protein by using magnetic streptavidin microbeads and then magnetic sorting.

First we used an AP-tagged standard protein, MBP-AviTag (Avidity), to test the purification efficiency of magnetic sorting. There are two versions of MBP-AviTag protein, one fully biotinylated (B-MBP), and the other un-biotinylated (UB-MBP). B-MBP (Figure 2, lane 2) was efficiently captured and purified by streptavidin magnetic microbeads (Figure 2, lane 3), while UB-MBP (Figure 2, lane 4) was mostly in the flow through (Figure 2, lane 5). Meanwhile we observed a high recovery of B-MBP after the sorting step (Figure 2, lane 2 vs lane 3).

We further examined the purification specificity of AP-tagged protein from different mammalian cell extracts by using magnetic microbeads. For the mixture of B-MBP with the lysate of natural killer NK92MI cells (Figure 2, lane 6), B-MBP was highly purified by magnetic microbeads while no nonspecific background proteins were detected (Figure 2, lane 7). As a contrast, from the NK92MI cellular lysate treated with BirA ligase *in vitro* (Figure 2, lane 8), no protein (Figure 2, lane 9) was detected, indicating that the NK92MI cellular proteins without AP tag were not biotinylated by BirA ligase. In addition, we tested the purification scheme using streptavidin magnetic microbeads for a variety of mammalian cells such as 293, 293T, and a liver cancer cell line treated with BirA ligase *in vitro*. Similar results as shown in Figure 2 were obtained. Taken all results above together, we have demonstrated that only AP-tagged protein could be specifically biotinylated and then specifically and efficiently purified by streptavidin magnetic microbeads in a single step.

We then utilized the biotinylation-streptavidin magnetic microbead scheme to obtain the 14-3-3 ϵ -interacting complex formed in mammalian cells. We constructed two plasmids that could express the 14-3-3 ϵ protein fused with AP-tag at either N- or C-terminal respectively as designated to pAP-1433 or p1433-AP.

Approximately 2.5×10^6 293T cells were transiently co-transfected with pAP-1433 and BirA plasmid or p1433-AP and BirA plasmid respectively. Similarly, we found that the expression level of biotin-tagged 14-3-3 ϵ (exogenous) was similar to that of between the endogenous 14-3-3 ϵ (Figure 1C, lanes 2 and 3). Forty hours after transfection, we isolated biotin-tagged 14-3-3 ϵ protein and its associates from the total cell lysate by using streptavidin magnetic microbeads. We then separated the pull-down complexes on 1D SDS-PAGE gel. As shown in Figure 3 (lanes 3 vs 4, lanes 5 vs 6), in the clearly stained bands at the size close to AP tagged 14-3-3 ϵ (Figure 1B) where the bait protein was identified,

indicating the effectiveness of biotin-tagging for enrichment of the bait protein. Meanwhile, multiple distinct bands visible on the gel (Figure 3, lanes 3 vs 4) suggested that possible 14-3-3 ϵ -interacting proteins were also enriched and pulled down along with the bait protein.

In comparison, we first transfected the same number of 293T cells with either pAP-1433 or p1433-AP alone, and then performed the biotinylation experiment on the cellular proteins *in vitro* 40 hours after transfection. After the purification of 14-3-3 ϵ protein complex, we noted that the yield of protein complex was significantly lower than what was obtained when using *in vivo* biotinylation method (Figure 3, lanes 3 & 4 vs lanes 5 & 6). Due to additional steps needed for *in vitro* biotinylation and purification (see Methods), we observed less numbers of the proteins recovered *in vitro* compared to those obtained by the *in vivo* biotinylation approach with direct purification by streptavidin magnetic microbeads. These results clearly demonstrated the high efficiency and specificity of *in vivo* biotinylation followed by direct purification by streptavidin magnetic microbeads.

Meanwhile, the isolated complex associating with AP-1433 or 1433-AP was separated on a 1D SDS gel respectively and the similar pattern of protein separation of each complex was observed (Figure 3, lane 3 vs lane 4). By using 1D SDS-LC-MS/MS approach as our previously described [9,10,13] we identified 78 and 72 proteins respectively in a several of parallel gel bands in lane 3 for AP-1433 vs lane 4 for 1433-AP (Figure 3, Supplemental Table 1). Among these proteins 61 mutual proteins were found in both pull-down complexes. These observations suggested that the 14-3-3 ϵ protein with the biotin tag either at N- or C-terminal has the equivalent ability to recruit interacting partners.

3.3 Unambiguous Identification of 14-3-3 ϵ Interacting Partners by Biotin Tagging Coupled with Amino Acid-coded Mass Tagging (BioCAT)

We then employed SILAC/AACT 'in-spectra' quantitative markers to establish the stringent threshold in distinguishing the specific interactors from the non-specific background in the pull-down complexes. The overall design of the BioCAT strategy for purification and identification of interacting proteins is illustrated in Figure 4. Approximate 2.5×10^6 parental 293T cells growing in the regular or 'light' medium (yellow dishes) were transiently transfected with pBirA plasmid expressing BirA ligase and pAP-14-3-3 expressing AP-14-3-3 ϵ , while the same number of cells growing in the leucine-d₃ containing or 'heavy' medium (green dish) were transiently transfected with only pBirA. Following cell lysis each of 'light' or 'heavy' protein extract was incubated with streptavidin magnetic microbeads respectively and the elutes of 'light' or 'heavy' origin were mixed prior to 1D-SDS-PAGE separation. The proteins in each of the visible bands were tryptic digested followed by LC-MS/MS for relative peptide quantitation and protein identification as our previously described [9, 10,13].

In one of the two BioCAT runs, we unambiguously identified 346 proteins in the AP-1433 complex from 293T cells (Supplemental Table 2) and the distribution of the light-to-heavy ratios (L/H) of the identified proteins is summarized in Figure 5. Theoretically, for those bait-interacting partners, their peptide abundances in the 'light' bait/biotin-specific pull-down complex should be significantly higher than their 'heavy' counterparts.

Among all proteins identified we found the peptides of many proteins showing the L/H ratio less than or close to 1 are non-specific contaminants such as tubulins and carboxylase enzymes (Figure 6A and 6B). Noted that tubulins are house-keeping proteins and carboxylase enzymes are known as the common biotinylated proteins in many mammalian cells [26]. In both light and heavy cultures these enzymes were equally biotinylated which led to the equal abundance of their light and heavy versions. In our dataset, 6 of 7 total isoforms of 14-3-3, including 14-3-3 β/α , γ , ϵ , η , θ and ζ/δ , were identified with a infinite L/H ratio (Supplemental Table 2 and Figure 6C, 6D, and 6F), which suggests their high affinity in binding to the 14-3-3 ϵ isomer in the complex. Meanwhile, 20 proteins previously known to interact with 14-3-3 ϵ were identified (Supplemental Table 2). Among them, the peptides of insulin receptor substrate 4 showed an approximate L/H of 4.4 which is the lowest ratio in comparing to other known 14-3-3 ϵ interactors. We therefore used this ratio value as the threshold to distinguish other 14-3-3 ϵ -specific interactors. As a result, a total of 266 proteins were distinguished as the 14-3-3 ϵ interacting partners from a single experimental run (Supplemental Table 2). Except for the 20 known interactors, a total of 246 proteins were newly identified as the 14-3-3 ϵ interacting partners. For example, non-pou domain-containing octamer-binding protein, NonO, was identified by 16 unique peptide signals with L/H ratio at an infinite value (Figure 6E), suggesting its specific enrichment with biotinylated 14-3-3 ϵ through streptavidin magnetic microbeads. To validate the accuracy of our proteomic results we performed co-transfection and co-immunoprecipitation experiments and found that NonO strongly interacts with 14-3-3 ϵ (Figure 6F).

Using currently available bioinformatics tools, STRING database, as shown in Supplemental Figure 1, close to 79% of our identified 14-3-3 ϵ interactors were found to be interconnected by the proteins involving in a variety of cellular processes such as signal transduction, RNA processing, cell cycle control, DNA repair, cellular energy metabolism, *etc.* (Table 1). Functional characterization of other novel 14-3-3 ϵ interacting proteins is under way and will be reported elsewhere.

4 Discussion

Here we demonstrate a novel integration of *in vivo* target-specific biotinylation, and efficient and high-yield affinity complex pull-down, and 'in-spectra' quantitative markers for precise screening of target protein-specific interactome. We have made significant improvements on our previously developed dual-tagging method [13]. Firstly, we substituted the epitope tag with relative low affinity such as Flag or Myc with an AP tag for *in vivo* biotinylation. This change leads to not only much stronger affinity for recognizing the target protein and therefore its associates in a complex, but also much higher efficiency and yield of affinity purification using streptavidin magnetic beads. Common TAP methods usually use relative long tags for affinity purification, which may inevitably interfere with protein interactions. To reduce possible effects on the existing native PPIs due to the size of an affinity tag here we used a shorter AP tag of 15 amino acids [18, 19, 24] for biotinylation than what was previously reported [17]. We then systematically evaluated the specificity of *in vivo* biotinylation of AP-tagged target protein and found highly specific biotinylation of target protein with minimum background biotinylation.

Secondly, to avoid the tedious step of establishing a stable cell line we adapted the strategy of transient co-transfection. A major concern for transient transfection is the possibility of the overexpression of the tagged target protein which may lead to the components of the isolated complex departure from those formed in physiological conditions. It was not case for our design of co-transfecting both AP-tagged target protein and BirA biotin ligase. Using this approach of transient co-transfection, it only takes 3 days to obtain a protein complex, including cell culture, transfection, and complex purification, which is much shorter than what needed for a method of taking the steps of established stable cells. More importantly, for the study of interaction proteomics in primary cells as stable transfection is obviously not suitable for primary cells, transient transfections are feasible alternatives.

Thirdly, due to the strong affinity between biotin and avidin with binding constant of K_d at 10^{-15} M, the biotin-tagged complex could be pulled down in high yield in competing with untagged endogenous counterpart for recruiting the interacting partners. As a result, we could significantly reduce the quality of the starting cells from 10^9 which are typically for the pull-down experiments using relative weak epitope tags [5] to 10^7 . Meanwhile those weak or transient interactions could be preserved. In this regard, even successful in transfection, primary cells are not amenable to any TAP approach because of the limited cellular materials. Because our BioCAT method needs very small quantities of starting cells, it could allow the characterization of complexes in primary cells if using high-efficiency transfection method, *e.g.*, lentivirus transfection, for these cells that are not easily transfected.

It is inevitable that non-specific contaminants may also be pulled down in an IP complex even with high affinity tags such as biotin or optimized TAP tags [17, 27]. Similar to our previous 'dual-tagging' scheme, we use the 'digitalized' AACT/SILAC tags to distinguish the specific interactors from those non-specific contaminants based on the quantitative L/H ratio (Supplemental Table 2). We observed an approximate 20% of totally identified proteins showing L/H close to 1 for non-specific contaminants which is much lower than what were found in the complex pulled down by anti-FLAG tag beads [13]. This suggested that high affinity isolation of IP complexes could result in less non-specific contaminants. Also we noticed that most of the specific interactors were identified with significantly higher L/H in the presence of biotin-tagged 14-3-3 ϵ (Figure 5). In this study we used an empirical and relatively stringent value, *i.e.*, 4.4-fold changes of particular protein abundance in the biotin-enriched complex, as the cut-off threshold for determining 14-3-3 ϵ interacting partners in high confidence. For the first time, 6 of total 7 isomers in the 14-3-3 family were simultaneously identified as the specific interactors in the same IP complex. In addition to 20 known interactors of 14-3-3 members, we have also identified 246 new interactors, which would shed light on the understanding of the undefined isomer-specific functions of 14-3-3 ϵ . The newly identified interactors of 14-3-3 ϵ by using BioCAT method allow us to extend the functional linkage of 14-3-3 ϵ to a variety of biological processes (Supplemental Figure 1). For example, we have identified a set of 14-3-3 ϵ interactors such as NonO at L/H 34.8 that was known to be involved in DNA damage response (Figure 6 and Table 1). Although many evidences suggest that only the 14-3-3 σ isomer is involved in DNA damage/repair [28, 29], little is evidenced for possible 14-3-3 ϵ involvement in DNA damage response. Our dataset of the profile of the 14-3-3 ϵ interactors related to DNA damage response have now

suggested the functional link between 14-3-3 ϵ and this particular biological process. Further, other interactors identified in the complex such as GBF1, ANKS1A, and API5, *etc*, indicated possible involvement of 14-3-3 ϵ in Golgi biogenesis and protein trafficking [30-33], in growth factor receptor signaling pathways [34], and in apoptosis pathways [35], respectively. Thereby the multi-functional nature of 14-3-3 ϵ isomers has then been illustrated by its interactions with the partners functional in diverse biological processes.

In summary, this BioCAT method represents a broadly applicable approach for precise screening of interaction proteome in mammalian cells. We conclude that this method has at least four major strengths including small quantities of the cells needed, short period of time for sample processing, high efficiency, and high MS signal specificity. Therefore, large-scale and high-throughput explorations of the human interactome (and hence, more importantly, the dynamic interactome) and cellular machinery should become more feasible. Furthermore, by integrating the *in vivo* quantitative proteomic approach, *e.g.*, the iTRAQ approach [36], our BioCAT method would provide an excellent tool for interaction proteomics in primary mammalian cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mr. J. Yao for his assistance in mass spectrometry analysis. This work was supported by grants from Shanghai Science and Technology Development Program (Grants 03DZ14024 and 07ZR14010) and the 863 High Technology Foundation of China (Grant 2006AA02A310). This work was also supported by US NIH 1R01AI064806-01A2, 5R21DK082706, and U.S. Department of Energy, the Office of Science (BER), Grant No. DE-FG02-07ER64422.

Abbreviations

IP	immunoprecipitation
TAP	tandem affinity purification
AACT	amino acid-coded mass tagging
SILAC	stable isotope labeling by amino acids in cell culture
HKE	human kidney embryonic
BioCAT	biotin tagging coupled with amino acid-coded mass tagging
AP	acceptor peptide
HRP	horseradish peroxidase conjugate
GO	gene ontology
PPIs	protein-protein interactions

5 References

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Figure 1. Analysis of the specificity and efficiency of the biotinylation of AP-tagged proteins through either *in vivo* or *in vitro* mechanism
The 293T cells were transiently transfected with the expression plasmid(s) as indicated. **(A)** Western blotting with streptavidin–HRP conjugate to detect biotinylated AP-tagged VAV1 protein (VAV1-AP). Lane 2 indicates the exogenously expressed VAV1-AP protein was biotinylated *in vivo* by the co-expressing BirA biotin ligase. Lane 4 suggests the proteins were biotinylated *in vitro* using the BirA biotin ligase. For lanes 2 and 4, densitometry analysis was performed and the relative density of the band was given underneath the lanes. **(B)** Western blotting with streptavidin–HRP conjugate to detect biotinylated AP-1433 or 1433-AP. Lane 1 indicates the cellular extract from nontransfected 293T cells. For lanes 7 and 8, the proteins were biotinylated *in vitro* using the BirA ligase. **(C)** Western blotting with an anti-14-3-3ε antibody to detect endogenous and tagged exogenous 14-3-3ε proteins.

Figure 2. Purification of AP-tagged proteins by streptavidin magnetic microbeads

Protein mixtures were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Lanes 1 and 10: protein size markers; Lane 2 indicates the purity of the biotinylated AP-tagged MBP-AviTag protein; Lane 4 shows the un-biotinylated AP-tagged MBP-AviTag protein; Lane 6 indicates a mixture of the protein in lane 2 and lane 8; Lane 8 and 20 indicate NK92MI cellular proteins treated with biotin ligase BirA *in vitro*; Lanes 3, 5, 7 and 9: the proteins purified by streptavidin magnetic microbeads from the same protein mixtures shown in lanes 2, 4, 6 and 8, respectively. The star indicates the streptavidin molecule (monomer) that was dissociated from the biotinylated proteins after boiled in the SDS-PAGE loading buffer. For lanes 2, 3 and 7, the densitometry analysis was performed and the relative density of the band was given underneath the bands.

Figure 3. Purification of biotin-tagged protein and the associating complex by streptavidin magnetic microbeads

Lane 1 – the cellular extract from nontransfected 293T cells; Lane 2 – markers; Lanes 3 and 4 - the AP-tagged proteins were biotinylated *in vivo* by co-expressed BirA. The proteins from the cells transfected with pAP-1433 or p1433-AP alone (lanes 5 and 6) were biotinylated *in vitro* using the BirA biotin ligase before purification as indicated in the Methods. The stars indicate the possible biotinylated AP-tagged 1433 proteins as compared to the results in Figure 1B and 1C. Following MS analysis, the protein identity in the bands indicated by rectangle was compared. For the purpose of comparison, the 293T cells were transfected with the expression plasmid pBirA or pAP-1433 alone, and then the protein mixtures were also purified by streptavidin magnetic microbeads. The purified elutes were then separated by SDS-PAGE (lanes 7 and 8).

Figure 4. Schematic representation of BioCAT strategy

Approximately 2.5×10^6 parental cells growing in the regular or 'light' medium (yellow dishes) were transiently transfected with an expression vector carrying the BirA gene (DS-BirA) and an expression vector carrying the gene coding for bait protein (DS-bait), which was fused with the AP DNA sequence (DS-AP), while the same number of cells growing in the leucine- d_3 containing or 'heavy' media (green dish) were transiently transfected with only the expression vector carrying the BirA gene. Following one-step purification using streptavidin magnetic microbeads, the immunoprecipitates from each cell pool were mixed and then separated by 1D-SDS-PAGE. The proteins in each gel band were tryptic digested followed by LC-MS/MS analysis. The peptides containing leu- d_3 should show the mass shifts of $3 \times n$ (n is the number of leucine in the peptide) with respect to the non-labeled or light counterparts. The proteins that specifically interact with the tagged bait protein as well as the nonspecific background binding proteins were distinguished by the light-to-heavy (L/H) ratio.

Figure 5. The distribution of L/H ratio of the identified proteins in the AP-1433 complex
 Each spot represents a MS-identified protein in Supplementary Table 2. The L/H ratio of each protein was shown with the empirical L/H threshold at 4.3 to determine those specific interactors.

Figure 6. MS spectra of the proteins identified by BioCAT and Validation of their interactions with 14-3-3ε

The peptide signals derived from tubulin β (A), acetyl-CoA carboxylase (B), 14-3-3ε (bait protein, (C)), 14-3-3ζ (D), and NonO (E) suggested those nonspecific binding (A, B) or those specifically enriched proteins (D, E). (F) Validation of the specifically enriched proteins in (D, E) by using western blotting.

Table 1Functional Categories of the Identified Interactors of 14-3-3 ϵ in 293T Cell

Category	Count
mRNA processing	42
cellular biosynthetic process	44
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	90
RNA localization	12
biopolymer metabolic process	106
establishment of RNA localization	11
nucleobase, nucleoside, nucleotide and nucleic acid transport	11
cellular macromolecule metabolic process	74
regulation of biosynthetic process	13
regulation of translation	12
protein metabolic process	75
intracellular transport	24
ribonucleoprotein complex biogenesis and assembly	11
establishment of cellular localization	25
regulation of protein metabolic process	12
response to DNA damage stimulus	12
organelle organization and biogenesis	27
regulation of metabolic process	53
cellular component assembly	15
regulation of cellular process	71
cell cycle process	17
cell cycle phase	10
mitotic cell cycle	9
protein transport	15
cell death	17
regulation of cell cycle	12
negative regulation of biological process	22

Gene annotations of those specific interactors listed in Supplemental Table 2 were performed using the Functional Annotation Tool at DAVID site.