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Analysis of the protein complex associated with 14-3-3 epsilon by a deuterated-leucine labeling quantitative proteomics strategy

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ABSTRACT

By using an unambiguous *in vivo* deuterated-leucine labeling quantitative proteomic approach, at close to the physiologically relevant level, we systematically profiled multiple proteins interacting with 14-3-3ε, the isoform with least characterized protein interactions in 14-3-3 family in mammalian cells. Among the 19 proteins interacting with 14-3-3ε identified, 6 of them including SKb1Hs, p54nrb, serine/threonine kinase 38, MEP50, 14-3-3θ and cofilin 2 were the previously unknown interacting partners with 14-3-3ε. The newly identified interactor cofilin 2 was also validated in co-transfection and co-immunoprecipitation. In contrast, with the same stringent criteria only three known partners were identified by conventional tandem affinity purification (TAP) approach. Therefore the 'in-spectra' quantitative marker of deuterated-leucine assisted to precisely identify those genuine interacting partners with minimum requirement of validation using other molecular approaches.

1. Introduction

The 14-3-3 proteins, seven isoforms including 14-3-3β, γ, ε, η, σ, θ (or τ) and ζ, belong to a family of conserved regulatory molecules expressed in all eukaryotic cells [1–2]. In mammals these isoforms have been identified to interact with a broad range of the human proteome including several signaling and proto-oncogene proteins [3,4]. Through interacting with 300 target proteins identified so far, these 14-3-3 proteins are known to be involved in widespread biological processes such as signal transduction, cell cycle control, apoptosis, cellular metabolism, proliferation, cytoskeletal regulation, transcription, and redox-regulation or stress response, etc. [1–3,5–7].

Several groups have applied various proteomic approaches to directly identify 14-3-3 interacting proteins [6,8–11]. Although Jin et al. identified 170 proteins associated with 14-3-3 family members in HEK293 cells using a combined affinity column-mass spectrometry (MS) approach [10], there were only approximately 25% overlap among those efforts from different groups [8,10]. More emerging data of identified isoform-specific interactions have suggested that an individual 14-3-3 isoform probably has its specific biological roles [8,11]. For example, 117 possible protein targets of 14-3-3σ have been identified by tandem affinity purification (TAP) and multidimensional MS/MS technology and most 14-3-3σ interactors were implicated to be involved in oncogenic signaling and cell cycle regulation [11]. Therefore, the precise characterization of the diverse interaction profile with each 14-3-3 isoform can facilitate our understanding the functional differences in the 14-3-3 family in regulating various cellular processes.

By now, many known binding proteins have been reported to interact with 14-3-3σ, β, γ, θ, and ζ isoforms respectively [9–11]. However little is known for the profile of interacting components with 14-3-3ε isoform in mammalian cells. Biologically interestingly, the isoform of 14-3-3ε is the most highly conserved member of 14-3-3 family, with conserved sequence from plant, yeast and mammalian [12,13]. In addition, the 14-3-3ε gene has been proposed to be a candidate tumor suppressor gene [1,14] and was also found to be involved in regulating carcinogenesis in various hepatocellular carcinoma cell lines [15,16] and lung cancers [17].

Previously, several reports identified a few proteins associated with 14-3-3ε through yeast two-hybrid approach or immunoprecipitation. For example, 14-3-3ε was found to interact with calmodulin in a yeast two-hybrid approach [18]. 14-3-3ε also formed heterodimers with 14-3-3β, γ, η and ζ [13,19]. Therefore, a systemic identification of novel 14-3-3ε protein interactions will provide new clues about possible target for diagnosis and therapeutic intervention.

To address the technical concerns about the sensitivity and accuracy of profiling protein–protein interactions for a specific

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isoform of 14-3-3 family, we expanded an *in vivo* dual-tagging proteomic approach [20] which combined a single epitope tagging for complex pull-down with deuterated-leucine (Leu-d_3)-based amino acid-coded tagging (AACT) for distinguishing quantitatively interacting partners with minimum false-positives in MS. This highly sensitive and accurate approach for protein complex analysis has first applied to study signaling protein interactions occurring in actual immune cells [20] and then to investigate the radiation-induced dynamic complex changes [21]. Here we are taking a first step to investigate the isoform-specific functions of 14-3-3ε on the context of specific protein–protein interactions occurring in HEK 293T cells by using the similar dual-tagging approach. Note that our aim has been to precisely identify those genuine interacting partners with minimum requirement of validation using other molecular/cellular approaches. Therefore, assisted by the 'in-spectra' quantitative markers of amino acid tags we were able to set more stringent threshold to distinguish the specific interactions in high precision which led to much lower numbers of 14-3-3ε interacting proteins in compared to what were reported previously [8,11]. Through the known function of these newly identified 14-3-3ε associating proteins, particular cellular processes regulated by specific 14-3-3ε interactions will be revealed.

2. Materials and methods

2.1. Plasmids and cell culture

The TAP vector pMIR-DFT contains double Flag peptides and a calmodulin binding peptide (CBP), spaced by a TEV protease cleavage site [20]. The PCR amplified 14-3-3ε cDNA was cloned into the *Bam* HI and *Xho* I site of pMIR-DFT. The recombinant plasmid was designated as pMIR-14-3-3ε. The HEK 293T cells were cultured with the DMEM media containing 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Generation of stable cells expressing the bait protein, 14-3-3ε

The pMIR-14-3-3ε was transfected into HEK 293T cells using GeneJammer transfection reagent (Stratagene # 204130) according to manufacture manual. 1 μg/ml puromycin was added to select for cells stably expressing the epitope-tagged 14-3-3ε. The expression level of both endogenous and Flag-tagged 14-3-3ε in stable cells was measured by western blot with both anti-Flag and 14-3-3ε antibodies respectively. The stable cells were cultured with deuterated-leucine (Leu-d_3, Cambridge Isotope Laboratories) labeled DMEM medium, in which only the Leu-d_3 replaced the regular leucine (Leu-d_0) component, while the control cells were cultured in regular DMEM medium. Cells were harvested when they became 80–100% confluence, and cell pellets were stored at −80 °C until further use.

2.3. Single-step affinity pull-down of dual-tagged complexes

Approximately 1×10^9 Leu-d_3-labeled stable cells expressing Flag-tagged 14-3-3ε or control cells, which only contained the empty vector pMIR-DFT, were respectively collected, and the cell pellets were lysed in the lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitor cocktail (Sigma #P-8340) to extract proteins. Then same quantity of proteins from two groups was mixed equally, following the protein mixture was incubated with 200 μl of anti-Flag M2 beads at 4 °C for 3–4 h. The beads were washed 3 times with 5 ml of TBS buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl), and the bound proteins were eluted with TBS buffer containing 250 μg/ml of 3 × Flag peptide (Sigma, F4799) twice. The eluted proteins were concentrated with trichloroacetic acid precipitation, then were run on 12% SDS-PAGE and visualized by silver staining.

2.4. Tandem affinity tag purification of bait-containing complexes

The total cellular proteins were extracted from about 1×10^9 stable cells expressing Flag-CBP-tagged 14-3-3ε cultured in regular DMEM medium. The first purification was performed using anti-Flag M2 Affinity Gel (Sigma #A2220). 20 ml of lysate was incubated with 200 μl of anti-Flag M2 beads at 4 °C for 3–4 h. The beads were washed 3 times with 5 ml of TBS buffer and followed by washing with 1 ml of TEV buffer (50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 0.1% NP-40, 0.1 mM DTT). The bound proteins were cut off from the beads using 200 unites of TEV protease (Invitrogen #12575-015) for cleavage overnight at 4 °C. The supernatants from the TEV reactions were performed for the second purification by binding in the Calmodulin Affinity Resin (Stratagene #214303) for 4 h at 4 °C. After removing the supernatant, the beads were washed 3 times with 10 ml of calmodulin binding buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM Imidazole, 2 mM $CaCl_2$, 10 mM β-ME), and the bound protein complex was eluted by boiling the beads for 3 min using 1x sample buffer. Proteins were separated by SDS-PAGE and visualized by silver staining.

2.5. Sample preparation for mass spectrometry analysis

Silver stained bands were excised, and in-gel digestion was performed mainly as described as follows. The gel slices were destained with the mixture of 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ and washed with deionized water for 3 times. The pieces were then dehydrated with acetonitrile (ACN) for 2–3 times. The dried slices were digested with 12.5 ng/μl of sequencing grade, modified trypsin in 20 mM ammonium bicarbonate overnight at 37 °C. Following digestion, tryptic peptides were extracted twice with 50% ACN/0.1% trifluoroacetic acid (TFA) for 15 min each time with moderate sonication. The extracted solutions were pooled and evaporated to dryness under vacuum. The dry peptide samples were redissolved in 0.1% TFA for the following MS analysis.

2.6. Protein identification by LC–MS/MS analysis

Protein identification of the complex components was performed by LC–nanospray–MS/MS analysis using a QSTAR XL mass spectrometer (Applied Biosystems, USA) as previously described by Gu et al. [22]. The MASCOT server was used to interpret the LC–MS/MS data by searching against the species of *homo sapiens* from NCBI database. The parameters for database searching were set including the mass tolerance at 0.2 Da for both MS and MS/MS, trypsin enzyme specificity and single missed cleavage allowed, variable modifications including phosphorylations of tyrosine/serine/threonine, oxidation of methionine and the Leu-d_3 tag.

2.7. Immunoprecipitation and Western blot

HEK 293T cells were co-transfected with CBP-tagged 14-3-3ε and Flag-tagged cofilin 2. 48 h after transfection, cell lysate was incubated with Calmodulin Affinity Resin for 3–4 h at 4 °C. The bound proteins were eluted by boiling the beads and separated by SDS-PAGE, then transferred to PVDF membrane for Western blotting. The primary antibodies were anti-Pan 14-3-3 (Chemicon, AB1671), anti-cofilin 2 (Abcam, ab14133). And the secondary antibody was alkaline phosphatase- conjugated antibody (Sino-American Biotech. Corp., China). The immunoblots were visualized by color development according to the manufacture's instruction.

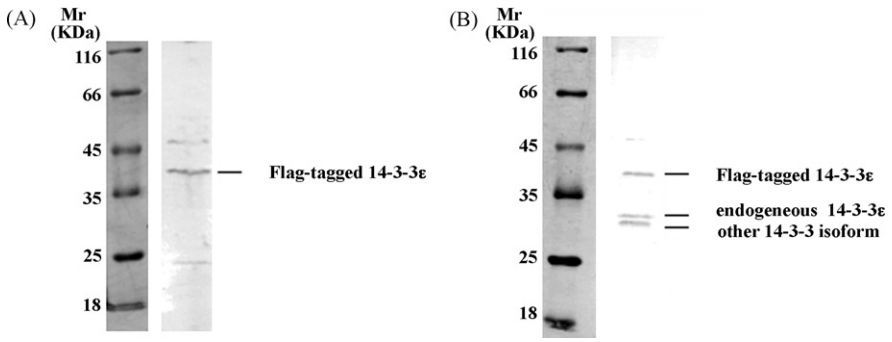

Fig. 1. The expression of Flag-tagged 14-3-3ε in stably transfected HEK 293T cells validated by Western blotting with using (A) anti-Flag M2 antibody and (B) anti-14-3-3 antibody respectively. The comparative expression level of Flag-tagged 14-3-3ε versus the endogenous 14-3-3ε was at similar level.

3. Results

3.1. Quantitative identification of 14-3-3ε interacting proteins using the dual-tagging proteomic strategy

First, the expression level of Flag-tagged 14-3-3ε was found at the similar natural level of its endogenous counterpart (Fig. 1). To avoid possible false-positives arisen from complex studies using transiently transfected 293T cells [11,23], the stable cells expressing the tagged bait protein at close to natural level were used in our study to ensure the precise identification of physiologically relevant complex components.

The strategy for purification and identification of interacting proteins associated with 14-3-3ε by dual-tagging proeomics was mainly illustrated in Fig. 2. The proteins specifically interacting with 14-3-3ε were distinguished from non-specific protein background in MS spectra according to the quantitative criteria established by our group [20,21]. The intensity ratio of heavy versus light isotope peaks reflects the binding strength of the identified protein to the bait 14-3-3ε. Theoretically, a heavy versus light isotope enrichment ratio greater than 1 corresponds to an increased abundance of a particular protein around the bait as the concentration of non-specifically distributed proteins surrounding the bait protein remains at similar level.

In our present study, a total of 42 proteins were unambiguously identified in the pull-down dual-tagged complex. Based on the average relative standard deviation (R.S.D.) obtained from quantification of the isotope enrichment ratio of all identified proteins, we conservatively set the cut-off ratio at 1.3 as the threshold for determining 14-3-3ε-interacting proteins as the ratios for those previously known 14-3-3 interacting partners including calmodulin, cofilin, 14-3-3 ζ, kinesin-related protein, etc., were in the range of 1.30–2.16. As the results of two reproducible experiments using the same approaches, 19 proteins were simultaneously identified to specifically interact with the bait 14-3-3ε (Table 1) and 23 were non-specific proteins (Supplementary Table 1).

Among the 19 specific binding proteins we identified, 13 were those proteins previously been found to associate with 14-3-3 family proteins by other methods [13,18,19,24,25], while the other 6 proteins including SKb1Hs, p54^{nrb}, serine/threonine kinase 38, MEP50, 14-3-3θ and cofilin 2 were newly identified 14-3-3 interacting partners in our study. Among the 13 known binding proteins (M2-PK, GAPDH, peroxiredoxin 6, thioredoxin, HSP90, HSP70, Bip, calmodulin, actin, 14-3-3ζ, cofilin1, kinesin-related protein and tropomyosin 3), only four proteins including calmodulin, 14-3-3ζ, cofilin1 and kinesin-related protein were known to associate with the isoform bait 14-3-3ε, while the other nine proteins were those previously identified by interacting with other 14-3-3 proteins [6,9]. Representative spectra of two specific partners previously unknown to interact with 14-3-3ε were shown in Fig. 3.

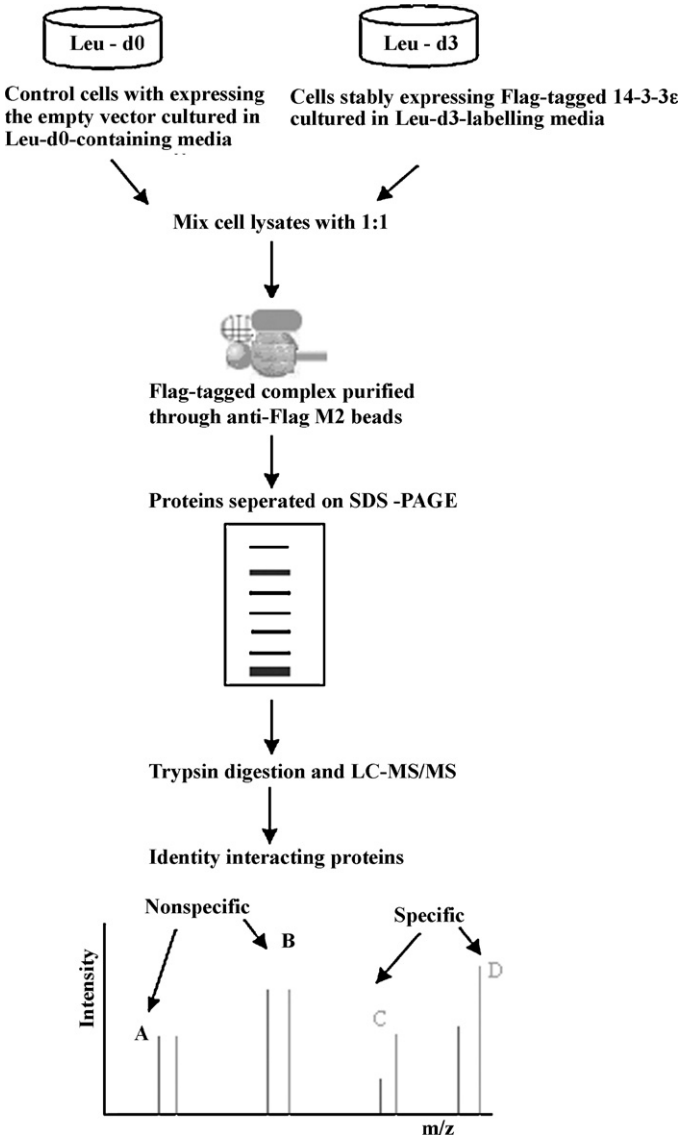

Fig. 2. The strategy of dual-tagging approach for purification and identification of interacting proteins associated with the bait protein, 14-3-3ε. The control cells were cultured in regular or 'light' DMEM media, and the stable cells expressing Flag-tagged 14-3-3ε were cultured in 'heavy' media. Equal concentration of cellular proteins extracted from unlabelled and labeled cells was mixed, and the mixture was affinity-purified with anti-Flag M2 beads. The proteins eluted from beads were separated on SDS-PAGE, and the peptide digests were analyzed by LC–MS/MS.

Table 1
Specific interacting proteins with 14-3-3ε identified by the dual-tagging approach.

Function	NCBI accession no	Protein name	Mean ratio[a]	S.D.[b]	No. of peptides[c]	No. of peptide information[d] (*m*/*z*, sequence)
	gi|30583161	Tyrosine 3/tryptophan 5-monoxygenase activation protein, epsilon polypeptide (bait protein)	4.75	1.24	5	457.28/454.25(2+): NLLSVAYK, 2 L 599.84/595.31(2+): DSTLIMQLLR, 3 L 608.32/607.31(3+): AASDIAMTELPPTHPIR, 1 L 630.30/628.79(2+): YLAEFATGNDR, 1 L 1047.66/1044.65(2+): AAFDDAIAELDTLSEESYK, 2 L
Adaptor molecule	gi|68084347	Tyrosine 3/tryptophan 5-monoxygenase activation protein, zeta polypeptide	1.80	0.17	3	599.85/595.32 (2+): DSTLIMQLLR, 3 L 654.35/652.84(2+): FLIPNASQAESK, 1 L 713.33/711.34(3+): TAFDEAIAELDTLSEESYK, 2 L
	gi|54696890	Tyrosine 3/tryptophan 5-monoxygenase activation protein, theta polypeptide	1.83	0.11	2	457.28/454.26(2+): NLLSVAYK, 2 L 599.86/595.33 (2+): DSTLIMQLLR , 3 L
Metabolism	gi|31645	Glyceraldehyde-3-phosphate dehydrogenase	2.31	0.19	3	707.90/706.39 (2+): GALQNIIPASTGAAK, 1 L 808.95/807.44(2+): LVINGNPITIFQER, 1 L 883.90/882.39 (2+): LISWYDNEFGYSNR, 1 L
	gi|189998	M2-type pyruvate kinase	1.61	0.04	4	572.80/571.30 (2+): GDLGIEIPAEK, 1 L 600.83/599.32 (2+): ITLDNAYMEK, 1 L 821.94/818.92 (2+): GVNLPGAAVDLPAVSEK, 2 L 893.45/890.42(2+): GADFLVTEVENGGSLGSK, 2 L
Protein folding and processing	gi|61656603	90 KD heat shock protein	1.66	0.07	6	418.28/415.26 (2+): ALLFIPR, 2 L 511.92/509.91 (3+): SLTNDWEDHLAVK, 2 L 624.86/621.84(2+): ADLINNLGTIAK, 2 L 641.33/638.31 (2+): ELISNASDALDK, 2 L 760.40/757.38 (2+): GVVDSEDLPLNISR, 2 L
	gi|24234686	Heat shock 70 KDa protein 8 isoform 2	1.65	0.07	5	491.27/489.26(3+): AQIHDLVLVGGSTR, 2 L 558.30/555.29 (2+): LLQDFFNGR, 2 L 602.35/599.33(2+): DAGVIAGLNVLR, 2 L 809.39/807.89(2+): AFYPEEISSMVLTK, 1 L 831.42/829.91 (2+): NQVALNPQNTVFDAK, 1 L
Stress response	gi|6900104	Glucose-regulated protein (Bip)	1.34	0.04	4	618.81/617.30 (2+): DAGTIAGLNVMR, 1 L 648.32/645.32(3+): DNHLLGTFDLTGIPPAPR, 3 L 700.89/699.38 (2+): ELEEIVQPIISK, 1 L 831.94/830.42 (2+): IINEPTAAAIAYGLDK, 1 L
Redox	gi|56204402	Peroxiredoxin 6	1.90	0.05	3	455.24/453.73 (2+): NFDEILR, 1 L 544.81/543.29 (2+): LPFPIIDDR, 1 L 599.35/596.33 (2+): LSILYPATTGR, 2 L
	gi|9508997	Thioredoxin	1.54	0.24	2	504.28/501.27(2+): LEATINELV, 2 L 670.31/668.80(2+): TAFQEALDAAGDK, 1 L
RNA binding	gi|2808511	p54[nrb]	1.37	0.05	2	546.78/543.78 (2+): VELDNMPLR, 2 L 933.46/930.45 (2+): LFVGNLPPDITEEEMR, 2 L
Cellular signalling	gi|4495062	Serine/threoninekinase38	1.42	0.08	3	460.60/457.58 (3+): DIKPDNLLLDSK, 3 L 690.35/688.34 (3+):VTLENFYSNLIAQHEER, 2 L 795.42/793.91 (2+): ETLTFPPEVPISEK, 1 L
	gi|2323410	SKb1Hs	2.10	0.18	3	557.83/554.81(2+): VPLVAPEDLR, 2 L 697.91/694.89(2+): AAILPTSIFLTNK, 2 L 1087.01/1085.51(3+): DDGVSIPGEYTSFLAPISSSK, 1 L
	gi|825635	Calmodulin	1.49	0.07	4	480.23/478.73(2+): EAFSLFDK, 1 L 586.60/585.60(3+): VFDKDGNGYISAAELR, 1 L 616.61/615.61(3+): EAFSLFDKDGDGTITTK, 1 L 634.79/633.29 (2+): DGNGYISAAELR, 1 L
Cytoskeleton	gi|14250401	Beta-actin	1.32	0.09	5	582.81/581.30 (2+): EITALAPSTMK, 1 L 655.06/652.05 (3+): VAPEEHPVLLTEAPLNPK, 3 L 897.54/896.03 (2+): SYELPDGQVITIGNER, 1 L 1111.26/1108.25(2+): DLYANTVLSGGTTMYPGIADR, 2 L 1119.25/1116.25(2+): DLYANTVLSGGTTMYPGIADR, 2 L
	gi|1155084	Kinesin-related protein	1.72	0.17	5	601.33/599.82 (2+): LDIPTGTTPQR, 1 L 618.81/617.30 (2+): ETTIDGEELVK, 1 L 747.92/743.38 (2+): EAGNINQSLLTLGR, 3 L 816.38/814.87 (2+): EEYITSALESTEEK, 1 L 844.44/839.92 (2+): NLNSLFNNMEELIK, 3 L
	gi|30582531	Cofilin 1	2.16	0.16	4	579.58/578.58 (3+): HELQANCYEEVKDR, 1 L 629.82/626.79 (2+): AVLFCLSEDKK, 2 L 675.38/670.85(2+): LGGSAVISLEGKPL, 3 L 1085.01/1083.50(2+): EILVGDVGQTVDDPYATFVK, 1 L
	gi|6831517	Cofilin 2	1.80	0.18	4	666.06/664.05(3+): KEDLVFIFWAPESAPLK, 2 L 670.79/669.29 (2+): YALYDATYETK, 1 L 695.97/691.44 (2+): LGGNVVVSLEGKPL, 3 L 1100.01/1098.50 (2+): QILVGDIGDTVEDPYTSFVK, 1 L

Table 1 (*Continued*)

Function	NCBI accession no	Protein name	Mean ratio[a]	S.D.[b]	No. of peptides[c]	No. of peptide information[d] (*m*/*z*, sequence)
	gi|55665780	Tropomyosin 3	1.53	0.10	5	569.31/566.30 (2+): MELQEIQLK, 2 L 581.84/578.82 (2+): LVIIEGDLER, 2 L 625.34/622.32(2+): IQLVEEELDR, 2 L 660.32/658.82 (2+): EQAEAEVASLNR, 1 L 823.40/821.89 (2+): IQVLQQQADDAEER, 1 L
Others	gi|13559060	MEP50	1.92	0.16	5	461.78/458.76(2+): ILLWDTR, 2 L 624.35/622.84(2+): ETPPPLVPPAAR, 1 L 708.40/702.36 (2+): SDGALLLGASSLSGR, 4 L 823.73/822.73(3+): YEHDDIVSTVSVLSSGTQAVSGSK, 1 L 835.44/832.42 (2+): VWDLAQQVVLSSYR, 2 L

[a] The mean ratio represents the intensity of Leu-d_3 labeled peptides versus that of the unlabeled ones. It is averaged when several peptides are available.
[b] S.D. is the standard deviation calculated by the isotopic intensity ratios from multiple leucine-containing peptides.
[c] Number of pairs of leucine-containing peptides for quantitation.
[d] The information of a pair of peptides (*m*/*z* and sequence) used to quantify proteins.

In this paper, our aim was to precisely identify those genuine interacting partners associated with 14-3-3 epsilon with minimum requirement of validation using other molecular/cellular approaches. Therefore, assisted by the 'in-spectra' quantitative markers of amino acid tags, we set more stringent threshold to distinguish the specific interactions in high precision which probably led to lose several weak interacting partners of 14-3-3ε in compared to what were reported previously to bind with other isoform. We found that some of 23 non-specific proteins classified in our case were identified as the 14-3-3-binding partners in other studies [13,18,19,24,25]. For example, the proteins including HSP60, actin binding protein (filamin), alpha actinin, spectrin beta, H^+-ATP synthase alpha subunit, H^+-ATP synthase beta subunit, ribosomal protein P0 and ribosomal protein P2, which were excluded from

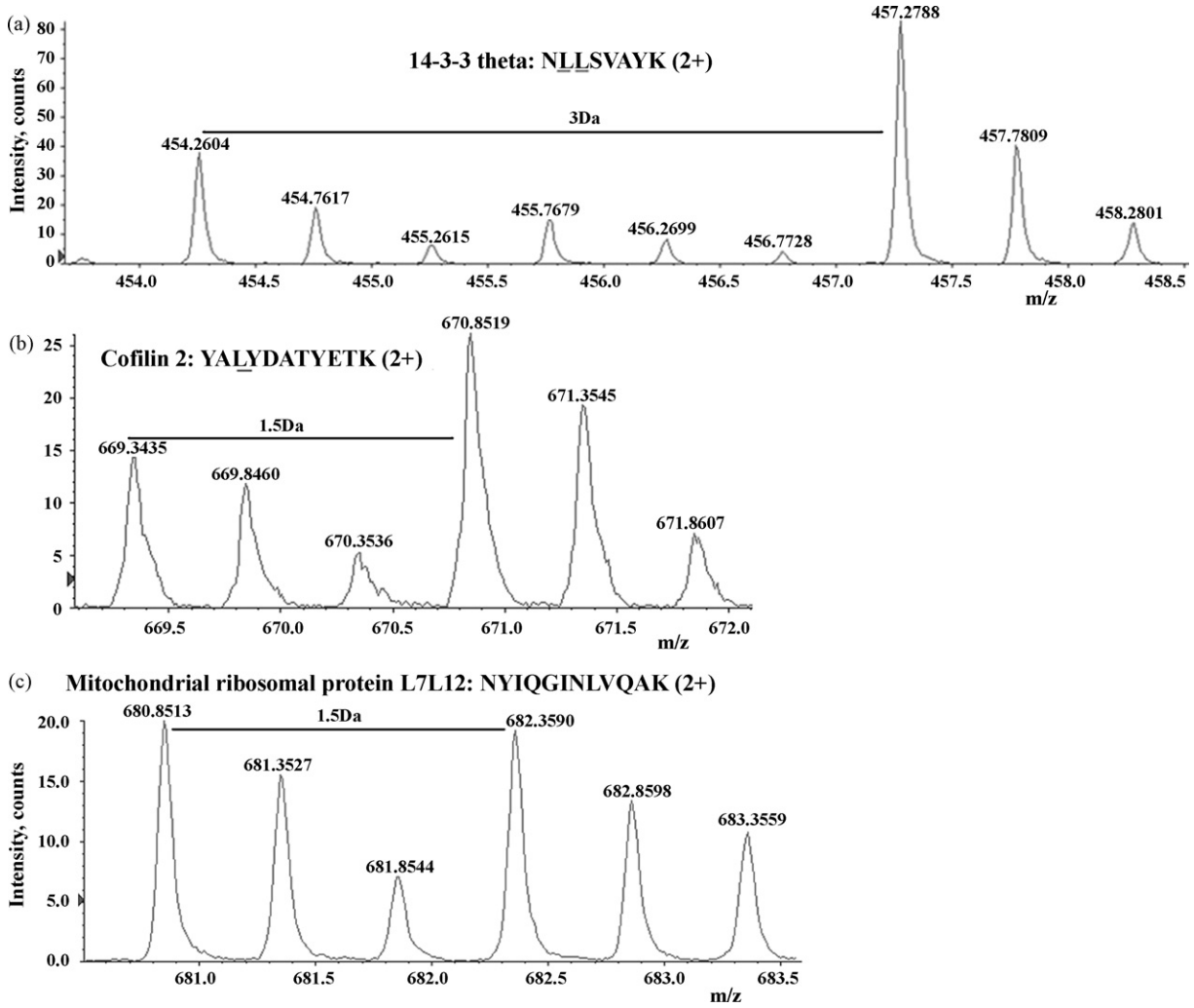

Fig. 3. The two representative MS spectra corresponding to the two specific binding proteins to the bait protein 14-3-3ε. (a) and (b) were one pair of isotope peaks of a peptide respectively from newly identified specific binding protein of 14-3-3 theta and cofilin 2. (c) was a pair of isotope peaks of one peptide from non-specific binding mitochondrial ribosomal protein L7L12.

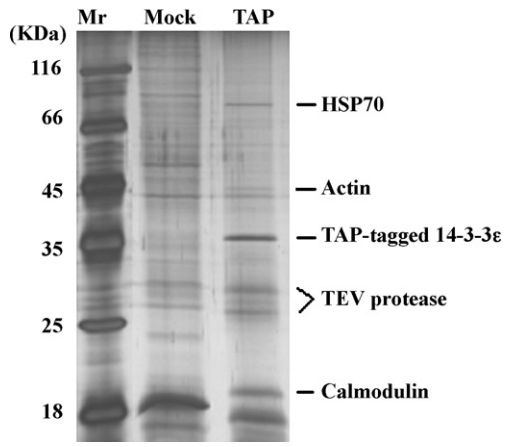

Fig. 4. Tandem affinity purification of the protein complex associated with TAP-tagged 14-3-3ε from stable transfected 293T cells. Total proteins extracted from 1×10^9 stable transfected cells were purified through two-step affinity purification. The protein complex was separated on SDS-PAGE by silver staining. HSP70, actin and calmodulin were identified as the specifically binding proteins with 14-3-3ε.

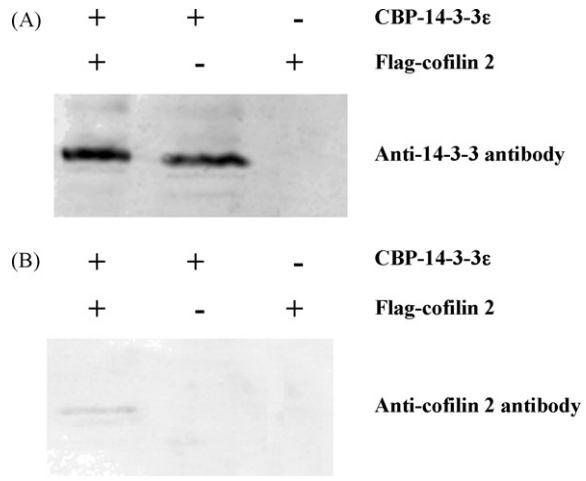

Fig. 5. Co-immunoprecipitation analysis of a newly identified binding protein of cofilin 2. The protein extracts from co-transfected and singlely transfected cells were both affinity-purified by calmodulin affinity beads, and the elutes were run on SDS-PAGE for Western blotting. (A) and (B) was indicated the co-IP results by using anti-14-3-3 and anti-cofilin 2 antibodies respectively.

specific-binding partners with the bait 14-3-3ε in our identification, were previously considered as the 14-3-3 interactors in other's report [9].

3.2. Comparison of detection sensitivity between TAP tag method and dual-tagging quantitative approach

Using the same stable 293T cells transfected by the double epitope-tagged 14-3-3ε construct, the direct comparison of the sensitivity and accuracy for detecting the components was made for the complexes isolated from single affinity tag and TAP tag strategy [26] respectively. In the TAP tag design, two-step washings were applied to obtain a stable complex. Following a 1D SDS-PAGE separation of complex components, the bands visualized by silver staining were determined as the 14-3-3ε-specific interactors (Fig. 4). In addition to identification of two strong non-specific bands associated with the two chains of remaining TEV protease from first elution from anti-Flag beads, here were only three specific proteins including heat shock protein (HSP) 70, actin, and calmodulin identified through the same stringent criteria we have established earlier. In TAP tag experiments, some superfluous TEV protease was always remained unavoidably in the protein complexes purified by TAP method [26,27].

As presented above, in comparison, assisted by the amino acid tags as the in-spectra quantitative marker, many more bait-specific proteins were unambiguously identified by our dual-tagging method in a much more precise way.

3.3. Validation of a newly identified 14-3-3ε-interacting partner

To validate the accuracy of the newly identified 14-3-3ε interactions in our proteomic datasheet, as an example, the interaction between 14-3-3ε and cofilin 2 in the complex was examined by co-immunoprecipitation and Western blotting. The co-transfection of the plasmids expressing CBP-tagged 14-3-3ε and Flag-tagged cofilin 2 respectively was affinity-purified through Calmodulin Affinity beads. As shown in Fig. 5, the eluted fraction showed positive responses toward both antibodies of 14-3-3 and cofilin 2 respectively, demonstrating clearly the existence of the interaction between these two proteins.

Based on our proteomic datasheet of 14-3-3ε interacting protein profile as summarized in Table 1, 14-3-3ε is now found to be involved in various cellular processed including signal transduction, protein folding, RNA binding, reduction–oxidation (redox) regulation, stress response, actin cytoskeleton, cellular energy metabolism, and cell growth.

4. Discussion

Currently, most of understanding of protein–protein interactions has been obtained from non-physiological systems where overexpression of particular genes was required for detection. There is a critical need for developing widely applicable and efficient purification procedures for characterizing protein complexes directly from mammalian cells. Immunoprecipitation (IP) is a commonly used method to analyze protein complexes, however, the difficulties associated with antibody generation, steric interference at the ligand binding site(s) due to antibody occupancy, and large non-specific background prevents IP for accurate complex analysis. Technically, to distinguish the diversity of 14-3-3 isoform-specific interaction profiles, the sensitivity and accuracy of TAP-based MS analysis of protein–protein interaction complexes have been proven to be insufficient as we have demonstrated in this study and in the investigation of various biological systems [20]. The basic concerns have been that (i) multiple washing steps are required in a TAP approach to obtain stable complex for analysis, hence, transient, low-abundant, and weak interactions of biological significance might be removed during the repetitive purification steps [21] and (ii) The determination of bait-interacting proteins fully relies on the visual bands developed from TAP-isolated complexes on gels, which leaves no other mechanisms to distinguish specific interactions from non-specific background. Based on our experiences, the elution efficiency of proteins from affinity beads can affect the recovery of protein complex. For example, the binding rate with calmodulin affinity beads for proteins can reach at 50% while the elution efficiency for mammalian target proteins can only be at 10–15% [23]. Our profiling result using TAP-tagged method is consistent with this conclusion. As some low-abundant regulatory proteins, such as STK38 and $p54^{nrb}$, precisely identified by our dual-tagging approach, were not detected in high confidence by TAP tag-based method.

In our this paper, we mainly expanded and tested the *in vivo* dual-tagging proteomic approach [20] for distinguishing quantitatively interacting partners associated with the isoform-specific bait protein, which shares a high sequence identity and similarity at amino acid sequences with other family members. This method

can effectively distinguish the cross-reactive effects in conventional IP for capturing protein complex, as well as reduce minimum false-positives in mass spectrometry compared with TAP approach. The typical feature of this Leu-d_3-based method is to introduce pair-wise isotope signals of each individual peptide containing a particular type of tagged amino acid (Leu-d_3) that originated from different cell states. For example, in this study, 14-3-3 epsilon, is one isoform of 14-3-3 family proteins, which share 77.41% homology at the amino acid level among the seven isoforms with very similar molecular weight. Maybe in conventional TAP, several interacting isoforms with the bait 14-3-3 epsilon are difficult to obviously display in SDS-PAGE, which affects the comparison from the band strength to determine if it is a specific-binding protein. Secondly, we modified the sample mixture strategy before purification. That is, equal concentration of two group of cellular proteins respectively extracted from unlabelled controls and labeled stable cells was mixed before purification, and the mixture was affinity-purified with anti-Flag M2 beads. Our "purification after mixture" method allowed purification was performed under same conditions at same time to reduce purification time. While, in previous dual-tagging method [20], each group of cellular proteins was separately purified to capture protein complex with anti-Flag M2 beads first, then the same amount of captured protein complex from two states of cells was mixed to run SDS-PAGE. The latter "mixture after purification" allows affinity purification to perform individually for each sample, and it is also difficult to monitor the concentration of each protein complex, which is always very little in quantity.

Because the Leu-d_3-based quantitative affinity purification provides an 'in-spectra' quantitative detection marker to distinguish specific binding proteins, single-step affinity pull down that could preserve the majority of complex components is sufficient. Therefore, our dual-tagging quantitative approach can characterize mammalian protein complexes and detect 'real-time' protein-protein interactions sensitively and efficiently. However, in either conventional TAP or Leu-d_3-based quantitative affinity purification, the epitope-tagging affinity purification is obviously the need to ectopically express the bait protein in cells. Although the artificially introduced tag may interfere with protein folding, protein function, or the ability to interact with other proteins [28,29]. Actually, the small size of epitope tags, like as the 8-amino acid-containing FLAG tag or the CBP with 34-amino acid residues, decreases the possibility of functional interference. For epitope tags, it is therefore advisable to create the N-terminal or C-terminal fusion with a bait protein in order to overcome this possible limitation. Optimal placement of the tag is protein-specific.

In our 14-3-3 epsilon tagging purification, the double FLAG and CBP tags were fused into the N-terminus of the bait protein 14-3-3ε. First, to avoid possible false-positives arisen from complex studies using transiently transfected 293T cells [11,23], the stable cells expressing the tagged bait protein at close to natural level were used in our study to ensure the precise identification of physiologically relevant complex components. The expression level of Flag-tagged 14-3-3ε was found at the similar natural level of its endogenous counterpart (Fig. 1), which was also indicated that the fusion expression did not affect the expression and secretion of the target protein. In addition, in order to remove non-specific associated proteins, including those only recognising the tag or the affinity matrix, a parallel control cells with only expressing the affinity tags were taken as the negative backgrounds for the conventional TAP and the Leu-d_3-based quantitative affinity purification.

Except the above-mentioned procedures, a general assay through a reporter gene, such as luciferase assay, is usually applied to monitor functions of the target protein and the protein interactions between two proteins. We will detect the newly identified interactions between 14-3-3ε and other proteins by the luciferase reporter assay in future, and will further discover the functions of these interactions. For example, how 14-3-3ε interacts with cofilin 2, directly or indirectly? And which amino acids are responsible for their binding? Further deletion analysis in cells or in the model organism was to perform to clarify these issues.

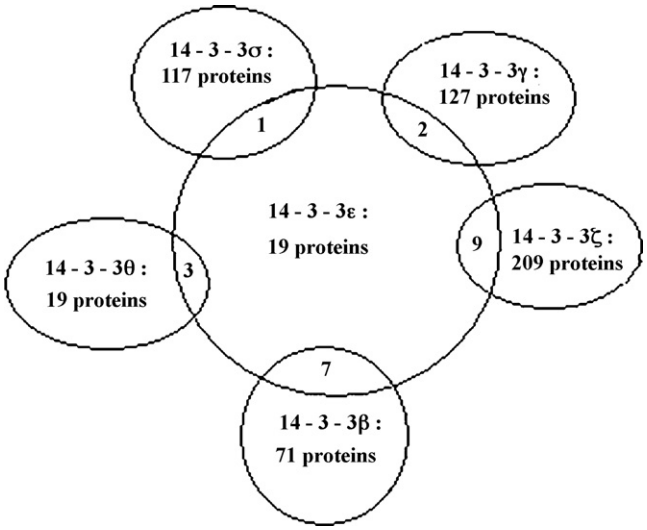

Fig. 6. Comparison of 14-3-3ε-interacting partners overlapped with proteins previously identified as binding to other isoforms. The number in the covered area between two circles means the common proteins between two isoforms' partners. Proteins associated with either ectopic 14-3-3 β, γ or θ in HEK293 cells are from Jin's report [10]. Proteins previously identified with 14-3-3 ζ are from the report by Meek et al. [9], and the 117 putative 14-3-3 σ-associated proteins are from Benzinger's report [11].

Among 19 of 14-3-3ε-interacting proteins, 13 specific partners (M2-PK, GAPDH, peroxiredoxin 6, thioredoxin, HSP90, HSP70, Bip, calmodulin, actin, 14-3-3ζ, cofilin1, kinesin-related protein and tropomyosin 3) representing 68.42% of all 14-3-3 family-specific proteins identified in our investigation were also found by other studies using 14-3-3 family-specific affinity purification coupled with mass spectrometry [6,9]. This indicated that most of the identified proteins are authentic 14-3-3ε interactors. The composition of 14-3-3 isoform-specific complexes may directly correlate with the functional diversity of 14-3-3 isoforms, i.e., the isoform-specific function determines the specificity in recruiting different sets of interacting proteins by different 14-3-3 isoforms. In comparison with the complex components of other specific 14-3-3 isoforms [9–11], 14-3-3ε-specific binding proteins were found to share 5–47% of the interactors with other isoforms(Fig. 6). 14-3-3 isoform-specific interacting proteins differ greatly, which shows 14-3-3 isoforms have broad functional difference and diversity. These differences are probably due to the different starting material (cell line), transfection (stable or transient transfection), purification method (one-step affinity purification or TAP) and MS-identification strategy etc. factors, but the more important reason is the result of isoform-specific binding proteins. For example, about 18% of all 117 proteins associated with 14-3-3σ [11] are same to those bound with 14-3-3ζ reported by Meek et al. [9], while approximately 8.5% (10 partners) with 14-3-3σ is overlapped with the interactors of 14-3-3β, which has 71 binding proteins in all [10]. These isoform-specific interactions reflect the biological functional differences among mammalian 14-3-3 family, which relates with the sequence specificity of individual isoform and its dimerization [30,31]. The most highly conserved residues between 14-3-3 isoforms lie within the phosphopeptide-binding groove and so any differences in isoform function are presumably not related to phosphopeptide binding. While the C-terminal and N-terminal regions of 14-3-3 proteins are not highly conserved. Therefore, the C and N termini are nearly unique for each isoform, supporting the potential for specific functions among isoforms [31]. Interestingly, each

individual isoform is more highly conserved among species than the conservation of isoforms within a single species. For example, human 14-3-3ε and *S. cerevisiae* BMH1 are approximately 70% similar at the amino acid level [1]. 14-3-3ζ in humans is 96–100% identical to ζ isoform in *Drosophila* and mouse, whereas the human 14-3-3ε and 14-3-3ζ isoforms show less than 60% identity [31]. Hence, the sequence difference and specificity of 14-3-3ε with those of other isoforms can partly explain the difference of 14-3-3ε-binding proteins between other isoform's associated ligands.

In summary, we have demonstrated an 'interaction- or function-relay' strategy through profiling protein-protein interactions to reveal the functional linkage of a particular uncharacterized protein, i.e., through the unambiguous identification of the interacting partners of a 14-3-3 isoform, those with known roles in particular function areas, 14-3-3ε involvement in regulating a variety of cellular functions has been inferred. This approach is generally applicable to characterize the proteins with unknown functions in mammalian cell complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.01.023.

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