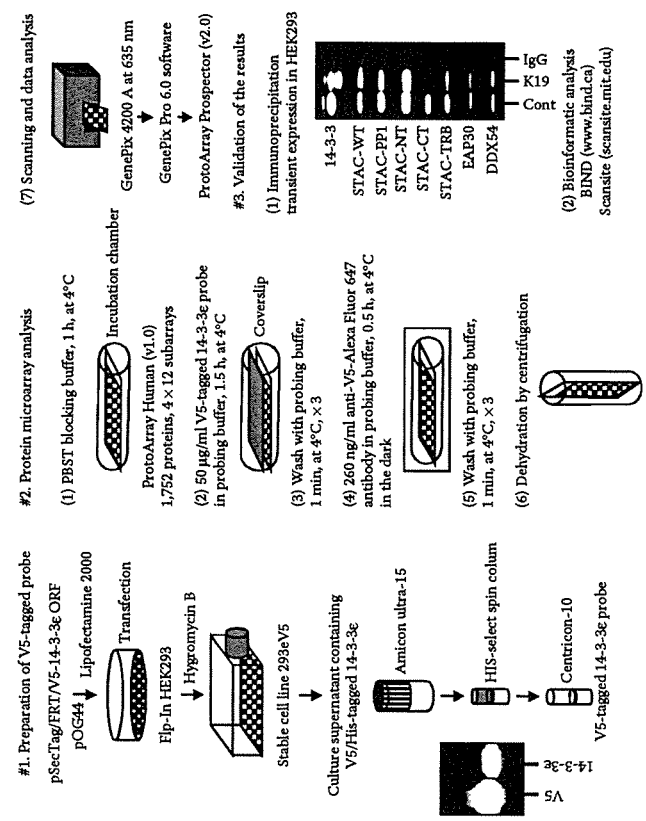

FIGURE 13.1 Protein microarray analysis of 14-3-3-binding proteins. The experimental protocol is comprised of the following three steps. (1) Preparation of V5-tagged probe. The recombinant human 14-3-3ε protein tagged with V5 was purified from the concentrated culture supernatant of a stable cell line 293eV5. The purity and specificity of the probe were verified by Western blot analysis using anti-V5 antibody or anti-14-3-3ε antibody. (2) Protein microarray analysis. After blocking nonspecific binding, the microarray containing 1752 human proteins was incubated with the probe, followed by incubation with anti-V5 antibody labeled with Alexa Fluor 647, and scanned on the microarray scanner. The significant binding was identified by analyzing the data with the ProtoArray Prospector software. (3) Validation of the results. The specific binding to 14-3-3 of STAC (WT, wild type; PP1, WT with inclusion of protein phosphatase-1 during protein extraction; NT, the N-terminal half; CT, the C-terminal half; TRB, the truncated form lacking both the RYYSSP motif and the cysteine-rich domain), EAP30 and DDX54 was validated by immunoprecipitation (IP) analysis of the corresponding recombinant proteins expressed in HEK293 cells. Lanes represent the input control (Cont), and IP with K-19 or normal rabbit IgG. The 14-3-3 interactors identified by protein microarray analysis were further evaluated by bioinformatic analysis of protein–protein interaction networks via BIND and Scansite database searches.

ultimate ORF clone collection (Invitrogen). They represent either the full-length or the partial fragment of recombinant proteins. They were expressed in Sf9 insect cells by using the Bac-to-Bac Baculovirus expression system (Invitrogen), purified under non-denaturating conditions by glutathione affinity chromatography in the presence of protease inhibitors, and processed for spotting on the slides (Invitrogen application note).

The proteins are spotted in an arrangement composed of 4 × 12 subarrays equally spaced in vertical and horizontal directions. Each subarray includes 16 × 16 spots, composed of 48 control spots (C), 80 human proteins (P), and 128 blanks (B) (Figure 13.2a). The controls include 14 positive control spots; four spots of an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2), six spots of a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 8; columns 3 to 8), and four spots of a concentration gradient of V5 protein (row 8; columns 13 to 16). They also include 34 negative control spots; six spots of a concentration gradient of bovine serum albumin (BSA) (row 1; columns 3 to 8), four spots of a concentration gradient of a rabbit anti-GST antibody (row 1; columns 9 to 12), four spots of a concentration gradient of calmodulin (row 1; columns 13 to 16), 16 spots of a concentration gradient of GST (row 2; columns 1 to 16), two spots of buffer only (row 8; columns 9,10), and two spots of an anti-biotin antibody (row 8; columns 11, 12).

Nonspecific binding was blocked by incubating the microarray for 60 min at 4in the PBST blocking buffer composed of 1% BSA and 0.1% Tween 20 in phosphate-buffered saline (PBS), as described previously (Figure 13.1).[56] Then, it was incubated for 90 min at 4 with the probe described above at a concentration of 50 μg/ml in the probing buffer composed of 1% BSA, 5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.05% Triton X-100, and 5% glycerol in PBS. The array was washed three times with the probing buffer, followed by incubation for 30 min at 4with mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (Invitrogen) at a concentration of 260 ng/ml in the probing buffer. The array was washed three times with the probing buffer, dehydrated by brief centrifugation, and then scanned by the GenePix 4200A scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm. The data in a format specified by the GenePix Pro 6.0 microarray data acquisition software (Axon Instruments) were analyzed by using the ProtoArray Prospector software v2.0 (Invitrogen) following acquisition of the microarray lot-specific information online (www.invitrogen.com/protoarray). The spots showing the background-subtracted signal intensity value greater than the median plus three standard deviations of intensities of all protein features were considered as having a significant binding.

The Z-Score was calculated by the following formula: $Z_k = (X_k - \mu_s)/\sigma_s$, where X_k represents the signal intensity value of the *k*th protein feature, μ_s is the mean signal intensity of all protein features, and σ_s expresses the standard deviation of intensities of all protein features. The Z-Score reflects a binding specificity determined by the definition how far and in what direction a signal from a specific protein feature deviates from the mean signal intensity of all the protein features.

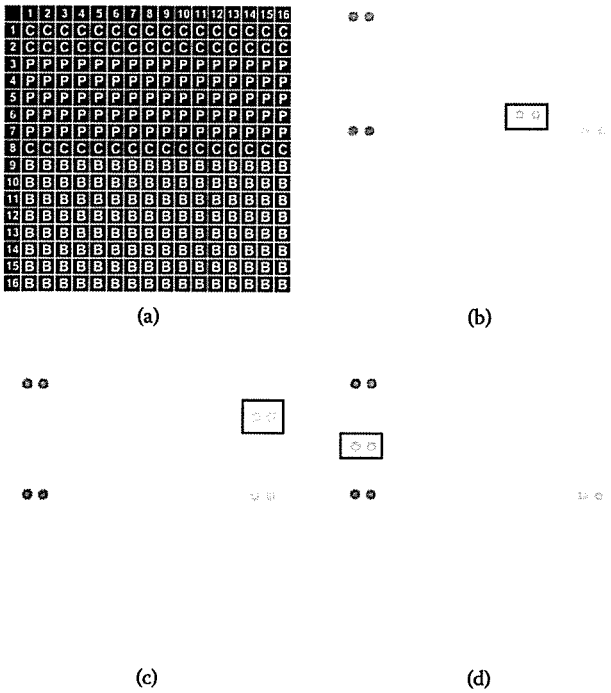

FIGURE 13.2 Detection of 14-3-3-binding proteins on protein microarray. The microarray we utilized contains 1752 distinct human proteins of various functional classes spotted in duplicate on a nitrocellulose-coated glass slide. They are printed in an arrangement of 4 × 12 subarrays equally spaced in vertical and horizontal directions. (a) Layout of the subarray. Each subarray includes 16 × 16 spots composed of 48 control spots (C), 80 human proteins (P), and 128 blanks (B). (b) EAP30 on the subarray 1. The spots of (row 7; column 1) and (row 7; column 12) indicated by a square represent EAP30. (c) DDX54 on the subarray 27. The spots of (row 3; column 15) and (row 3; column 16) indicated by a square represent DDX54. (d) STAC on the subarray 39. The spots of (row 5; column 1) and (row 5; column 2) indicated by a square represent STAC. In these subarrays (b–d), the positive control spots represent an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2) that provides the strong signals, a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody labeled with Alexa Fluor 647 (row 8; columns 3 to 8), and a concentration gradient of V5 protein (row 8; columns 13 to 16). The signals are only visible at the higher concentration in the latter two.

VALIDATION AND EVALUATION OF THE RESULTS OF PROTEIN MICROARRAY ANALYSIS

Transient Expression of Recombinant Proteins in HEK293 Cells

To verify the results of protein microarray analysis, the ORF of the genes encoding EAP30 subunit of ELL complex (EAP30, NM_007241, amino acid residues 2 to 258), dead box polypeptide 54 (DDX54, NM_024072, amino acid residues 2 to 881), and

src homology three (SH3) and cysteine rich domain (STAC, NM_003149, amino acid residues 2 to 402, full-length) were amplified by PCR using PfuTurbo DNA polymerase and the specific primer sets (5'caccgccgcggggtgggagctggc3' and 5'tcaggggagggcttctctg-gcctc 3' for EAP30; 5'gcggccgacaagggcccggcggct3' and 5'tcacatcctcttccgcatcttgcc3' for DDX54; and 5'atccctccgagcagcccccgcgag3' and 5'tcagatgttttctagtacatcaag3' for STAC). The N-terminal half of STAC (amino acid residues 2 to 333, NTF), the C-terminal half of STAC (amino acid residues 234 to 402, CTF), and two distinct truncated forms of STAC (amino acid residues 2 to 164 named TRA and amino acid residues 2 to 105 named TRB) were amplified using the corresponding primer sets (5'atccctccgagcagcccccgcgag3' and 5'tcaaagatctgaagtagaggttct3' for NTF; 5'gtggaggt-tcctgaggaagccaat 3' and 5'tcagccacctggatgcagaccagc3' for CTF; 5'atccctccgagcagc-ccccgcgag 3' and 5'tcatggcagcttgcccatgcaccg3' for TRA; and 5'atccctccgagcag- ccccc gcgag 3' and 5'tcagccacctggatgcagaccagc3' for TRB).

They were then cloned into a mammalian expression vector pcDNA4/HisMax-TOPO (Invitrogen) to produce a fusion protein with an N-terminal Xpress tag. To express the STAC mutant with a single amino acid substitution S172A (the single mutant; SMT) or with double amino acid substitutions S172A and S173A (the double mutant; DMT), the pcDNA4/HisMax-TOPO vector containing the full-length wild-type (WT) STAC gene was modified by consecutive site-directed mutagenesis using QuikChange II site-directed mutagenesis kit (Stratagene) and the primer sets (5'gtttcggcgttactacgcctccccttgctcattc3' and 5'gaatgagcaaggggaggcgtagtaacgccgaaac 3' for SMT and 5'cggcgttactacgccgcccccttgctcattcat3' and 5'atgaatgagcaaggggcggc gtagtaacgccg3' for DMT). All these vectors were transfected in HEK293 cells by Lipofectamine 2000 reagent.

Coimmunoprecipitation Analysis

For coimmunoprecipitation analysis, total protein extract was prepared by homogenizing the cells in M-PER lysis buffer (Pierce, Rockford, IL) supplemented with a cocktail of protease inhibitors (Sigma), either with inclusion of phosphatase inhibitors (Sigma) to maintain the protein phosphorylation status or with inclusion of recombinant protein phosphatase-1 (PP1) catalytic subunit α-isoform (5 U/ml; Sigma) instead of phosphatase inhibitors to induce the protein dephosphorylation reaction.[57] The homogenate was centrifuged at 12,000 rpm for 20 min at 4. After preclearance, the supernatant was incubated for 3 hours at 4 with 30 μg/ml rabbit polyclonal anti-14-3-3 protein antibody (K19)-conjugated agarose (Santa Cruz Biotechnology, Santa Cruz, CA) or the same amount of normal rabbit IgG-conjugated agarose (Santa Cruz Biotechnology). After several washes, the immunoprecipitates were processed for Western blot analysis using mouse monoclonal anti-14-3-3 protein antibody (H-8, Santa Cruz Biotechnology) and mouse monoclonal anti-Xpress antibody (Invitrogen). K-19 and H-8 antibodies recognize all 14-3-3 isoforms. The specific reaction was visualized by using a chemiluminescent substrate (Pierce).

Please verify symbol.

Please verify symbol.

Bioinformatic Analysis

In addition to validation of the specific interactions by wet experiments, we evaluated them by bioinformatic analysis. The information on known 14-3-3 interactors, molecular

function, molecular weight, and subcellular localization was obtained from Biomolecular Interaction Network Database (BIND; www.bind.ca), Human Protein Reference Database (HPRD; www.hprd.org), Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequence Database (PSORT II; psort.ims.u-tokyo.ac.jp), and PubMed Database (www.pubmed.gov). The 14-3-3-binding consensus motif mode I (RSXpSXP) located in target proteins was surveyed by the Scansite 2.0 Motif Scanner (scansite.mi.edu),[58] which assesses the probability of a site matching the candidate motif under high, medium or low stringent conditions (Figure 13.3).

RESULTS

Protein Microarray Analysis Identified 20 Distinct 14-3-3-Interactors

Western blot analysis verified the purity and specificity of the recombinant 14-3-3ε protein tagged with V5 (Figure 13.1). Among 1752 proteins on the microarray, 20 were identified as the proteins showing significant binding to the probe, all of which were previously unreported 14-3-3-binding partners by the BIND search.[56] Seven were categorized into hypothetical clones of uncharacterized function, derived from either the Mammalian Genome Collection (MGC) or the Full-Length Long Japan (FLJ). They include FLJ10415 (GenBank accession number NM_018089), LOC57228 (NM_020467), MGC17403 (NM_152634), LOC137781 (BC032347), LOC92345 (NM_138386), FLJ10156 (NM_019013), and FLJ25758 (NM_001011541). Thirteen proteins with annotation are as follows:

1. *EAP30 subunit of ELL complex (EAP30; NM_007241)* (Figure 13.2b). This is a 30-kDa component of the ELL complex (estimated MW is 28,866 suggested by HPRD; putative subcellular location is cytoplasmic suggested by PSORT II), which confers derepression of transcription by RNA polymerase II.[59] EAP30 is also named VPS22, a component of the ESCRT-II endosomal sorting complex that plays a key role in the multivesicular body (MVB) pathway.[60] The 14-3-3-binding consensus motif mode I is not identified by the Scansite Motif Scanner, although the Z-Score of two corresponding spots on the array shows the highest values, 22.9 and 24.6 respectively. The similarity in the scores between distinct spots supports the reproducibility of the results of protein microarray analysis.
2. *Lymphocyte cytosolic protein 2 (LCP2; NM_005565).* This is a 72-kDa protein (MW 60,191; nuclear), alternatively named SH2 domain-containing leukocyte protein of 76kD (SLP76), which associates with the Grb2 adaptor protein and provides a substrate of the ZAP-70 protein tyrosine kinase.[61] LCP2 plays a key role in promoting T cell development and activation. It contains three mode I motifs with low stringency; pS297 (TTERHERSSPLPGKK), pS376 (SSFPQSASLPPYFSQ), and pT456 (DSSKKTTTNPYVLMV).

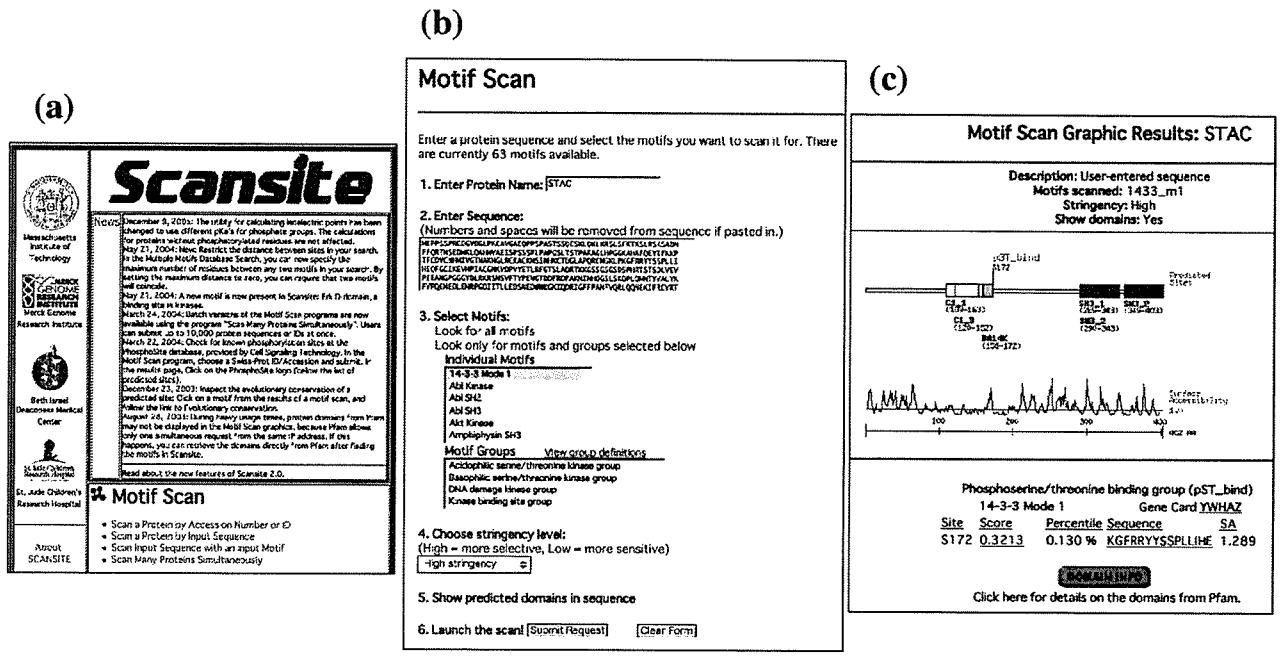

FIGURE 13.3 The Scansite Motif Scanner. The 14-3-3-binding consensus motif mode I (RSXpSXP) located in target proteins was surveyed by the Scansite Motif Scanner which assesses the probability of a site matching the candidate motif under high, medium or low stringent conditions. (a) The opening menu (cited from the website of http://scansite.mi.edu). (b) The motif scan menu. After entering the protein sequence of STAC, 63 different motifs including 14-3-3 mode I could be processed for searching. (c) The graphic view of the results. The candidate for the 14-3-3-binding motif of STAC under the high stringent condition (pST_bind S172) is indicated on the predicted domain structure of the protein (upper panel), accompanied by the relative scores for the interaction of pS172 domain: KGFRRYYSSPLLIHE with 14-3-3 protein (lower panel). A plot of the surface accessibility suggests the residues located near the protein surface with a capacity to interact with target proteins (upper panel).

3. *Methionine aminopeptidase 2 (METAP2; NM_006838).* This is a 67-kDa protein (MW 52,894; cytoplasmic) that interacts with eukaryotic initiation factor-2 (eIF-2) and regulates protein synthesis [62]. It contains two mode I motifs with low stringency; pT113 (KRGPKVQTDPPSVPI) and pS152 (TAAWRTTSEEKKALD).
4. *Melanoma antigen family B, 4 (MAGEB4; NM_002367).* This is a member of the MAGEB family (MW 38,925; nuclear) expressed abundantly in testis whose function remains unknown.[63] It contains three mode I motifs; T18 (AREKRQRTRGQTQDL) with medium stringency, and pT194 (GNQSSAWTLPRNGLL) and pS339 (SAYSRATSSSSSQPM) with low stringency.
5. *Chondroitin 4 sulfotransferase 11 (CHST11; NM_018413).* This is a member of the HNK1 sulfotransferase family GalNAc 4-*O*-sulfotransferase (MW 41,557; endoplasmic reticulum and mitochondria) that plays a role in chondroitin sulfate and dermatan sulfate biosynthesis.[64] It contains three mode I motifs; pS93 (TDTCRANSATSRKRR) with medium stringency, and pS56 (DICCRKGSRSPLQEL) and S194 (EPFERLVSAYRNKFT) with low stringency.
6. *Zinc finger, C3HC-type containing 1 (ZC3HC1; NM_016478).* This is a 60-kDa protein (MW 55,258; nuclear) that interacts with anaplastic lymphoma kinase (ALK) and plays an antiapoptotic role in nucleophosmin-ALK signaling event.[65] The 14-3-3-binding consensus motif mode I is not found.
7. *Minichromosome maintenance deficient 10 (MCM10; NM_018518).* This is a key component of the pre-replication complex (pre-RC) (MW 98,188; nuclear) essential for the initiation of DNA replication.[66] It contains five mode I motifs; pS90 (AQPPRTGSEFPRLEG) with medium stringency, and pS35 (KPAIKSISASALLKQ) S55 (LEMRRRKSEEIQKRF), pS302 (PCGNRSISLDRLPNK), and T329 (DGMLKEKTGPKIGGE) with low stringency.
8. *DEAD box polypeptide 54 (DDX54; NM_024072)* (Figure 13.2c). This is a 97-kDa RNA helicase (DP97) (MW 98,601; nuclear) that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes.[67] It contains two mode I motifs with low stringency; pT95 (EDKKKIKTESGRYIS) and pS102 (TESGRYISSSYKRDL).
9. *Heterogeneous nuclear ribonucleoprotein C (HNPRC; NM_004500).* This is a member of heterogeneous nuclear ribonucleoproteins (hnRNPs) (MW 33,291; nuclear) involved in pre-mRNA processing, mRNA metabolism and transport.[68] It contains four mode I motifs; pS125 (DYYDRMYSYPARVPP) with high stringency, and pS158 (NTSRRGKSGFNSKSG), pS170 (KSGQRGSSKSGKLKG), and pS240 (ETNVKMESEGGADDS) with low stringency.
10. *Fibroblast growth factor 12 (FGF12; NM_004113).* This is a member of the FGF family (MW 27,401; nuclear) that plays a role in nervous system development and function.[69] It contains two mode I motifs with low

stringency; pS150 (VCMYREQSLHEIGEK) and pS165 (QGRSRKSSGTPTMNG).

11. *Glutathione S-transferase M3 (GSTM3; BC030253).* This is a cytoplasmic glutathione *S*-transferase of the mu class (MW 26,561; cytoplasmic) that plays a role in detoxification of carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress.[70] It contains one mode I motif with low stringency; pS64 (GIKLRSFSV).
12. *Src homology three (SH3) and cysteine rich domain (STAC; NM_003149)* (Figure 13.2d). This is a 47-kDa protein containing a SH3 domain and a cysteine-rich domain (MW 44,556; nuclear) that plays a role in the neuron-specific signal transduction pathway.[71] It contains seven mode I motifs; pS172 (KGFRRYYSSPLLIHE) with high stringency (Figure 13.3c), pS56 (TKSLRSKSADNFFQR) and pS255 (DLRKRSNSVFTYPEN) with medium stringency, and pS46 (QKLKRSLSFKTKSLR), pS51 (SLSFKTKSLRSKSAD), pS66 (NFFQRTNSEDMKLQA), and pS253 (GYDLRKRSNSVFTYP) with low stringency.
13. *ATPase, H^+ transporting, lysosomal, 21 kD, V0 subunit C" (ATP6V0B; NM_004047).* This is a 23-kDa component of vacuolar ATPase (MW 21,408; endoplasmic reticulum) that mediates acidification of intracellular organelles.[72] The 14-3-3-binding consensus motif mode I is not found.

Immunoprecipitation Analysis Validated the Specific Binding to 14-3-3

EAP30, DDX54, and STAC were selected to verify the results of microarray analysis in view of higher Z-Score values.[56] The recombinant proteins were expressed in HEK293 cells that constitutively express a substantial amount of endogenous 14-3-3 protein. The cells were homogenized in the lysis buffer either with inclusion of phosphatase inhibitors or with inclusion of recombinant protein phosphatase-1 (PP1) instead of phosphatase inhibitors. Total cell lysate was processed for immunoprecipitation (IP) with rabbit anti-14-3-3 protein antibody (K-19) or with normal rabbit IgG. K19 coimmunoprecipitated 14-3-3 and STAC from the lysate of HEK293 cells that express the recombinant STAC protein, whereas normal rabbit IgG did not pull down these proteins (Figure 13.1). K-19 immunoprecipitated EAP30 and DDX54 from the lysate of HEK293 cells that express the recombinant EAP30 or DDX54 protein, respectively (Figure 13.1). These results indicate that EAP30, DDX54 and STAC interact with the endogenous 14-3-3 protein in HEK293 cells where the corresponding recombinant proteins were expressed.

STAC has the highly stringent 14-3-3-binding consensus motif RYYSSP in amino acid residues 169 to 174 (pS172) by the Scansite Motif Scanner search (Figure 13.3). Therefore, a possible involvement of this motif in binding to 14-3-3 was further investigated by IP analysis of a panel of mutant and truncated STAC proteins. K-19 immunoprecipitated the full-length wild-type (WT) STAC consisting of amino acid residues 2 to 402 (Figure 13.1). K-19 also pulled down the S172A mutant (SMT), and the S172A and S173A double mutant (DMT) from the lysate of HEK293 cells that express the corresponding recombinant proteins.[56] K-19 immunoprecipitated

the N-terminal half (NTF; amino acid residues 2 to 233) but not the C-terminal half (CTF; amino acid residues 234 to 402) of STAC (Figure 13.1). These observations indicate that the RYYSSP motif is not involved in binding of STAC to 14-3-3. This was confirmed by the observations that K-19 immunoprecipitated the truncated STAC protein lacking the RYYSSP motif (TRA; amino acid residues 2 to 164)[56] and the shortest form lacking both the RYYSSP sequence and the cysteine-rich domain (CRD) (TRB; amino acid residues 2 to 105) (Figure 13.1). Finally, K-19 pulled down the full-length WT STAC, EAP30, and DDX54 under the dephosphorylated condition (PP1) (Figure 13.1). These observations indicate that the 14-3-3-interacting domain is located in the N-terminal segment spanning amino acid residues 2 to 105 of STAC. The interaction of 14-3-3 with STAC, EAP30, and DDX54 is independent of serine/threonine-phosphorylation of the binding domains.

DISCUSSION

Protein Microarray Analysis Effectively Identifies 14-3-3-Binding Proteins

Protein microarrays provide a valuable tool for global proteome analysis with a wide range of applications, particularly to identification and characterization of protein function and molecular pathways closely associated with disease markers and therapeutic targets.[39–43] The great advantage of this technology exists in low reagent and sample consumption, rapid interpretation of the results, and the ability to easily manipulate experimental conditions.

The present study was designed to identify 14-3-3-binding proteins by using a high-density human protein microarray. The array contains 1752 proteins derived from multiple gene families of biological importance, including cell-signaling proteins, kinases, membrane-associated proteins, and metabolic proteins. The entire procedure could be accomplished within five hours of obtaining a specific probe. By probing with V5-tagged 14-3-3ε, we identified twenty 14-3-3 interactors, most of which were previously unreported except for glutathione *S*-transferase M3 (GSTM3) that was reported previously.[36] Unexpectedly, the highly stringent 14-3-3-binding consensus motifs (STAC and HNPRC) were identified only in two by the Scansite Motif Scanner search. The specific binding to 14-3-3 of EAP30, DDX54 and STAC was validated by coimmunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results indicate that protein microarray is an effective tool for the rapid and systematic identification of protein–protein interactions, including those not predicted by the Database searching.

Potential Problems Remain to Be Solved in the Present Study

In general, protein microarray has its own limitations associated with the efficient expression and purification of native target proteins.[40,41] The target proteins spotted on the microarray we utilized were expressed by a baculovirus expression system and purified under non-denaturating conditions to maximize the preservation of native folding, posttranslational modifications, and proper functionality. In contrast,

bacterially expressed proteins lack glycosylation and phosphorylation moieties, and are often misfolded during purification. Post-translational modifications play a pivotal role in a range of protein–protein interactions. Immuno-labeling with anti-phosphotyrosine (pTyr) antibody showed that approximately 10 to 20% of the proteins on the array are phosphorylated (Invitrogen, unpublished data). When it was utilized for kinase substrate identification, most of known kinases immobilized on the array are enzymatically active with the capacity of autophosphorylation, suggesting that they are certainly phosphorylated on tyrosine residues, probably on serine and threonine residues (Invitrogen application note). However, we could not currently validate the precise level of serine and threonine phosphorylation of individual target proteins due to a lack of anti-phosphoserine (pSer) and anti-phosphothreonine (pThr) antibodies suitable for detection on glass slides.

The protein microarray we utilized includes 11 known 14-3-3-binding proteins, such as PCTAIRE protein kinase 1 (PCTK1),[73] protein kinase C zeta (PRKCZ),[74] keratin 18 (KRT18),[75] myosin light polypeptide kinase (MYLK),[76] v-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1),[77] v-akt murine thymoma viral oncogene homolog 1 (AKT1),[78] epidermal growth factor receptor (EGFR),[79] cell division cycle 2 (CDC2),[80] mitogen-activated protein kinase kinase kinase 1 (MAP3K1),[81] mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2),[82] and stratifin (SFN).[37] However, none of these were identified as positive. Therefore, there exists the possibility that some 14-3-3 binding partners were not detected due to imperfect phosphorylation of target proteins, inaccessibility by a sterical hindrance of epitope tags,[83] or a 14-3-3 isoform-specific binding ability. Calmodulin, another known 14-3-3 interactor,[84] is included as a negative control on the array. It was found as negative in the present study, because the calcium-dependent interaction between 14-3-3 and calmodulin could not be detected under the calcium-free conditions we employed. Recently, by using two dimensional (2-D)-gel electrophoresis and mass spectrometry, we showed that vimentin, an intermediate filament protein, interacts with 14-3-3ε in cultured human astrocytes.[20] More recently, we found that heat shock protein Hsp60 and the cellular prion protein PrPC interact with 14-3-3ζ in human neurons in culture and brain tissues.[85] Unfortunately, the protein microarray we examined here includes neither vimentin, Hsp60 nor prion protein.

Repetition of "kinase" OK here?

Recent evidence indicates that 14-3-3-binding phosphorylation sites do not exactly fit the consensus motif,[1,25,75] and an accessory site is required to enhance a stable 14-3-3-target interaction.[4,86] Furthermore, 14-3-3 interacts with a set of target proteins in a phosphorylation-independent manner.[26–29] We found that the interaction is independent of serine/threonine-phosphorylation of the binding sites of EAP30, DDX54 and STAC, supporting this possibility.

Biological Roles of 14-3-3-Interacting Proteins

Among the 14-3-3 interactors we identified, several proteins are categorized as a component of multimolecular complexes involved in transcriptional regulation. ELL is a human oncogene encoding a RNA polymerase II (Pol II) transcription factor that promotes transcription elongation. EAP30 is a component of the ELL complex where EAP30 mediates derepression of transcription by Pol II,[59] although the PSORT

II search suggests that its putative location is cytoplasmic. A recent study showed that EAP30 interacts with the tumor susceptibility gene TSG101 product, a cellular factor that mediates packaging of HIV virions.[87] DDX54 is a RNA helicase that interacts with estrogen receptor (ER) and represses the transcription of ER-regulated genes.[67] A chromatin immunoprecipitation (ChIP) assay showed that hepatocyte nuclear factor 4-alpha (HNF4α), a master regulator of hepatocyte gene expression, interacts with the DDX54 gene promoter, together with Pol II.[88] HNPRC belongs to a member of heterogeneous nuclear ribonucleoproteins (hnRNPs) involved in pre-mRNA processing, mRNA metabolism and transport.[68] Increasing evidence indicates that the 14-3-3 protein and its targets are widely distributed in nearly all subcellular compartments, including the nucleus.[3,35]

STAC has a cysteine-rich domain (CRD) of the protein kinase C family in the N-terminal half (NTF) and a src homology three (SH3) domain in the C-terminal half (CTF), suggesting its role as an adapter on which divergent signaling pathways converge.[71,89] STAC is expressed predominantly in the brain with the distribution in a defined population of neurons.[71] IP analysis of mutant and truncated forms of STAC argued against an active involvement of the most stringent motif RYYSSP (pS172) in its binding to 14-3-3, and indicated that the interacting motif is located in the N-terminal amino acid residues 2 to 105 without requirement of serine/threonine phosphorylation.

FUTURE DIRECTIONS

Protein microarrays are a powerful tool for the rapid and systematic identification of protein–protein and other biomolecule interactions. However, they are still under development in methodological aspects. The strict quality controls of analytical procedures,[90] validation of the results by different methods, and evaluation of enormous data by bioinformatic approaches are highly important. The applications of protein microarrays include characterization of antibody specificity and autoantibody repertoire, and identification of novel biomarkers and molecular targets associated with disease type, stage and progression, leading to establishment of personalized medicine.[44–51] Theoretically, this technology could determine all of the binding partners at once, consisting of "the whole interactome" in a subset of cells responding to specific treatment. It would open up a new avenue of drug discovery research. Development of an ultrahigh-density protein microarray containing all spliced variants of target proteins could facilitate achievement of this purpose. A cell-free transcription and translation-coupled system might provide an effective tool for producing ideal proteins.[83] At present, the most advanced version of human protein microarray contains approximately 5000 GST-tagged proteins, commercially available from Invitrogen (ProtoArray v3.0), accompanied by an upgraded version of the analytical software (ProtoArray Prospector). It seems highly efficient to screen a large number of protein–protein interactions in human cells, including those unrecognized by the conventional methods such as Y2H.[91,92] However, when faced with a huge amount of data, bioinformatic and statistical analyses become crucial (visit the useful website of Pathguide for a comprehensive pathway resource list; cbio.mskcc.org/prl). Recently, an ultrahigh sensitive detection method armed with

silicon-nanowire field-effect sensors has come into use with its application to protein microarray analysis.[93] This promising technology could detect the low-femtomolar range of interacting proteins, and greatly increase the detection sensitivity and specificity.

SUMMARY AND CONCLUSIONS

The 14-3-3 protein family consists of acidic 30-kDa proteins composed of seven isoforms in mammalian cells, expressed abundantly in neurons and glial cells of the CNS. The 14-3-3 isoforms form a dimer that acts as a molecular adaptor interacting with key signaling components involved in cell proliferation, transformation, and apoptosis. Until present, more than 300 proteins have been identified as 14-3-3-binding partners, although most of previous studies focused on a limited range of 14-3-3-interacting proteins. In this chapter we describe a comprehensive profile of 14-3-3-binding proteins by analyzing a high-density protein microarray (1752 proteins; ProtoArray v1.0) using recombinant human 14-3-3ε protein as a probe. We identified twenty 14-3-3 interactors, most of which were previously unreported 14-3-3-binding partners, although eleven known 14-3-3-binding proteins on the array, including KRT18 and MAPKAPK2, were undetected. The assay required less than five hours. Unexpectedly, highly stringent 14-3-3-binding consensus motifs, such as STAC and HNPRC, were identified only in two proteins by the Scansite Motif Scanner search. The specific binding to 14-3-3 of EAP30, DDX54 and STAC was verified by coimmunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results suggest that protein microarray is a valuable tool for rapid and comprehensive profiling of 14-3-3-binding proteins.

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Rapid identification of 14-3-3-binding proteins by protein microarray analysis

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Abstract

The 14-3-3 protein family consists of acidic 30-kDa proteins composed of seven isoforms in mammalian cells, expressed abundantly in neurons and glial cells of the central nervous system (CNS). The 14-3-3 isoforms form a dimer that acts as a molecular adaptor interacting with key signaling components involved in cell proliferation, transformation, and apoptosis. Until present, more than 300 proteins have been identified as 14-3-3-binding partners, although most of previous studies focused on a limited range of 14-3-3-interacting proteins. Here, we studied a comprehensive profile of 14-3-3-binding proteins by analyzing a high-density protein microarray using recombinant human 14-3-3 epsilon protein as a probe. Among 1752 proteins immobilized on the microarray, 20 were identified as 14-3-3 interactors, most of which were previously unreported 14-3-3-binding partners. However, 11 known 14-3-3-binding proteins, including keratin 18 (KRT18) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), were not identified as a 14-3-3-binding protein. The specific binding to 14-3-3 of EAP30 subunit of ELL complex (EAP30), dead box polypeptide 54 (DDX54), and src homology three (SH3) and cysteine rich domain (STAC) was verified by immunoprecipitation analysis of the recombinant proteins expressed in HEK293 cells. These results suggest that protein microarray is a powerful tool for rapid and comprehensive profiling of 14-3-3-binding proteins.

Keywords: 14-3-3-Binding protein; Immunoprecipitation; Protein microarray; Protein–protein interaction; STAC

1. Introduction

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30-kDa proteins composed of seven isoforms named β, γ, ε, ζ, η, θ, and σ in mammalian cells. A homodimeric or heterodimeric complex composed of the same or distinct isoforms constitutes a large cup-like structure possessing an amphipathic groove with two ligand-binding capacity (Fu et al., 2000; van Hemert et al., 2001). The dimeric complex acts as a molecular adaptor that interacts with key signaling molecules involved in cell differentiation, proliferation, transformation, and apoptosis. It regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity, and protecting them from dephosphorylation or proteolysis (Dougherty and Morrison, 2004; MacKintosh, 2004). Although 14-3-3 is widely distributed in neural and non-neural tissues, it is expressed most abundantly in neurons in the central nervous system (CNS), where it represents 1% of total cytosolic proteins (Berg et al., 2002). Aberrant expression and impaired function of 14-3-3 in the CNS are associated with pathogenetic mechanisms of Creutzfeldt–Jacob disease, Alzheimer disease, Parkinson disease, spinocerebellar ataxia, amyotrophic lateral sclerosis, and multiple sclerosis (Chen et al., 2003; Kawamoto et al., 2002; Layfield et al., 1996; Malaspina et al., 2000; Satoh et al., 2004; Zerr et al., 1998).

In general, the 14-3-3 protein interacts with phosphoserine-containing motifs of the ligands such as RSXpSXP (mode I) and RXXXpSXP (mode II) in a sequence-specific manner (Dougherty and Morrison, 2004; MacKintosh, 2004). Previously, more than 300 proteins have been identified as being 14-3-3-binding partners. They include key signaling components, such as Raf-1 kinase, Bcl-2 antagonist of cell death (BAD), protein kinase C (PKC), phophatidylinositol 3-kinase (PI3K), and cdc25 phosphatase (Fu et al., 2000; van Hemert et al., 2001). Binding of 14-3-3 to Raf-1 is indispensable for its kinase activity in the Ras-MAPK signaling pathway, and the interaction of 14-3-3 with BAD, when phosphorylated by

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a serine/threonine kinase Akt, inhibits apoptosis. Recent studies indicate that the 14-3-3 protein could interact with a set of target proteins in a phosphorylation-independent manner (Dai and Murakami, 2003; Henriksson et al., 2002; Zhai et al., 2001). Increasing knowledge of interactions between 14-3-3 and interacting molecules would help us to understand the biological function and pathological implication of the 14-3-3 protein networks.

The yeast two-hybrid (Y2H) system is a powerful approach to identify novel protein–protein interactions. However, Y2H screening requires a lot of time and effort, and is often criticized for detecting the interactions unrelated to the physiological setting and obtaining high rates of false positive interactors caused by spontaneous activation of reporter genes and self-activating bait proteins (Vidalain et al., 2004; Zhang et al., 2004). Affinity purification coupled with mass spectrometry (APMS) is an alternative approach to identify the components of protein complexes on a large scale. This approach has been taken to identify a wide variety of 14-3-3-interacting proteins involved in cell proliferation, metabolism, and survival (Benzinger et al., 2005; Jin et al., 2004; Meek et al., 2004; Pozuelo Rubio et al., 2004). Although the APMS procedure detects binding partners of physiological significance, it is laborious and has a difficulty in detecting transmembrane proteins and loosely associated components that might be lost during purification (von Mering et al., 2002). Recently, protein microarray technology has been established for rapid, systematic, and less expensive screening of thousands of protein–protein, protein–lipid, and protein–nucleic acid interactions in a high-throughput fashion. This approach has important applications in the areas not only of basic biological research but also of drug discovery research, including identification of the substrates of protein kinases and the protein targets of small molecules (Chan et al., 2004; MacBeath and Schreiber, 2000; Michaud et al., 2003; Zhu et al., 2001).

The present study was designed for the first time to identify a comprehensive profile of human 14-3-3-binding proteins by analyzing a high-density protein microarray.

2. Materials and methods

2.1. Preparation of a probe for microarray analysis

Human embryonic kidney cells HEK293 whose genome was modified for the Flp-In system (Flp-In 293) were obtained from Invitrogen, Carlsbad, CA. Flp-In 293 cells contain a single Flp recombination target (FRT) site targeted for the site-specific recombination, integrated in a transcriptionally active locus of the genome, where it stably expresses the *lacZ*-Zeocin fusion gene driven from the pFRT/*lac*Zeo plasmid under the control of SV40 early promoter. Flp-In 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (feeding medium) with inclusion of 100 μg/ml of Zeocin (Invitrogen) as described previously (Satoh and Yamamura, 2004).

To prepare the probe for protein microarray analysis, the open reading frame (ORF) of the human 14-3-3ε gene (YWHAE) was amplified from cDNA of NTera2-N cells (Satoh and Kuroda, 2000) by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the primer sets listed in Table 1. The PCR product was then cloned into a mammalian expression vector pSecTag/FRT/V5-His TOPO (Invitrogen) to produce a fusion protein with a C-terminal V5 (GKPIPNPLLGLDST) tag, a C-terminal polyhistidine (6 × His) tag, and an N-terminal Ig κ-chain secretion signal. This vector, together with the Flp recombinase expression vector pOG44 (Invitrogen), was transfected in Flp-In 293 cells by Lipofectamine 2000 reagent (Invitrogen). A stable cell line was established after incubating the transfected cells for approximately 1 month in the feeding medium with inclusion of 100 μg/ml of Hygromycin B (Invitrogen). It was named 293 eV5. The recombinant protein was secreted into the culture medium of 293 eV5 cells after the Ig κ-chain secretion signal sequence was processed by an endogenous signal peptidase-mediated cleavage.

To purify the recombinant 14-3-3ε protein, the culture supernatant of 293 eV5 cells incubated for 48 h in the serum-free DMEM/F-12 medium was harvested and concentrated at an 1/40 volume by centrifugation on an Amicon Ultra-15 filter (Millipore, Bedford, MA). It was then purified by the HIS-select spin column (Sigma, St. Louis, MO) and concentrated at an 1/10 volume by centrifugation on a Centricon-10 filter (Millipore). The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA). The purity and specificity of the probe were verified by Western blot analysis using mouse monoclonal anti-V5 antibody (Invitrogen) and rabbit polyclonal antibody specific for the 14-3-3ε isoform (IBL, Gumma, Japan).

2.2. Protein microarray analysis

ProtoArray human protein microarray (v1.0) commercially available from Invitrogen was utilized in the present study. It contains 1752 human proteins of various functional classes spotted in duplicate on a nitrocellulose-coated glass slide. To prepare target proteins immobilized on the microarray, an N-terminal glutathione-*S* transferase (GST)-6 × His fusion protein derived from the genes selected from the human ultimate ORF clone collection (Invitrogen) was expressed in Sf9 insect cells by using the baculovirus expression system (Invitrogen). Either the full-length or the partial fragment of recombinant proteins, was purified under native conditions by glutathione affinity chromatography in the presence of protease inhibitors, then processed for spotting on the slides. The proteins were printed in an arrangement composed of 4 × 12 subarrays equally spaced in vertical and horizontal directions (Fig. 1a). Each subarray included 16 × 16 spots, composed of 48 control spots (C), 80 human proteins (H), and 128 blanks (B) (Fig. 1c). The control proteins (C) were composed of 14 positive control spots and 34 negative control spots. The former includes four spots of an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2), six spots of a concentration gradient of a biotinlyated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 8; columns 3–8), and four spots of a concentration gradient of V5 protein (row 8; columns 13–16). The latter includes six spots of

Table 1
Primers utilized for PCR-based cloning and site-directed mutagenesis

Genes	Proteins (amino acid residues)	GenBank accession no.	Sense primers	Antisense primers	Cloning vector
YWHAE	14-3-3ε Isoform (2-255)	NM_006761	5′gatgatcgagaggatctggtgtac3′	5′ctgatttcgtcttccacgtcctg3′	pSecTag/FRT/V5-His TOPO
EAP30	EAP30 subunit of ELL complex (2-258)	NM_007241	5′cacgccgcgggtggagctggc3′	5′tcagggaggcttctctggccic3′	pcDNA4/HisMax-TOPO
DDX54	Dead box polypeptide 54 (2-881)	NM_024072	5′gcggccgacaaggccccggcggct3′	5′tcacatcctcttccgcatcttgcc3′	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-402, full length)	NM_003149	5′atccctccgagcagcccccgcgag3′	5′tcagatgttttctagtacatcaag3′	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-402 with S172A; SMT)	NM_003149	5′gtttcggcgttactacgcctcccccttgctcattc3′	5′gaatgagcaaggggaggcgtagtaacgccgaaac3′	pcDNA4/HisMax-TOPO modified by site-directed mutagenesis
STAC	src homology three and cysteine rich domain (2-402 with S172A and S173A; DMT)	NM_003149	5′cggcgttactacgccgccccttgctcattcat3′	5′atgaatgcgcaaggggcggcgtagtaacgccg3′	pcDNA4/HisMax-TOPO modified by site-directed mutagenesis
STAC	src homology three and cysteine rich domain (2-233, N-terminal half; NTF)	NM_003149	5′atccctccgagcagcccccgcgag3′	5′tcaaagatctgaagtagaggttct3′	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (234-402, C-terminal half; CTF)	NM_003149	5′gtggaggttcctgaggaagccaat3′	5′tcagccacctgatgcagaccagc3′	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-164, truncated form A; TR-A)	NM_003149	5′atccctccgagcagcccccgcgag3′	5′tcatggcagcttgcccatgcaccg3′	pcDNA4/HisMax-TOPO
STAC	src homology three and cysteine rich domain (2-105, truncated form B; TR-B)	NM_003149	5′atccctccgagcagcccccgcgag3′	5′tcagccacctggatgcagaccagc3′	pcDNA4/HisMax-TOPO

The PCR product was cloned into a vector pSecTag/FRT/V5-His TOPO to express a fusion protein with a V5 tag or into a vector pcDNA4/HisMax-TOPO to express a fusion protein with a Xpress tag in HEK293 cells.

a concentration gradient of bovine serum albumin (BSA) (row 1; columns 3–8), four spots of a concentration gradient of a rabbit anti-GST antibody (row 1; columns 9–12), four spots of a concentration gradient of calmodulin (row 1; columns 13–16), 16 spots of a concentration gradient of GST (row 2; columns 1–16), two spots of buffer only (row 8; columns 9,10), and two spots of an anti-biotin antibody (row 8; columns 11,12). The complete list of 1752 target proteins immobilized on the microarary is shown in Supplementary Table 1 online.

Non-specific binding was blocked by incubating the microarray for 90 min in the PBST blocking buffer composed of 1% BSA and 0.1% Tween 20 in phosphate-buffered saline (PBS). Then, it was incubated for 30 min at 4 °C with the probe described above at a concentration of 50 μg/ml in the probing buffer composed of 1% BSA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.05% Triton X-100, and 5% glycerol in PBS. The array was washed three times with the probing buffer, followed by incubation for 30 min at 4 °C with mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (Invitrogen) at a concentration of 260 ng/ml in the probing buffer. The array was washed three times with the probing buffer, and then scanned by the GenePix 4200A scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm. The data were analyzed by using the ProtoArray Prospector software v2.0 (Invitrogen) following acquisition of the microarray lot-specific information online, including inter-lot variations in protein concentrations (http://www.invitrogen.com/protoarray). According to the default setting of the software, the spots showing the background-subtracted signal intensity value greater than the median plus three standard deviations of all the fluorescence intensities were considered as having a significant binding. The Z-score, an indicator for statistical evaluation of binding specificity, was calculated as the background-subtracted signal intensity value of the target protein minus the average of the background-subtracted signal intensity value from the negative control distribution, divided by the standard deviation of the negative control distribution. All the procedure described above could be accomplished within 5 h. The 14-3-3-binding consensus motif mode I (RSXpSXP) sequence located in target proteins was surveyed by the Scansite Motif Scanner, which assesses the probability of a site matching the candidate motif under high, medium, or low stringent conditions (Obenauer et al., 2003). The information on known 14-3-3 interactors was obtained from Biomolecular Interaction Network Database (BIND; http://www.bind.ca) and PubMed database search.

2.3. Transient expression of 14-3-3-binding proteins in HEK293 cells

To verify the results of microarray analysis, the ORF of the genes encoding EAP30 subunit of ELL complex (EAP30), dead box polypeptide 54 (DDX54), and src homology three (SH3) and cysteine rich domain (STAC) were amplified by PCR using PfuTurbo DNA polymerase and the primer sets listed in Table 1. They were then cloned into a mammalian expression vector pcDNA4/HisMax-TOPO (Invitrogen) to produce a fusion protein with an N-terminal Xpress tag. To express the STAC mutant