Role of 14-3-3γ in FE65-dependent Gene Transactivation Mediated by the Amyloid β-Protein Precursor Cytoplasmic Fragment*[^5]

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The amyloid β-protein precursor intracellular domain fragment (AICD) is generated from amyloid β-protein precursor by consecutive cleavages. AICD is thought to activate FE65-dependent gene expression, but the molecular mechanism remains under consideration. We found that dimeric 14-3-3γ bound both AICD and FE65 simultaneously, and this binding facilitated FE65-dependent gene transactivation by enhancing the association of AICD with FE65. 14-3-3γ bound to the 666VTPEER[^72] motif of AICD and, most interestingly, the phosphorylation of AICD at Thr-668 in this motif inhibited the interaction with 14-3-3γ and blocked gene transactivation. 14-3-3γ required a sequence between the WW domain and the first phosphotyrosine interaction domain of FE65 for association with FE65. Deletion of this region blocked 14-3-3γ binding to FE65 and suppressed AICD-mediated FE65-dependent gene transactivation, although the deletion mutant FE65 was still able to bind Tip60, a histone acetyltransferase that forms a complex with FE65 in the nucleus. Taken together, these data demonstrate that 14-3-3γ facilitates FE65-dependent gene transactivation by forming a complex containing AICD and FE65, and phosphorylation of AICD down-regulates FE65-dependent gene transactivation through the dissociation of 14-3-3γ and/or FE65 from AICD. Our findings suggest that multiple interactions of AICD with FE65 and 14-3-3γ modulate FE65-dependent gene transactivation.

Amyloid β-protein precursor (APP)[^1] is thought to be a causative factor of Alzheimer disease (AD), and it is hypothesized that generation of β-amyloid from APP and its aggregation are central to the pathogenesis of AD (1). APP is cleaved consecutively, first at the extracellular juxtamembrane region by α- or β-secretase and second at the intramembrane region by γ-secretase (2). Following the first cleavage, sAPPα or sAPPβ (large extracellular amino-terminal domains truncated at the α and β sites, respectively) is secreted, and then following the second cleavage, p3 or β-amyloid peptides are secreted, together with release of the cytoplasmic fragment into the cytoplasm. The metabolism of APP resembles that of Notch, a cell surface receptor essential for the commitment to cell differentiation (3, 4). Many type I membrane proteins, including CD44, ErbB4, neuregulin-1, and alcaldein, have been found recently to be cleaved first at an extracellular juxtamembrane region and subsequently at an intramembrane region by γ-secretase (5–8). Cleavage of these type I membrane proteins generates and releases their cytoplasmic domain, which is thought to play an important role, together with other transcriptional regulatory factors, in gene transactivation (9, 10).

The cytoplasmic domain fragment derived from APP (AICD) activates the transcriptions of a reporter gene in the presence of the neuron-specific adaptor protein FE65 (11, 12) and the histone acetyltransferase Tip60, but the molecular mechanisms mediating activation of transcription are not clear. Several hypotheses have been presented and remain under consideration (13–19). The AICD contains several functionally important motifs. The 654^YTSI656 motif (human APP695 isoform numbering) is the basolateral sorting signal of APP in Madin-Darby canine kidney epithelial cells (20). The 666^VTPEER[^72] motif contains the phosphorylation site Thr-668 and controls the stability of the overall structure of AICD (21–23). The 681^GYENPTY[^67] motif regulates metabolism and transport of APP through interactions with various adaptor proteins such as X11s, FE65s, JIPs, and AIDA-1 (24–31). Thus, the 681^GYENPTY[^67] motif is thought to be essential for AICD function in FE65-dependent gene transactivation. However, it remains uncertain whether other motifs regulate AICD function and whether other protein factors play an important role in the regulation of AICD-mediated FE65-dependent gene transactivation. To reveal the role of AICD in FE65-dependent gene transactivation may contribute to understanding the physiological roles of APP and the pathogenesis of AD. In the present study, we found that the 666^VTPEER[^72] motif regulates AICD function in FE65-dependent gene transactivation through the phosphorylation of Thr-668 and interactions with the 14-3-3γ protein.

Mammalian 14-3-3 proteins are ubiquitously expressed gene family with seven distinct isoforms that are involved in various signal transduction pathways (32). 14-3-3γ is one of the most abundant isoforms in brain, skeletal muscle, and heart (33, 34). We found that association of the 14-3-3γ dimer with both nonphosphorylated AICD and FE65 facilitates gene expression, whereas the phosphorylation of AICD interferes with 14-3-3γ binding and down-regulates FE65-dependent gene transactivation. These findings, taken together with our previous report that the phosphorylation of AICD suppresses its interaction with FE65 (21),

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[^9]: The abbreviations used are: APP, amyloid β-protein precursor; AICD, APP intracellular domain fragment; AD, Alzheimer disease; Gal8BD, Gal4 DNA-binding domain; IP, immunoprecipitate; N2a, Neuro-2a; WT, wild type; PI, phosphotyrosine interaction; JNK, c-Jun NH2-terminal kinase; JIP, JNK-interacting protein; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; RNAI, RNA interference; S-rich, Ser-rich.
suggest that the AICD \(^{66-71}\)VTPEER\(^{67-72}\) motif is an important regulatory region for AICD-mediated FE65-dependent gene transactivation.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—cDNAs encoding the six human 14-3-3 isoforms used in this study, \(\beta\) (GenBank\(^{TM}\) accession number 21328444), \(\epsilon\) (GenBank\(^{TM}\) accession number 21328449), \(\gamma\) (GenBank\(^{TM}\) accession number 21646100), \(\eta\) (GenBank\(^{TM}\) accession number 21646102), \(\theta\) (GenBank\(^{TM}\) accession number 21646103), and \(\zeta\) (GenBank\(^{TM}\) accession number 21735623), were amplified by PCR. The entire coding sequence was inserted into the Nhel/Xhol sites of pcDNA3.1-N-FLAG to generate pcDNA3.1-14-3-3. pcDNA3.1-FLAG-FE65 into the NheI/NotI sites of pcDNA3.1-N-FLAG to generate pcDNA3.1-FLAG-FE65, pcDNA3.1-EGFP-AICD, respectively. The cDNA encoding amino-terminal EGFP sequence to produce pcDNA3.1-EGFP-Tip60 amplified by PCR and subcloned into the pcDNA3.1 vector with an amino-terminal 3Myc tag to produce pcDNA3.1–3Myc-14-3-3. pcDNA3.1-APP-T668D, substitution of Asp for Thr-668 was prepared by PCR using pcDNA3.1-APP or pcDNA3.1-APP-T668D as template and cloned into the NheI/XhoI sites of the pcDNA3.1 vector. The cDNA encoding deletion constructs pcDNA3.1-14-3-3-N160 (lacking the carboxyl-terminal 160 amino acids of 14-3-3) was inserted into the Nhel/Xhol sites of pcDNA3.1 vector with an amino-terminal 3Myc tag to produce pcDNA3.1–3Myc-14-3-3-N160. cDNA encoding a typical nuclear localization signal (NLS, the NH2-DPKKKRKV-COOH sequence was used) tandemly repeated three times was inserted into the NheI/XhoI sites of pcDNA3.1 vector with an amino-terminal 3Myc tag to produce pcDNA3.1–3Myc-14-3-3.

Plasmids encoding proteins fused with green fluorescent protein variants were prepared as follows. cDNA encoding AICD(C44) was cloned into the Nhel/Xhol sites of pcDNA3.1-N-FLAG to generate pcDNA3.1-14-3-3-N-FLAG. cDNA encoding human Tip60 (GenBank\(^{TM}\) accession number 36287048) and 87 amino acids of 14-3-3 was inserted into the NheI/XhoI sites of pcDNA3.1-N-FLAG to generate pcDNA3.1-14-3-3-N-FLAG. The cDNA encoding human Tip60 (GenBank\(^{TM}\) accession number 36287048) and AICD (carboxyl-terminal 44 amino acids of human APP695, C44) were cloned into the NheI/XhoI sites of pcDNA3.1 vector with an amino-terminal 3Myc tag to produce pcDNA3.1–3Myc-14-3-3-N-FLAG. pcDNA3.1-APP-T668D mutant mouse congenic to C57BL/6 (12-week-old males) and APP-T668D were homogenized on ice in a 4-fold volume of buffer (10 mM HEPES (pH 7.4), 0.32 M sucrose, 5 \(\mu\)g/ml chymotrypsin, 5 \(\mu\)g/ml leupeptin, and 5 \(\mu\)g/ml pepstatin A) containing bovine serum albumin (1 mg/ml). The GST fusion protein was recovered by pull down with glutathione beads, and the precipitates were analyzed by Western blotting with the indicated antibodies.

**Coimmunoprecipitation and Western Blot Analysis**—Neuro-2a (N2a) cells (4.5 \(\times\) 10\(^{5}\) cells) were transfected with the indicated plasmids, as described previously (37). The cells were cultured for 24 h and lysed in HBS-N buffer on ice. After centrifugation (15,000 \(\times\) g for 10 min), antibody was added to the supernatant of the lysate. The immunoprecipitates were subjected to Western blot analysis using specific antibodies. Brains of wild type mouse (C57BL/6, 8-week-old males) and APP-T668D mutant mouse congenic to C57BL/6 (12-week-old males) were homogenized in ice on a 4-fold volume of buffer (10 mM HEPES (pH 7.4), 0.32 M sucrose, 5 \(\mu\)g/ml chymotrypsin, 5 \(\mu\)g/ml leupeptin, and 5 \(\mu\)g/ml pepstatin) by 10 strokes of a loose-fitting Dounce homogenizer. The homogenates were centrifuged at 1,000 \(\times\) g for 7 min, and the supernatants were then further centrifuged at 2,000 \(\times\) g for 30 min. The resulting precipitates were lysed for 1 h on ice in HBS-N buffer. After centrifugation at 10,000 \(\times\) g for 1 h at 4 °C, 0.3 \(\mu\)l of UT116 antigen serum or 1 \(\mu\)g of nonimmune rabbit IgG was added to the supernatant of the HBS-N lysate together with protein G-Sepharose. The immunoprecipitate was eluted by addition of the UT116 antigen peptide (1.5 mg/ml), and the eluate was analyzed by Western blotting with specific antibodies.

**Gene Transactivation Assays**—The cDNA of APP695 was inserted into the Nhel/NotI sites of pBIND-C-Gal4BD to construct pBIND-APP-Gal4BD, which has a Gal4 DNA-binding domain inserted into the 3’ end of APP. To construct pBIND-AICD(C44)-Gal4BD, pBIND-C30-Gal4BD, pBIND-C20-Gal4BD, pBIND-C49-Gal4BD, and pBIND-AICD(T668D)-Gal4BD, the cDNAs that encode the respective amino acids at positions 652–695, 666–695, and 676–695 of APP695 were generated by PCR using pcDNA3.1-APP or pcDNA3.1-APP-T668D as templates and cloned into the Nhel/Xhol sites of pBIND-C-Gal4BD. To construct pBIND-S-rich-C20-Gal4BD, the cDNA that encodes amino acids 191–220 of XB51\(_\alpha\) (37) was generated by PCR using pcDNA3.1-FLAG-XB51\(_\alpha\) as a template and inserted into the 5’ end of pBIND-C20-Gal4BD. The cDNA of Tip60 was recloned into the Nhel/Xhol sites of pBIND-N-Gal4BD to construct pBIND-N-Gal4BD-Tip60, which had a Gal4 DNA-binding domain inserted into the 5’ end of Tip60. N2a cells (1.8 \(\times\) 10\(^{5}\) cells) plated in 96-multiwell plates were transiently transfected, using LipofectAMINE2000 (Invitrogen), with the indicated amounts of pBIND plasmids and pG5luc (Promega), a reporter luciferase gene having a Gal4 DNA-binding sequence on its promoter, in the presence of various cDNAs inserted into the pcDNA3.1 vector. To standardize the overall amount of plasmid used, empty vector was added. The transcriptional activity of the reporter gene was analyzed by using the dual luciferase assay system (Promega). All of the combinations were tested in quadruplicate, and the luciferase activity was normalized according to the manufacturer’s protocol to eliminate the effect of transfection efficiency differences.

**In Vitro Binding Assay**—The cDNA encoding 14-3-3 was recloned into pGEX-4T-1 (Amersham Biosciences) to produce a GST-14-3-3 fusion protein. The synthetic AICD(C47) peptide (8.3 pmol) with or without phosphate at Thr-668 (21) was mixed with 3 pmol of GST-14-3-3 or 6 pmol of GST alone in HBS-N buffer (10 mM HEPES (pH 7.6), 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 5 \(\mu\)g/ml chymotrypsin, 5 \(\mu\)g/ml leupeptin, and 5 \(\mu\)g/ml pepstatin A) containing bovine serum albumin (1 mg/ml). The GST fusion protein was recovered by pull down with glutathione beads, and the precipitates were analyzed by Western blotting with the indicated antibodies.
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200 ng of pSuper14-3-3\(\gamma\) or pSuper14-3-3\(\varepsilon\) in Lipofectamine 2000, and the expression level of endogenous 14-3-3\(\gamma\) was examined by Western blot analysis with the UT116 antibody.

Intracellular Localization of Fluorescent Protein Fusion Proteins—N2a cells were transfected with the indicated plasmids encoding GFP fusion proteins using Lipofectamine 2000 (Invitrogen). The cells were cultured for 24 h and viewed using a confocal laser scanning microscope (LSM510, Carl Zeiss).

RESULTS

Association of 14-3-3\(\gamma\) with Nonphosphorylated APP and AICD—Comprehensive screening of a human brain cDNA library with the yeast two-hybrid system using the cytoplasmic domain of APP as bait resulted in isolation of cDNA clones encoding a part of the 14-3-3\(\gamma\) protein (data not shown). Thus, we prepared complete cDNA clones encoding 14-3-3 isoforms evolutionarily close to 14-3-3\(\gamma\), except for 14-3-3\(\varepsilon\) (Fig. 1a), and we examined their association with APP by coimmunoprecipitation assays (Fig. 1b). N2a cells expressing amino-terminal FLAG-tagged 14-3-3\(\beta\), \(-\varepsilon\), \(-\gamma\), \(-\eta\), or \(-\theta\) isoforms and APP were lysed and subjected to coimmunoprecipitation assays with anti-FLAG antibody. The immunoprecipitates (IP) and cell lysates (lysate) were analyzed by Western blotting with anti-FLAG and anti-APP antibodies. APP was recovered in FLAG-14-3-3\(\gamma\) and FLAG-14-3-3\(\eta\) immunoprecipitates, but 14-3-3\(\beta\), 14-3-3\(\varepsilon\), and 14-3-3\(\varepsilon\) failed to coimmunoprecipitate APP. Control antibody from nonimmunized animals did not recover either 14-3-3\(\gamma\) or APP, indicating that the intracellular interaction between 14-3-3\(\gamma\) and APP is specific (Fig. 1c). Because FLAG-14-3-3\(\gamma\) binding to APP was stronger than that of FLAG-14-3-3\(\eta\) (Fig. 1b) and both 14-3-3\(\gamma\) and 14-3-3\(\varepsilon\) have been associated with AD (38), we focused our analysis on the role of 14-3-3\(\gamma\) in APP function. Among the postulated APP functions, such as providing a cargo receptor on transport vesicles (27, 39), or mediating neurite extension on differentiating neuronal cells (40), gene transactivation mediated by AICD may be an important function after APP processing (11–19). AICD and FE65 are thought to associate with several proteins to stimulate gene transactivation. Thus, we analyzed the interaction of AICD with 14-3-3\(\gamma\). A GST-14-3-3\(\gamma\) fusion protein was incubated with a synthetic AICD peptide (649–695 of APP695, C47), with or without phosphorylation of the Thr-668 residue, which is a major phosphorylation site in brain and cultured cells (41–43). Many 14-3-3 proteins bind to phosphorylated target proteins and regulate their function and/or structure (44). The GST-14-3-3\(\gamma\), however, bound the nonphosphorylated AICD peptide but did not bind AICD peptide phosphorylated at Thr-668 (Fig. 1d). The physiological significance of this interaction was confirmed using mouse brain samples (Fig. 1e). The 14-3-3\(\gamma\)-specific antibody UT116 (supplemental Fig. 1) was used to immunoprecipitate the protein from mouse brain lysates, and the immunoprecipitate was analyzed by Western blotting for 14-3-3\(\gamma\), APP, APP phosphorylated at Thr-668, and synaptotagmin 1. The 14-3-3\(\gamma\) antibody coimmunoprecipitated APP but not Thr-668-phosphorylated APP and synaptotagmin 1, indicating that the association of 14-3-3\(\gamma\) with APP occurs in brain and the phosphorylation of APP interferes with the interaction (Fig. 1e, left). Because we have established a mutant mouse line, in which the phosphorylation site Thr-668 in APP is altered to Asp-668 and Ala-668,4 we examined the association of 14-3-3\(\gamma\) with APP in the T668D mutant mouse brain. 14-3-3\(\gamma\) was immunoprecipitated from a mutant mouse brain lysate using the 14-3-3\(\gamma\)-specific antibody UT116, and the immunoprecipitate did not contain the T668D APP (Fig. 1e, right). AICD was not detected in these assays, and this may

4 K. Seki, S. Takeda, Y. Sano, T. Nakaya, E. Kawaguchi, T. Suzuki, and S. Itohara, submitted for publication.
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be due to the small amounts or very rapid catabolism of AICD in brain.

We have reported previously that phosphorylation of Thr-668 in the 667VTPEER672 motif suppressed the interaction of AICD with FE65, which recognizes the 681GYENPTY687 motif, because the phosphorylation of Thr-668 affects the amino-terminal helix-capping-box structure composed of 667VTPEER672 and the helical state of the following amino acid sequence that includes the 681GYENPTY687 FE65-binding motif (21–23). To confirm that 14-3-3γ binds to APP through the 667VTPEER672 motif, we examined 14-3-3γ binding to other motifs. 14-3-3γ failed to bind to APP when a region containing the 667VTPEER672 motif was deleted, but not when regions containing the 653YSIT658 or 681GYENPTY687 motifs were deleted (supplemental Fig. 3). Therefore, our observations confirm that 14-3-3γ binds to the 667VTPEER672 motif, that Thr-668 in this motif is important for the 14-3-3γ-APP interaction, and that the phosphorylation of Thr-668 suppresses the association of APP and AICD with 14-3-3γ.

Association of 14-3-3γ with AICD Facilitates FE65-dependent Gene Transactivation—Because 14-3-3γ associated with AICD, we explored the effect of 14-3-3γ on FE65-dependent gene transactivation mediated by AICD. pBIND-AICD(C44)-Gal4BD and pG5luc were transfected in N2a cells expressing different isoforms of 14-3-3, in the presence (+) or absence (−) of FE65. The AICD-Gal4BD fusion protein showed FE65-dependent gene transactivation (Fig. 2a, compare lane 2 with lane 1), as reported previously (11). Coexpression of 14-3-3γ or 14-3-3β enhanced this activity to 2–2.5-fold, respectively (Fig. 2a, compare lanes 5 and 6 with lane 2), which is consistent with the affinity of their binding to APP (Fig. 1b).

AICD(C49), a product of the e-site cleavage of APP (45), is the form predominantly observed in cells (46). Similar to AICD(C44) (Fig. 1b and Fig. 2a), AICD(C49) mediated FE65-dependent gene transactivation, and transactivation was enhanced by 14-3-3γ (supplemental Fig. 4). Transactivation by other 14-3-3 isoforms was absent or minimal (Fig. 2a, compare lanes 3, 4, 7, and 8 with lane 2). The effect of 14-3-3γ addition was confirmed using APP instead of AICD (Fig. 2b, and also see Fig. 3a). N2a cells expressing APP-Gal4BD with pG5luc in the presence (+) or absence (−) of FE65 and 14-3-3γ were examined for gene transactivation mediated by AICD generated endogenously from APP. 14-3-3γ expression increased APP-mediated FE65-dependent gene transactivation by 1.5–2-fold. Furthermore, we performed a knockdown study of 14-3-3γ expression using RNA interference (RNAi) because 14-3-3γ is ubiquitously expressed in many types of cells (33), and the endogenously expressed 14-3-3γ may contribute to FE65-dependent gene transactivation (Fig. 2c). Cells in which RNAi to 14-3-3γ almost completely abrogated 14-3-3γ expression (see panel in Fig. 2c), retained ~50% of the AICD-mediated FE65-dependent gene transactivation (right columns). RNAi to 14-3-3γ and EGFP had no effect (Fig. 2c). These results suggest that many studies (11–19) assaying AICD-mediated FE65-dependent gene transactivation have included the effect facilitated by endogenous 14-3-3γ. A previous report indicated that Tip60 accelerates FE65-dependent gene transactivation of APP (11), and we therefore assayed the effect of Tip60 in the presence of 14-3-3γ (Fig. 2d). In N2a cells expressing Gal4BD-Tip60, 14-3-3γ facilitated APP-mediated FE65-dependent gene transactivation by 2-fold. Taken together, these data indicate that APP-mediated FE65-dependent gene transactivation involves 14-3-3γ in addition to Tip60 to express higher activity.

The 667VTPEER672 Motif Containing the Thr-668 Phosphorylation Site of AICD Is Important for FE65-dependent Gene Transactivation Activity—To determine the region of AICD-mediated up-regulation of gene transactivation activity by 14-3-3γ, we prepared various AICD-Gal4BD fusion proteins (Fig. 3a). The C30-Gal4BD contains both the
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FIGURE 3. Regulation of 14-3-3γ interactions with AICD and effects on FE65-dependent gene transactivation. a, schematic structure of APP-Gal4BD and AICD-Gal4BD constructs. S-rich indicates the serine-rich sequence (amino acids 191–220) of the XB51α protein, which interacts with 14-3-3γ (see supplemental Fig. 5). Gal4BD indicates the DNA-binding domain of Gal4. b, effect of 14-3-3γ on AICD-Gal4BD-mediated reporter gene transactivation. N2a cells (1.8 × 10^5 cells) were transfected with pG5 luc (40 ng) and 4 ng of pBIND-Gal4BD, pBIND-C30-Gal4BD, pBIND-C20-Gal4BD, pBIND-S-rich-C20-Gal4BD, or pBIND-S-rich-Gal4BD, in the presence (+) or absence (−) of pcDNA3.1-FE65 (20 ng). The transactivation values are the ratios of transactivation by the indicated plasmid combinations to that of pBIND alone (Gal4BD, left of the left column set). To standardize the plasmid amounts, empty vector (−) was added to yield a total of 64 ng of plasmid. The error bars indicate the standard deviation. c, interaction of 14-3-3γ with APP (WT) and APP carrying a Thr-668→Asp mutation (T668D). N2a cells (4.5 × 10^5 cells) were transiently cotransfected with 0.5 ng of pcDNA3.1-14-3-3γ (amino acids 191–220) can associate with 14-3-3γ. Moreover, we have confirmed that C20 and S-rich-C20 bind to FE65, but these interactions are weak compared with C30 binding (data not shown). These studies clearly showed that association of 14-3-3γ with AICD facilitated FE65-dependent gene transactivation. These observations supported our hypothesis that AICD-mediated FE65-dependent gene transactivation requires binding of 14-3-3γ to the 667VTPEER672 motif to express higher activity.

Moreover, we have confirmed that C20 and S-rich-C20 bind to FE65, but these interactions are weak compared with C30 binding (data not shown). These studies clearly showed that association of 14-3-3γ with AICD facilitated FE65-dependent gene transactivation. These observations supported our hypothesis that AICD-mediated FE65-dependent gene transactivation requires binding of 14-3-3γ to the 667VTPEER672 motif to express higher activity.

Phosphorylation of Thr-668 in the 667VTPEER672 motif blocked the interaction of AICD and APP with 14-3-3γ (Fig. 1, d and e). Thus, we examined the relationship between Thr-668 and FE65-dependent gene transactivation activity. When lysates of N2a cells expressing both FLAG-14-3-3γ and the wild type or T668D mutant of APP, in which Thr-668 is altered to Asp668, were analyzed by coimmunoprecipitation, the anti-FLAG antibody failed to recover the T668D mutant APP (Fig. 3c). The T668D AICD-Gal4BD also lost the activity to facilitate FE65-dependent gene transactivation by 14-3-3γ (Fig. 3d, right columns). In this study, the T668D mutation decreased its activity slightly in the

A. Sumioka, unpublished observations.
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absence of 14-3-3γ (Fig. 3d, middle columns) because the mutation at the Thr-668 residue causes a conformational change in the carboxyterminal region containing the FE65-binding motif (21). We induced the phosphorylation of AICD in cells by expressing a JNKK2-JNK1 fusion protein, which is a constitutively active Jun kinase (Fig. 3f) (35).

Because recent findings reported that JNK could phosphorylate APP at the Thr-668 residue (36), a conformational change in the carboxyterminal region containing the FE65-binding motif (21).

Expression of wild type JNKK2-JNK1 (KM) which carries a Lys419Met mutation on JNKK2 and has no kinase activity (35) was coexpressed, no phosphorylation of AICD-EGFP was observed. Using this system the effect of AICD phosphorylation on FE65-dependent gene transactivation was measured (Fig. 3f). In the presence of mutant JNKK2-JNK1 (KM), 14-3-3γ facilitated AICD-mediated FE65-dependent gene transactivation. However, the expression of wild type JNKK2-JNK1 blocked this effect (Fig. 3f, right columns). These observations indicate that the phosphorylation of Thr-668 in the YVPEER motif inhibits the 14-3-3γ enhancement of AICD-mediated FE65-dependent gene transactivation, which is due to dissociation of 14-3-3γ from AICD.

Dimerization of 14-3-3γ and Association with AICD—The 14-3-3 proteins form dimers (48), and we therefore examined the dimerization

expression of the JNKK2-JNK1 fusion protein is expected to increase the phosphorylation of AICD at Thr-668 in cells. When AICD-EGFP was expressed in N2a cells with wild type JNKK2-JNK1 (WT), phosphorylated AICD-EGFP (pAICD-EGFP) appeared regardless of the coexpression of FE65 and 14-3-3γ (Fig. 3e). When a mutant JNKK2-JNK1 (KM) which carries a Lys419Met mutation on JNKK2 and has no kinase activity (35) was coexpressed, no phosphorylation of AICD-EGFP was observed. Using this system the effect of AICD phosphorylation on FE65-dependent gene transactivation was measured (Fig. 3f). In the presence of mutant JNKK2-JNK1 (KM), 14-3-3γ facilitated AICD-mediated FE65-dependent gene transactivation. However, the expression of wild type JNKK2-JNK1 blocked this effect (Fig. 3f, right columns). These observations indicate that the phosphorylation of Thr-668 in the YVPEER motif inhibits the 14-3-3γ enhancement of AICD-mediated FE65-dependent gene transactivation, which is due to dissociation of 14-3-3γ from AICD.

Dimerization of 14-3-3γ and Association with AICD—The 14-3-3 proteins form dimers (48), and we therefore examined the dimerization

FIGURE 4. Functional characterization of 14-3-3γ dimer formation, interaction with APP and FE65-dependent gene transactivation. a, schematic diagram of 14-3-3γ constructs used and summary of their functions. Amino-terminal FLAG-tagged 14-3-3γ (pcDNA3.1-FLAG-14-3-3γ), FLAG-tagged 14-3-3γ lacking the amino-terminal 70 amino acids (pcDNA3.1-FLAG-14-3-3γ71C), and FLAG-tagged 14-3-3γ lacking the carboxy-terminal 87 amino acids (pcDNA3.1-FLAG-14-3-3γN160) are shown (left), along with their functional activity, active (+) or inactive (−) (right). b, dimer formation of 14-3-3γ. N2a cells (4.5 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1 vector (−), pcDNA3.1-FLAG-14-3-3γ WT, pcDNA3.1-FLAG-14-3-3γ71C (71C), and pcDNA3.1-FLAG-14-3-3γN160 (N160), with (+) or without (−) pcDNA3.1-Myc-14-3-3γ (0.5 μg). The cells were lysed, and the 14-3-3γ proteins were recovered by immunoprecipitation with anti-FLAG antibody M2. Cell lysates (Lysate) and the immunoprecipitates (IP) were analyzed by Western blotting with M2 and anti-Myc antibody. c, interaction of APP with 14-3-3γ proteins. N2a cells (4.5 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1-FLAG−71C protein. The asterisk indicates a nonspecific band, which overlapped with the 14-3-3γ band (48), along with their functional activity, active (+) or inactive (−) (right). Dimer formation of 14-3-3γ was evaluated by the formation of dimers (48), and we therefore examined the dimerization

expression of wild type JNKK2-JNK1 (KM) which carries a Lys419Met mutation on JNKK2 and has no kinase activity (35) was coexpressed, no phosphorylation of AICD-EGFP was observed. Using this system the effect of AICD phosphorylation on FE65-dependent gene transactivation was measured (Fig. 3f). In the presence of mutant JNKK2-JNK1 (KM), 14-3-3γ facilitated AICD-mediated FE65-dependent gene transactivation. However, the expression of wild type JNKK2-JNK1 blocked this effect (Fig. 3f, right columns). These observations indicate that the phosphorylation of Thr-668 in the YVPEER motif inhibits the 14-3-3γ enhancement of AICD-mediated FE65-dependent gene transactivation, which is due to dissociation of 14-3-3γ from AICD.

Dimerization of 14-3-3γ and Association with AICD—The 14-3-3 proteins form dimers (48), and we therefore examined the dimerization
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of 14-3-3\(\gamma\) using FLAG- or 3Myc-tagged amino-terminal (71C) and carboxyl-terminal (N160) deleted constructs with the full-length 14-3-3\(\gamma\)(WT) (Fig. 4a). When FLAG-14-3-3\(\gamma\)(WT) and 3Myc-14-3-3\(\gamma\) were coexpressed in cells and coimmunoprecipitated with an anti-FLAG antibody, both 3Myc-14-3-3\(\gamma\) and FLAG-14-3-3\(\gamma\) (WT) were found in the immunoprecipitate, indicating that 14-3-3\(\gamma\) tends to form dimers (Fig. 4b), although we cannot rule out the possibility that 14-3-3\(\gamma\) also forms oligomers. When the FLAG-N160 and 3Myc-14-3-3\(\gamma\) were coimmunoprecipitated with the anti-FLAG antibody, the immunoprecipitate contained 3Myc-14-3-3\(\gamma\), showing that N160 retains the ability to dimerize. However, FLAG-71C failed to coimmunoprecipitate 3Myc-14-3-3\(\gamma\) in identical assays (Fig. 4b). These results indicate that 14-3-3\(\gamma\) dimerization and/or oligomerization requires the amino-terminal region composed of 70 amino acids. By using these same constructs, we examined APP binding (Fig. 4c). FLAG-14-3-3\(\gamma\)(WT), 71C, or N160 were expressed in cells with APP, and coimmunoprecipitation was performed with the anti-FLAG antibody. FLAG-71C coimmunoprecipitated APP as did FLAG-14-3-3\(\gamma\), but FLAG-N160 could not coimmunoprecipitate APP. The nonimmunized control mouse IgG did not immunoprecipitate FLAG proteins or APP (data not shown). These results indicated that 14-3-3\(\gamma\) binds to APP via its carboxyl-terminal half. These 14-3-3\(\gamma\) mutants were also examined for their ability to facilitate AICD-mediated FE65-dependent gene transactivation (Fig. 4d). Expression of wild type 14-3-3\(\gamma\)(WT) resulted in a 2-fold facilitation of transactivation compared with cells lacking 14-3-3\(\gamma\) (−) (Fig. 4d, compare column 3 with column 2), as has already been shown in Fig. 2a, whereas neither 71C nor N160 enhanced transactivation. The 71C and N160 proteins caused a slight inhibition of FE65-dependent gene transactivation (Fig. 4d, compare columns 4 and 5 with column 2), perhaps by acting as dominant-negative interactors with endogenous 14-3-3\(\gamma\). These observations suggested that both the dimer formation of 14-3-3\(\gamma\) and the binding ability to AICD are essential for facilitation of AICD-mediated FE65-dependent gene transactivation. A table summarizing the activity of the 14-3-3\(\gamma\) proteins is shown in Fig. 4a.

14-3-3\(\gamma\) Associates with FE65 at a Region Differing from the Region Interacting with Tip60—A previous study reported that deletion of the first phosphotyrosine interaction (P11) domain of FE65 blocked AICD-mediated FE65-dependent gene transactivation (11). However, the sequence (amino acids 314–470 of rat FE65) removed by Cao and Südhof (11) did not include the carboxyl-terminal sequence of the P11 domain and includes extra amino acids at the amino terminus compared with the full-length P11 domain (360–510 of rat FE65). Because our preliminary studies showed that FE65 proteins with a complete deletion of P11 (370–509 of human FE65, which corresponds to amino acids 360–510 of rat FE65) retained AICD-mediated FE65-dependent gene transactivation (supplemental Fig. 6), we focused on the amino acid sequence adjacent to the amino terminus of the P11 domain as an important region for transactivation. We explored whether this region, composed of amino acids 313–356 of FE65, is needed for binding 14-3-3\(\gamma\) by using coimmunoprecipitation assays (Fig. 5, a and b). FLAG-FE65 (WT) or FLAG-FE65\(\Delta\)1 (Δ1 lacks amino acids 313–356) was expressed in cells with 3Myc-14-3-3\(\gamma\); FE65 was immunoprecipitated with the anti-FLAG antibody, and the immunoprecipitates were analyzed by Western blotting. The 3Myc-14-3-3\(\gamma\) was not recovered with FLAG-FE65\(\Delta\)1, indicating that amino acids 313–356 of human FE65 are needed for binding 14-3-3\(\gamma\). To exclude the possibility that FE65 may associate with 14-3-3\(\gamma\) through binding to endogenous APP and/or AICD, the interaction between FLAG-FE65\(\Delta\)P12 and 3Myc-14-3-3\(\gamma\) was examined (supplemental Fig. 7a). FE65\(\Delta\)P12, which cannot interact with APP or AICD, bound to 14-3-3\(\gamma\) in coimmunoprecipitation assays. Therefore, we concluded that FE65 binds to 14-3-3\(\gamma\) independently of APP and/or AICD. Further analysis was performed to identify the FE65 region to which 14-3-3\(\gamma\) binds (supplemental Fig. 7b). Constructs with deletions within the 313–356-amino acid region of FE65, FLAG-FE65\(\Delta\)313–326, FLAG-FE65\(\Delta\)327–356, and FLAG-FE65\(\Delta\)342–356, were expressed in cells, and interactions with 14-3-3\(\gamma\) were assayed. Each of these deletion proteins coimmunoprecipitated 14-3-3\(\gamma\), but the recovery was substantially lower than wild type FE65. These observations suggested that the conformation of the 313–356 region of FE65 may contribute to 14-3-3\(\gamma\) binding. Next, we examined whether the deletion of FE65 amino acids 313–356 affects facilitation of AICD-mediated FE65-dependent gene transactivation (Fig. 5c). Again, 14-3-3\(\gamma\) facilitated FE65-dependent gene transactivation 2-fold in the presence of wild type FE65 (Fig. 5c, compare lane 4 with lane 3), but both facilitation in the presence of 14-3-3\(\gamma\) (lane 6) and basal activity in the absence of 14-3-3\(\gamma\) (lane 5) were almost completely lost in cells expressing the FE65\(\Delta\)1 mutant. These observations indicated that amino acids 313–356 of FE65 are needed for FE65-dependent gene transactivation, and the interaction of FE65 with 14-3-3\(\gamma\) is significant for facilitation of FE65-dependent gene transactivation.

We further investigated whether deletion of FE65 amino acids 313–356 affects association with Tip60 (Fig. 5d), because FE65-Tip60 interactions have been reported previously (11). FLAG-FE65 or FLAG-FE65\(\Delta\)1 was expressed in cells with EGFP-Tip60, and lysates were coimmunoprecipitated with the anti-FLAG antibody. Western blot analysis to detect immunoprecipitates revealed that Tip60 bound both wild type (WT) FE65 and FE65\(\Delta\)1, indicating the FE65\(\Delta\)1 retains the ability to bind Tip60.

Previous analysis demonstrated that the Gal4BD-Tip60 fusion protein (Gal4BD-Tip60) displays transcriptional activity in the presence of FE65 and AICD or APP (11, 15). Thus, we analyzed whether Gal4BD-Tip60 expresses transcriptional activity in the presence of the FE65\(\Delta\)1 mutant (Fig. 5e). The result showed that Gal4BD-Tip60 did not express transcriptional activity in the presence of FE65\(\Delta\)1 (Fig. 5e, compare lane 7 with lane 6), even though FE65\(\Delta\)1 could bind to Tip60 (Fig. 5d). We confirmed that FE65\(\Delta\)1 could bind to AICD, but the binding was weaker than wild type FE65 (Fig. 5f) and elicited concomitantly weaker FE65-dependent gene transactivation activity (Fig. 5g). These data are consistent with the hypothesis that FE65\(\Delta\)1 possesses a region (261–307 amino acids) essential for gene transactivation (17). Additionally, FE65\(\Delta\)1 had reduced interactions with endogenous 14-3-3\(\gamma\) (Fig. 5h), which resulted in a decreased interaction between FE65\(\Delta\)1 and AICD (Fig. 5f). The lack of association between FE65 and endogenous 14-3-3\(\gamma\) decreased the interactions between FE65 and AICD (see Fig. 6b). These observations support the hypothesis that FE65 binding to endogenous 14-3-3\(\gamma\), but not Tip60, is needed for Tip60-dependent activation of transcription mediated by APP.

Finally, we investigated the effect of 14-3-3\(\gamma\) in association of FE65 with AICD (Fig. 6, a and b). N2a cells expressing plasmid combinations of FLAG-FE65, EGFP-AICD, and 3Myc-14-3-3\(\gamma\) were used for coimmunoprecipitation assays. FLAG-FE65 was immunoprecipitated with the anti-FLAG antibody, and the cell lysate and IP were analyzed by Western blotting. From the lysates of cells expressing FLAG-FE65 and EGFP-AICD, the FLAG antibody coimmunoprecipitated EGFP-AICD and FLAG-FE65 (Fig. 6a, lane 2), and the recovery of EGFP-AICD was remarkably improved by coexpression of 3Myc-14-3-3\(\gamma\) (Fig. 6a, compare lane 4 with lane 2). From lysates of cells expressing FLAG-FE65 and 3Myc-14-3-3\(\gamma\), the FLAG antibody coimmunoprecipitated a small amount of 3Myc-14-3-3\(\gamma\) together with FLAG-FE65 (Fig. 6a, lane 3), but recovery was greatly improved in the presence of EGFP-AICD (Fig. 6a).
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6a, compare lane 4 with lane 3). These interactions among the three proteins are specific because none of the proteins were immunoprecipitated with the anti-FLAG antibody in the absence of FLAG-FE65 (Fig. 6a, lane 1). It is obvious that 14-3-3\(\gamma\) binds FE65 independently of endogenous and exogenous AICD (supplemental Fig. 7a). In cells in which endogenous expression of 14-3-3\(\gamma\) was knocked down, coexpression of EGFP-AICD with FLAG-FE65 decreased, but this decrease was not observed in cells in which the expression of 14-3-3\(\gamma\) was suppressed (Fig. 6b). Taken together, coexpression of three proteins, FE65, AICD, and 14-3-3\(\gamma\), enhances their respective associations and may stabilize a functional complex composed of FE65, AICD, and 14-3-3\(\gamma\), which facilitates AICD-mediated FE65-dependent gene transactivation.

CELLULAR LOCALIZATION OF FE65 AND AICD IN THE PRESENCE OF 14-3-3\(\gamma\) was examined (Fig. 6c). When YFP-14-3-3\(\gamma\) was expressed with RFP-FE65 (Fig. 6c, upper panels) or CFP-AICD (middle panels), the majority of FE65 localized in the cytoplasm, whereas AICD localized in both the

FIGURE 5. Identification of a functional region in FE65-mediating interactions with 14-3-3\(\gamma\) and Tip60, and the effect of this region on gene transactivation. a, schematic structures of amino-terminal FLAG-tagged human FE65 (WT) and FE65\(\Delta\) (Δ313–356), WW, WW domain; PI, phosphotyrosine interaction domain. Gene transactivation activity is indicated (+ or −). The PI1 domain corresponds to amino acids 370–509 in human FE65 and 360–510 in rat FE65. b, interaction of FE65 protein constructs with 14-3-3\(\gamma\). N2a cells (4.5 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1-FLAG vector (−), pcDNA3.1-FLAG-FE65 (WT), or pcDNA3.1-FLAG-FE65\(\Delta\) (Δ1), and pcDNA3.1-3Myc-14-3-3\(\gamma\) (0.5 μg). The cells were lysed, and FLAG-FE65 proteins were recovered by immunoprecipitation with the anti-FLAG antibody M2. Cell lysates (lysate) and the immunoprecipitates (IP) were analyzed by Western blotting with M2 and anti-EGFP antibody. c, effect of FE65 proteins on AICD-mediated gene transactivation. N2a cells (1.8 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1-14-3-3\(\gamma\) (20 ng) and 20 ng of pcDNA3.1-14-3-3\(\gamma\) (WT) or pcDNA3.1-14-3-3\(\gamma\) (Δ1). The transactivation values are the ratios of transactivation of the indicated plasmid combinations to that of pBIND-AICD-Gal4BD alone (lane 2). To standardize the plasmid amounts, empty vector (−) was added to yield a total of 84 ng of plasmid. d, interaction of FE65 protein constructs with Tip60. N2a cells (4.5 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1-FLAG vector (−), pcDNA3.1-FLAG-FE65 (WT), or pcDNA3.1-FLAG-FE65\(\Delta\) (Δ1), and pcDNA3.1-EGFP-Tip60 (0.5 μg). The cells were lysed, and FLAG-FE65 proteins were recovered by immunoprecipitation with the anti-FLAG antibody M2. Cell lysates (lysate) and the immunoprecipitates (IP) were analyzed by Western blotting with M2 and anti-EGFP antibody. e, effect of FE65 proteins on gene transactivation by Tip60. N2a cells (1.8 × 10⁵ cells) were transiently cotransfected with (+) or without (−) pcDNA3.1-Gal4BD-Tip60 (4 ng) and pcDNA3.1-APP (20 ng) in the presence (+) or absence (−) of 20 ng of pcDNA3.1-14-3-3\(\gamma\) (WT) or pcDNA3.1-14-3-3\(\gamma\) (Δ1) and pG5luc (40 ng). The transactivation values are the ratios of transactivation of the indicated plasmid combination to that of pBIND-Gal4BD alone (lane 1). To standardize the plasmid amounts, empty vector was added to yield a total of 84 ng of plasmid. f, interaction of FE65\(\Delta\) (Δ313–356) with AICD. N2a cells (4.5 × 10⁵ cells) were transiently cotransfected with 0.5 μg of pcDNA3.1-EGFP-AICD and 0.5 μg of pcDNA3.1-FLAG-FE65 (WT) or pcDNA3.1-14-3-3\(\gamma\) (Δ1). The cells were lysed, and FLAG-FE65 or FLAG-FE65\(\Delta\) (Δ1) were recovered by immunoprecipitation with the anti-FLAG M2 antibody. Cell lysates (lysate) and the immunoprecipitates (IP) were analyzed by Western blotting with M2 and anti-EGFP antibody. g, demonstrates the expression of pcDNA3.1-14-3-3\(\gamma\) (CTF vector), importance of FE65 amino acids 313–356 in AICD-mediated FE65-dependent gene transactivation. N2a cells were transfected with pcDNA3.1-EGFP-AICD (C44) (20 ng), pG5luc (40 ng), and 4 ng of pBIND-Gal4BD, pBIND-Gal4BD-FE65, or pBIND-Gal4BD-14-3-3\(\gamma\), and reporter gene transactivation was assayed. The transactivation values are indicated as the ratio of transactivation to that of pBIND-Gal4BD alone (left). To standardize the plasmid amounts, empty vector was added to yield a total of 84 ng of plasmid.  

6a, compare lane 4 with lane 3). These interactions among the three proteins are specific because none of the proteins were immunoprecipitated with the anti-FLAG antibody in the absence of FLAG-FE65 (Fig.
cytoplasm and nucleus. 14-3-3\(\gamma\) localized to the cytoplasm (Fig. 6c, upper and middle panels), and 14-3-3\(\gamma\) did not colocalize with AICD in the nucleus (middle panel). However, when the three proteins were coexpressed, 14-3-3\(\gamma\) forms a complex with FE65 and AICD in the nucleus and facilitates AICD-mediated FE65-dependent gene transactivation.

DISCUSSION

The 14-3-3 protein family is known to play an important role in many intracellular processes, including apoptosis, the cell cycle, and signal transduction (32, 44). Interaction of target proteins with 14-3-3 has been useful in characterizing the molecular mechanisms of these cellular events, because 14-3-3 proteins are expressed abundantly in various tissues and provide scaffolding for many intracellular reactions. In this report, we demonstrated that interactions between AICD and 14-3-3\(\gamma\) are regulated by phosphorylation of AICD at Thr-668. We proposed that the 14-3-3\(\gamma\) protein contributes AICD-mediated FE65-dependent gene transactivation (Fig. 7). As a first step, AICD associates with FE65, and the dimeric 14-3-3\(\gamma\) binds to these proteins to form an active complex composed of AICD, FE65, and 14-3-3\(\gamma\), which mediates FE65-dependent gene transactivation. When AICD was phosphorylated at Thr-668, 14-3-3\(\gamma\) fails to associate with the initial complex composed of AICD and Fe65, because phosphorylated AICD cannot bind to 14-3-3\(\gamma\), or suppresses the association with FE65 (21). Thus, the phosphorylation of AICD at Thr-668 results in failure to form the active ternary complex composed of AICD, FE65, and 14-3-3\(\gamma\) and down-regulates AICD-mediated FE65-dependent gene transactivation. Therefore, the phosphorylation of AICD at Thr-668 may act as a molecular switch for FE65-dependent gene transactivation mediated by AICD. The complex may be initially formed in the cytoplasm and translocated into the nucleus, because all three proteins colocalized in both the cytoplasm and nucleus when coexpressed.

The cytoplasmic domain of APP and AICD has been reported to interact with several adaptor proteins. X11 and X11L regulate the metabolism of APP (24–28); FE65 interacts with AICD and is thought to activate gene transcription (11), and JIP connects APP to kinesin light chain (27) and/or JIP may function in AICD-mediated transcription (30,
31). Here we found that 14-3-3-3 interacts with AICD in vitro and in vivo. Overexpression of 14-3-3-3 has little effect on the metabolism of APP and generation of 3-amylloid peptides, suggesting that 14-3-3-3 is not a metabolic regulator of APP. However, 14-3-3-3 enhanced activation of reporter gene transcription in the presence of AICD and FE65. The results of gene transactivation assays in cells depleted of 14-3-3-3 by RNAi, and in cells expressing deletion constructs of AICD and FE65 that lacked the ability to interact with 14-3-3-3, demonstrate that 14-3-3-3 is an important component in gene transactivation mediated by AICD and FE65.

Several recent studies have reported that AICD shows transactivation activity in the presence of FE65 in cellular models, but the mechanism of AICD modulation of FE65-dependent gene transactivation remains unclear. During the preparation of this manuscript, Cao and Südhof (15) reported that gene transactivation mediated by Tip60 depends on the APP holoprotein but is independent of AICD. However, in our study, using a construct in which the PI1 domain of FE65 is deleted, AICD-mediated FE65-dependent gene transactivation was observed despite the lack of interaction between FE65 and Tip60. Furthermore, in the gene transactivation assays in our study, using the FE65Δ1 construct, which is not able to interact with 14-3-3-3, AICD-mediated FE65-dependent gene transactivation was almost completely lost. Taken together, these data suggest that AICD-mediated gene transactivation depends on 14-3-3-3 rather than Tip60. Therefore, new models of the mechanism of FE65-dependent gene transactivation may be needed. We demonstrated that expression of 14-3-3-3 enhanced the interaction between AICD and FE65, and the down-regulation of endogenous 14-3-3-3 expression by RNAi suppressed this interaction. 14-3-3-3 expression was correlated with the facilitation of gene transactivation activity, and the lack of 14-3-3-3 was correlated with suppression of transactivation. The molecular mechanism by which these regulated interactions controlled gene transactivation will be the subject of future studies. Further analyses will be needed to reveal the molecular mech-

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