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Association of a 14-3-3 Protein with CMP-NeuAc:GM1 α2,3-Sialyltransferase[1]

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CMP-NeuAc:GM1 α2,3-sialyltransferase (ST-IV) was purified to homogeneity from rat brain. Microsequencing of the tryptic peptides derived from the purified enzyme revealed two amino acid sequences homologous to the 14-3-3 proteins. A polyclonal antibody was raised against purified ST-IV. A 33 kDa protein was co-immunoprecipitated from rat brain extracts with the anti-(ST-IV) antibody as detected by Western blot analysis. This protein was identified as a subtype of 14-3-3 family by an anti-(14-3-3) antibody. Screening of a rat brain λgt11 library using the anti-(ST-IV) antibody resulted in the identification of a cDNA clone coding for the subtype of 14-3-3 protein. These results indicate an association of the 14-3-3 protein with the sialyltransferase. Since the 14-3-3 protein has PKC inhibitor activities and the activity of sialyltransferases is, at least in part, regulated by PKC, the association of the 14-3-3 protein with ST-IV may indicate a role for this protein in the post-translational regulation of the sialyltransferase activity through the processes of phosphorylation and dephosphorylation.

14-3-3 proteins were first described nearly 30 years ago in brain extracts as a family of acidic proteins with molecular weights in the neighborhood of 30 kDa (1). They are most abundant in brain, although recent studies indicate that they are a family of highly conserved and widespread eukaryotic proteins; at least seven 14-3-3 isoforms with molecular weights ranging from 29 to 33 kDa have been identified (2, 3). 14-3-3 proteins possess PKC inhibitor activities (4, 5) and activate tyrosine and tryptophan hydroxylases in the presence of Ca^{2+}/calmodulin-dependent protein kinase II (6). Recently, it has been reported that the 14-3-3 proteins are co-immunoprecipitated with polyomavirus middle tumor antigen (7), oncogene products Raf-1 (8), or Bcr and Bcr-AbI (9) by the corresponding antibodies, respectively, and are tightly associated with cdc25 phosphatases (10). Although the *in vivo* biological roles of the 14-3-3 proteins remain uncertain, these results indicate that these acidic proteins interact with other proteins, and may be involved in the regulation of enzyme activities and modulation of signal transduction in the cell (3, 11).

We have recently purified CMP-NeuAc:GM1 (Galβ1-3GalNAc) α2-3 sialyltransferase (ST-IV)(EC 2.4.99.2) to homogeneity (12). We have further demonstrated that the activity of the sialyltransferase may be regulated by phosphorylation and dephosphorylation (13). Here, we report that a 33 kDa protein is associated with ST-IV and this protein was identified as an isoform of the 14-3-3 family.

[1] The nucleotide sequence reported in this article has been submitted to the GenBank/EMBL with Accession Number U53882.

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Abbreviations: CDP, cytidine diphosphate; GM1, Galβ1,3GalNAcβ1,4[α2,3]Galβ1,4Glcβ1,1′Cer; PKC, protein kinase C; SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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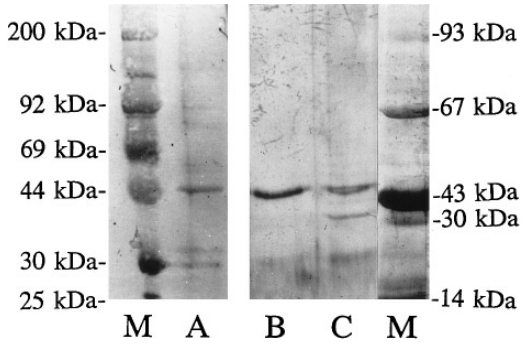

FIG. 1. Presence of the 33 kDa protein during purification of ST-IV. ST-IV was purified from rat brain microsomal extracts through a CDP-Sepharose affinity column (lane A) and then a 'GM1-acid'-Sepharose affinity column (lanes B and C). The eluents were collected in 0.5 ml/tube. Samples on lanes B and C represent the fractions of the second 3 ml and the fractions of the first 5 ml eluted from the 'GM1-acid'-Sepharose column, respectively. The bands shown on the SDS–polyacrylamide gel (8%) were visualized by silver staining. (M) molecular weight markers.

MATERIALS AND METHODS

Protein purification. Purification of ST-IV from rat brains was carried out as previously described (12). In brief, a microsomal fraction was prepared from brains of 14-day-old Wistar rats (Harlan Sprague-Dawley, Indianpolis, IN, U.S.A.) by several centrifugation steps. A crude enzyme extract was obtained from the microsomal fraction by addition of 1% Triton X-100, and then applied to a CDP-Sepharose affinity column followed by a ''GM1 acid''-Sepharose affinity column. The eluents were collected in 0.5 ml/tube, and the enzyme activity and protein contents were determined.

Preparation of polyclonal antibody. A polyclonal antibody against rat brain ST-IV was raised by immunizing a male New Zealand white rabbit. The enzyme preparation of the single 45 kDa band was mixed with Freund's complete adjuvant and injected subcutaneously at several sites on the back. After six weeks, booster injections were given every three weeks. The anti-(ST-IV) antibody was partially purified by chromatography on a protein A-Sepharose column.

Microsequencing. The purified protein was subjected to SDS-PAGE, electroeluted onto a polyvinylidine difluoride membrane (Immobilon-P, Millipore, Bedford, MA, U.S.A.), and stained with Coomassie blue. The stained bands were excised from the membrane and partially digested with trypsin. The tryptic peptides were separated on a Vydac C4 column, and the amino acid sequences of the HPLC-separated peptides were determined by an Applied Biosystems 477A Protein/Peptide Sequencer.

Immunoprecipitation and Western blot analysis. Immunoprecipitates were prepared with the anti-(ST-IV) antibody or preimmune serum, collected with protein A-agarose, analyzed on SDS-PAGE, and immunoblotted with the anti-(14-3-3) ϵ antibody (a gift from Dr. D. C. Pallas, Dana Farber Cancer Institute, Boston, USA). Western blot analysis was performed as described (14). Secondary antibody was HPR-conjugated goat anti-rabbit IgG and the immunoreactive bands were detected using enhanced chemiluminescence reagent kit (Amersham, Arlington Heights, IL, U.S.A.).

Screening of rat brain cDNA libraries. A rat fetal brain λgt11 cDNA library (Clontech, Palo Alto, CA, U.S.A.) was screened with the anti-(ST-IV) antibody using the CLIK kit (Clontech, Palo Alto, CA, U.S.A.) as described by the manufacturer. In addition, two degenerate oligodeoxynucleotide probes were synthesized based on the peptide sequences (Table 1) and contained deoxyinosine substitution at appropriate positions. A rat brain pcDNAI (Invitrogen, San Diego, CA, U.S.A.) library was screened with these probes. Positive clones from both libraries were sequenced on double-stranded templates by the dideoxynucleotide chain termination method (15).

Expression of cDNAs for 14-3-3 protein. The entire cDNA in pcDNAI vector was tranfected into COS-1 cells using the Lipofectin reagent (Gibco BRL, Gaithersburg, MD, U.S.A). The cells were collected 3 days after transfection and the gene product was analyzed by Western blot. The entire cDNA was also subcloned into pQE vector (QIAGEN, Chatsworth, CA, U.S.A) and expressed in E. coli.

RESULTS AND DISCUSSION

ST-IV was purified to homogeneity as a single polypeptide of 45 kDa (Fig. 1, B) by affinity columns as previously described (12). A 33 kDa protein seemed present in the fractions containing the sialyltransferase activity (Fig. 1, A and C). This protein was completely elimi-

TABLE 1
Amino Acid Sequences of Peptides from CMP-NeuAc:GM1 (Galβ1-4GalNAc) α2,3-Sialytransferase Homologous to the 14-3-3 Protein

Peptide	Sequence	Identical to the 14-3-3 at
88	DNLTLWTSDTQGDEAXXGEGXEN	223–245
94	GIVDQSQQAYQEAFEISKK	140–153

X represents the unidentified residue.

nated in the purified enzyme fractions (Fig. 1, B). However, the enzyme activity of the purified ST-IV decreased when the 33 kDa protein was completely separated (data not shown). Microsequencing of the tryptic peptides derived from the purified 45 kDa enzyme protein revealed two amino acid sequences out of four tryptic peptides homologous to the 14-3-3 family (Table 1). These results might indicate a role of the 33 kDa protein in modulating the enzyme activity and/or a related gene structure between the two proteins.

A polyclonal antibody against the rat brain ST-IV was raised using the purified 45 kDa enzyme protein. The results from immunoblotting showed that the anti-(ST-IV) antibody specifically recognized the 45 kDa ST-IV protein (Figure 2, A). Interestingly, immunoprecipitates obtained from crude brain extracts with the anti-(ST-IV) antibody showed a strong 33 kDa band recognized by the anti-(14-3-3) antibody (Figure 2, C). This band was not seen in immunoprecipitates prepared with the preimmune serum (Figure 2, B). Therefore, co-immunoprecipitation of the 14-3-3 protein with the anti-(ST-IV) antibody may indicate an association of the two proteins in the crude extracts.

The anti-(ST-IV) antibody was then used to screen a λgt11 library. After three round of screening, 2 of 5 positive clones were identified as cDNAs for the 14-3-3 ϵ protein by DNA sequencing. At the same time, two oligodeoxynucleotide probes were synthesized based on the amino acid sequence information (Table 1) and used to screen a rat brain pcDNAI library. DNA sequencing indicated that 5 of 7 positive clones were fragments or entire cDNA (clone 3-2-2) for the 14-3-3 ϵ isoform. The clone 3-2-2 contained 1771 bp, showing 92.5% or 96.8% homology to human (10) or rat pineal ϵ isoform (16), respectively. The clone 3-2-2 in pcDNAI vector was transiently expressed in COS-1 cells (Figure 3, lane A), or subcloned into pQE vector and expressed in E. coli (Figure 3, E). The gene products expressed in both cells were recognized by the anti-(14-3-3) antibody (Figure 3, A and E).

Hui et al. (17) reported that the purified 105 kDa puromycin-sensitive aminopeptidase (PSA)

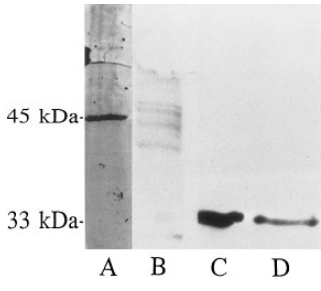

FIG. 2. Western blot analysis. ST-IV fraction eluted from the CDP-Sepharose column (A), immunoprecipitates prepared with preimmune serum (B) or the anti-(ST-IV) antibody (C), and rat brain microsomal extracts (D) were separated by SDS–PAGE. Western blot analysis was performed with the polyclonal anti-(ST-IV) antibody (A) or the anti-(14-3-3 ϵ) antibody (B–D).

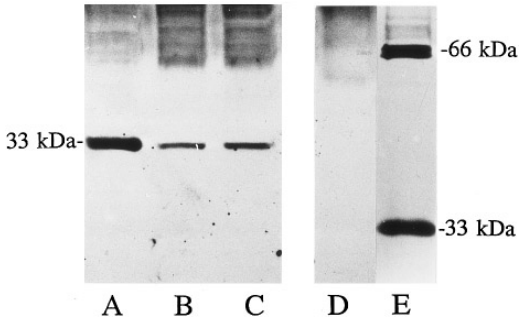

FIG. 3. Expression of cloned 14-3-3 cDNA in mammalian cells and E. coli. The clone 3-2-2 obtained from pcDNAI library was transiently expressed in COS-1 cells (A) or subcloned into pQE vector and expressed in E. coli (E). (B) COS-1 cells transfected with pcDNAI vector containing no insert; (C) untransfected COS-1 cells; (D) untransformed E. coli cells. The 66 kDa band represents the dimeric form of the 14-3-3 ϵ protein.

isozymes exhibit high similarity to the 14-3-3 protein. They found that 9 sequences out of 17 tryptic peptides of PSA are identical to the 14-3-3 protein within the region of 145-246, and suggested that PSA is related to the 14-3-3 protein in their gene structures. Here, we report that microsequencing of purified ST-IV revealed two amino acid sequence of four tryptic peptides homologous to the 14-3-3 protein. Our efforts in molecular cloning resulted in the identification of a cDNA for a subtype of the 14-3-3 protein, using either the anti-(ST-IV) antibody or the oligonucleotide probes derived from the homologous sequences. However, considering that the 14-3-3 protein contributes to 1% of total proteins in brain (2), and thus a large number of its cDNA clones in the libraries, it is not surprising that we failed in the identification of ST-IV cDNA by the antibody. Therefore, it remains unknown whether there is similarity in their gene structures between the 14-3-3 protein and ST-IV.

It has been reported that the ζ and ϵ isoforms of the 14-3-3 family co-purify with the pineal serotonin N-acetyltransferase (16) and that the 14-3-3 protein binds to histone (18). Recently, it has been shown that the 14-3-3 protein associates with cdc25 phosphatases (10). These investigators attempted to identify the interacting proteins of cdc25 phosphatases by two-hybrid screening which resulted in cloning of cDNAs coding for human β and ϵ isoforms of the 14-3-3 family. In this report, a subtype of the 14-3-3 protein was co-immunoprecipitated with the sialyltransferase, indicating that an ϵ protein of the 14-3-3 family is associated with ST-IV. This association may be functionally important based on the following observation. We recently reported that treatment of several sialyltransferases by PKC down-regulates their activities in a time-dependent manner and the enzyme activity can be partially recovered by treatment of the phosphorylated sialyltransferases with rat brain protein phosphatase (13). Thus, sialyltransferase activities are likely modulated by PKC and protein phosphatase. Association of the 14-3-3 protein with sialyltransferases, as shown in this report, should indicate a functional role of the 14-3-3 protein ϵ in modulating sialyltransferase activities through the processes of phosphorylation and dephosphorylation. Further studies are in progress to elucidate the generality and mechanisms for this regulatory role, as well as their gene structures.

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