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Phosphorylation of GAP-43 and the Effect on APP Processing

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ABSTRACT The phosphorylation of Growth Associated Protein may affect the occurrence of interaction between GAP-43 and the Amyloid Precursor Protein. By synthesizing mutants of the GAP-43 protein, the amount of C-terminal fragments of APP that results from the interaction could be observed under various conditions. An alanine mutant (S41A) and an aspartate mutant (S41D) and the wild type GAP-43 were all co-expressed with APP in mouse neuroblastoma cells and analyzed on a Western blot. The mutant used to block phosphorylation, S41A, showed a greater occurrence of interaction with APP while the mutant that was created to mimic the effect of phosphorylation, S41D, showed less interaction. Further study of this research would include verifying the interaction of the two proteins through a co-immunoprecipitation experiment and determining the mechanism by which GAP-43 mediates changes in APP metabolism.

INTRODUCTION Alzheimer’s Disease (AD) is the sixth leading causes of death in the United States and the most common form of dementia, causing problems with memory, cognition, and behavior (5). The disease is characterized by a buildup of amyloid plaques in the brain, which is associated with a decrease in efficiency of neuronal communications (6). There is no cure or effective treatment for the disease (Figure 1). The plaques that damage the brain’s tissues are formed by fibrils of a metabolite of a protein called Amyloid Precursor Protein (APP).

Located on the membrane of cells, the neuronal form of APP is a 695 amino acid protein that can be cleaved in several locations by complex metabolic processing (7). If the protein is cleaved at the α-site by the enzyme α-secretase, then a second cleavage by γ-secretase yields a soluble protein that is non-pathogenic to the cells around it (Figure 2). However, if the protein is cleaved first at the β site by β-secretase, then subsequent cleavage by γ-secretase releases β-amyloid (Aβ) peptides, which have a propensity to aggregate and form fibrils and plaques, leading to pathologies related to AD (7;9). Thus, it is of significant interest to identify and explore proteins that may bind to and influence the processing of APP (2).
Growth Associated Protein (GAP-43) is a cytosolic protein involved in cell growth, neuronal axon extension, and molecular mechanisms of learning and memory (3). Recently, our lab has found that GAP-43 can interact with APP and alter its rate of metabolism, an effect that suggests dependence on membrane localization (1;3). One factor that affects the functioning of GAP-43 is phosphorylation on a serine residue, which has a regulatory effect on GAP-43 membrane localization (4;8). Thus, since APP is a transmembrane protein, I hypothesize that phosphorylation of GAP-43 may be a critical factor in its modulatory effect on APP metabolism.

**OBJECTIVES**

The purpose of this research is to determine whether GAP-43 phosphorylation plays a role in modulation of APP processing. This is tested by producing phosphorylation deficient and mimetic mutants of GAP-43 and co-expressing them with APP in cultured cells. Following that, an assay will be used to measure the impact of the Wild-type, phospho-deficient, and phospho-mimetic GAP-43 on the APP metabolic processing by comparing the amount of C-terminal fragments that result for each given sample. A reduction of C-terminal fragments would indicate a greater interaction between APP and GAP-43.

**METHODS**

To test the effect of phosphorylation on the GAP-43 protein, molecular and cell culture techniques were used to co-express APP with a GAP-43 that carries a mutation rendering it unable to be phosphorylated. The metabolism of APP in this condition will be compared to samples in which APP has been co-expressed with a wild type form of GAP-43. As an additional condition, a form of GAP-43 that has been mutated to mimic the phosphorylated state will also be tested.

To begin the experiment, site-directed mutagenesis was enacted to change the phosphorylation site of GAP-43. Mutagenesis is a laboratory technique that changes the genetic information on a strand of DNA in order to alter the makeup and functions of the protein that is expressed from the DNA code (Figure 3). For this experiment the DNA was mutated to alter a serine residue at amino acid 41 (the consensus phosphorylation site of GAP-43) to an alanine, which does not possess the required hydroxyl group onto which the phosphate is added via an enzyme-mediated dehydration reaction, thus preventing the mutant protein from being phosphorylated. In parallel reactions, the same codon was changed to aspartate that has a similar charge and structural profile to that of phosphate and can act as a phospho-mimetic (D). Once
cloning of the mutants was completed, the GAP-43 mutant and the wild type GAP-43 proteins were then co-expressed with APP in cultured mouse neuroblastoma (N2a) cells and the metabolites of APP were measured by Western blot analysis. This procedure is conducted through polyacrylamide gel electrophoresis (SDS-PAGE), which separates the proteins by their relative size, allowing for the identification of the cleaved APP products by their lengths. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane and incubated with antibodies that bind to APP. Then, using chemiluminescence, the antibodies that were attached to the APP metabolites were detected and quantified, and compared.

Figure 3. Polymerase Chain Reaction (PCR) was used to amplify specific DNA sequences of the GAP-43 open reading frame.

RESULTS

The first step of the procedure was to amplify the specific DNA sequences of the GAP-43 open reading frame (ORF). Primers included codons to mutate S41 to either alanine (S41A) or aspartate (S41D). Gels were run and imaged to confirm that each mutant contained both the forward and reverse primers from the first round of PCR, and the full ORF transcript in the second round of the reaction. The images from the gel are shown for the alanine mutant below (Figure 4)(Figure 5). Each fragment was confirmed based on its length in base pairs. Then, each sample was digested to run on a gel in order to confirm the presence of the inserts in each sample. As shown below, the first, third, and fourth samples from each mutant contained the templates and were suitable for the subsequent procedures (Figure 6).

From there, the samples were sent to a laboratory to have their sequences confirmed. Chromatograms were returned for each sample, showing the specific nucleotide bases in each piece of DNA. For the wild type GAP-43, the codon at the forty-first residue is AGC codes for a serine amino acid. However, in the first sample the codon is GAC. This confirmed the aspartate codon in the first sample (Figure 8). Similarly the codon in the second sample was GCC, which codes for alanine (Figure 7).

With the sequences confirmed, the wild type and mutants were co-expressed with APP in cultured mouse neuroblastoma (N2a), and the metabolites were measures with a Western Blot analysis. Additionally, the presence of GAP-43 was confirmed in the Western Blot procedure to ensure that all samples contained the protein. The chemiluminescence image is shown below (Figure 9). The Western Blot shows that there was a greater reduction of C-terminal fragments when the APP was co-expressed with S41A than when it was co-expressed with the GAP-43 wild type. The S41A mutant was unable to be phosphorylated, which resulted in a reduction of C-terminal fragments than the wild type GAP-43 protein. This would suggest that there was an increase in interaction between the mutated protein and APP than with the wild type GAP-43.

Attempting to mimic the effects of phosphorylation in the S41D mutant did not result in the same amount of functional interaction as wild type GAP-43, as shown by the increase of C-terminal fragments of APP on the Western Blot. These speculatie data readings suggest that GAP-43 associates with APP primarily in its non-phosphorylated form. Phosphorylation at serine 41 may thus serve as a
regulatory modification that inhibits the APP/GAP-43 complex formation.

Further study of the topic would include a replication of the preliminary results. A quantitative determination of the relative population of phosphorylated and unphosphorylated GAP-43 would be calculated, and a co-immunoprecipitation of APP with wild-type and mutant GAP-43 would be conducted.

Figure 4. PCR Round 1, Generating S41A mutant containing DNA

Figure 5. PCR Round 2, Combining round 1 products as a template to generate full ORF transcript.

Figure 6. Screening of S41A and S41D clones.

Figure 7. The chromatogram indicates that the codon for serine (AGC), was replaced with GCC which codes for an alanine.
Figure 8. The chromatogram indicates that the codon for serine (AGC), was replaced with GAC which codes for an aspartate.

Figure 9. The Western Blot shows that there was a greater reduction of C-terminal fragments when the APP was co-expressed with S41A than when it was co-expressed with the GAP-43 wild type. The presence of GAP-43 was confirmed in the bottom bands. The band in between APP CTF and APP-Full length present in the mutants was determined to be background and noise and excluded from further experimentation.

EXPERIMENTAL DESIGN

Generation of Mutant GAP-43:
Crossover PCR mutagenesis was performed to generate GAP-43 mutants from wild-type GAP-43. Template DNA for the reactions was the plasmid pAG3 containing the ORF of GAP-43. Primers containing mutations at codons indicated in poster were used for the amplification reactions in the first round, the products of which were used as the template for the second cross-over round using flanking primers. Products of round 2 were digested and ligated into pAG3 vector, transformed into DH5α cells and incubated over night (o/n) at 37°C on LB-agar plates containing the selective antibiotic ampicillin. Isolated colonies were selected grown in 3mL LB Broth, with 50µg/mL ampicillin at 37°C on a shaker at 250rpm overnight. Plasmids were isolated using alkali lysis followed by matrix capture using the GeneJET Plasmid Miniprep Kit (Fermentas). Plasmid DNA was digested with the enzyme BamHI and HindIII to detect the presence of an insert. Positive colonies were submitted for sequencing at the University of Chicago sequencing facility.

Cell culture: N2a mouse neuroblastoma cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin and incubated at 37°C under 5% CO₂. Cells were passaged 1:10 every 3-4 days.

Transfection: N2a cells were transiently cotransfected with APP and either GAP-43, GAP-43 S41A, or GAP-43 S41D or GFP as a control protein. Transfection was performed in 30mm culture plates at a cell confluence of approximately 80% using Lipofectamine 2000 (Invitrogen). Coexpression was allowed for 40hrs at 37°C under 5% CO₂.

Cell lysis: Cells were lysed on ice using 200uL lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% IGEPAL, 0.5%
Na:DOC and a protease inhibitor cocktail). Insoluble material was removed by centrifugation at 16,000 x g and the resultant supernatants were transferred into new microcentrifuge tubes and the pellets discarded. The lysis contents were analyzed for protein concentration using the BCA method (Pierce).

**Western blot:** Lysates samples were boiled in Laemmli sample buffer and analyzed by Western blot after poly-acrylamide gel electrophoresis, on hand-poured gradient tris/tricine gels. Proteins were transferred onto polyvinylide fluoride membranes (Bio-Rad) and washed 3 times with phosphate buffered saline containing 0.1% tween-20 detergent (PBST) for 5 min each and then blocked with 5% milk in PBST for 1hr at room temperature with rocking. The membranes were then transferred to containers containing the appropriate primary antibody and incubated on a rocker at 4°C o/n and then washed 3x for 5 min each with PBST and incubated with the appropriate secondary antibody with rocking for 1hr at room temperature. The membranes were then washed 4 times for 5 minutes each with PBST and rinsed with distilled water. Immunoreactivity was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and read using FluorChem HD2 software or OmegaLum G CCD imager.

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