Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6218–6221, August 1988
Neurobiology

Phosphorylation of Alzheimer disease amyloid precursor peptide by protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II

(dementia/phosphoproteins/receptor internalization)

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Contributed by Paul Greengard, May 19, 1988

ABSTRACT **The amino acid sequence of the Alzheimer disease amyloid precursor (ADAP) has been deduced from the corresponding cDNA, and hydropathy analysis of the sequence suggests a receptor-like structure with a single transmembrane domain. The putative cytoplasmic domain of ADAP contains potential sites for serine and threonine phosphorylation. In the present study, synthetic peptides derived from this domain were used as model substrates for various purified protein kinases. Protein kinase C rapidly catalyzed the phosphorylation of a peptide corresponding to amino acid residues 645–661 of ADAP [ADAP peptide(645–661)] on Ser-655. Ca^{2+}/calmodulin-dependent protein kinase II phosphorylated ADAP peptide(645–661) on Thr-654 and Ser-655. This peptide was virtually ineffective as a substrate for cAMP-dependent protein kinase, cGMP-dependent protein kinase, casein kinase II, or insulin receptor protein-tyrosine kinase. When a homogenate of rat cerebral cortex was used as the source of protein kinase, phosphorylation of ADAP peptide(645–661) was stimulated by calcium/phosphatidylserine/diolein to a level 4.6-fold above the basal level of phosphorylation, consistent with a prominent stimulation by protein kinase C. Using rat cerebral cortex synaptosomes prelabeled with $^{32}P_i$, a ^{32}P-labeled phosphoprotein of ≈135 kDa was immunoprecipitated by using antisera prepared against ADAP peptide(597–624), consistent with the possibility that the holoform of ADAP in rat brain is a phosphoprotein. Based on analogy with the effect of phosphorylation by protein kinase C of juxtamembrane residues in the cytoplasmic domain of the epidermal growth factor receptor and the interleukin 2 receptor, phosphorylation of ADAP may target it for internalization.**

Hydropathic analysis of the deduced primary sequence of the Alzheimer disease amyloid precursor (ADAP) suggests a structure similar to that of a receptor protein (1–4), having a single membrane-spanning region and putative extracellular, transmembrane, and cytoplasmic domains. The primary sequence determinants that account in part for the substrate specificity exhibited by several protein kinases have been established (5–10). Based on these consensus sequences and the deduced amino acid sequence of ADAP, we identified potential phosphorylation sites in the putative cytoplasmic domain of ADAP. Synthetic peptides, corresponding to ADAP amino acid residues 645–661 [ADAP peptide(645–661)] and ADAP amino acid residues 668–683 [ADAP peptide(668–683)], which contained these potential phosphorylation sites were prepared and tested as model substrates for a number of purified protein kinases. Phosphorylation of the ADAP peptides was compared with that of peptide substrates derived from holoproteins whose phosphorylation has been demonstrated to be physiologically significant.

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MATERIALS AND METHODS

Bovine brain L-α-phosphatidyl-L-serine, diolein, and 8-Br-cGMP were obtained from Sigma. [γ-^{32}P]ATP was obtained from New England Nuclear. Cellulose TLC plates were obtained from Eastman Kodak. The ADAP peptide(645–661) was obtained from Cambridge Research Biochemicals (Cambridge, England). The ADAP peptide(668–683) and the peptide corresponding to phosphorylation site 3 of bovine synapsin I (synapsin I phosphorylation site 3 peptide) (7) were prepared, respectively, by the Rockefeller University Protein Sequencing Facility and Meng Ho (Rockefeller University, New York). The latter two peptides were purified in our laboratory by HPLC. Synthetic peptides were analyzed by amino acid composition and mass spectrometry to confirm their structure. The myelin basic protein peptide [Ala^{105}]MBP(104–118) (11) was the gift of Bruce Kemp (University of Melbourne, West Heidelberg, Australia). The amino acid sequences of the various synthetic peptides are shown in Fig. 1.

Bovine brain synapsin I was prepared as described (7). Purified calmodulin (CaM) and some protein kinases and substrate proteins were gifts from investigators in our laboratory [rat and mouse brain protein kinase C and the 87-kDa protein (12), Katherine Albert; bovine lung cGMP-dependent protein kinase and the catalytic subunit of bovine heart cAMP-dependent protein kinase (6), Angus C. Nairn; bovine brain DARPP-32 (6), Hugh C. Hemmings, Jr.; sheep brain CaM and rat brain Ca^{2+}/CaM-dependent protein kinase II (Ca^{2+}/CaM kinase II) (13), Yvonne Lai and Fred Gorelick; bovine brain casein kinase II (14), Jean-Antoine Girault]. The human placental insulin receptor protein kinase (15) was a gift from Jules Shafer (University of Michigan, Ann Arbor, MI) and Dennis Pang (our laboratory).

Phosphorylation reactions were carried out at 30°C. All enzyme activities were measured under initial rate conditions. The purified protein kinases tested were as follows: protein kinase C (specific activity, 2.8 μmol of phosphate transferred to histone H1·min^{-1}·mg^{-1}) (12), the catalytic subunit of cAMP-dependent protein kinase (specific activity, 0.6 μmol of phosphate transferred to DARPP-32·min^{-1}·mg^{-1}) (6), cGMP-dependent protein kinase (specific activity, 31 nmol of phosphate transferred to DARPP-32·min^{-1}·mg^{-1}) (6), Ca^{2+}/CaM kinase II (specific activity, 2.9 μmol of phosphate transferred to synapsin I·min^{-1}·mg^{-1}) (13), casein kinase II (activity, 15.7 pmol of phosphate transferred to DARPP-32 per min per ml of assay mixture) (ref. 14; J.-A. Girault, personal communication), and insulin receptor protein kinase (activity, 8.0 pmol of phosphate transferred to histone H2B per min per ml of assay mixture) (15).

Abbreviations: ADAP, Alzheimer disease amyloid precursor; CaM, calmodulin; Ca^{2+}/CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II.

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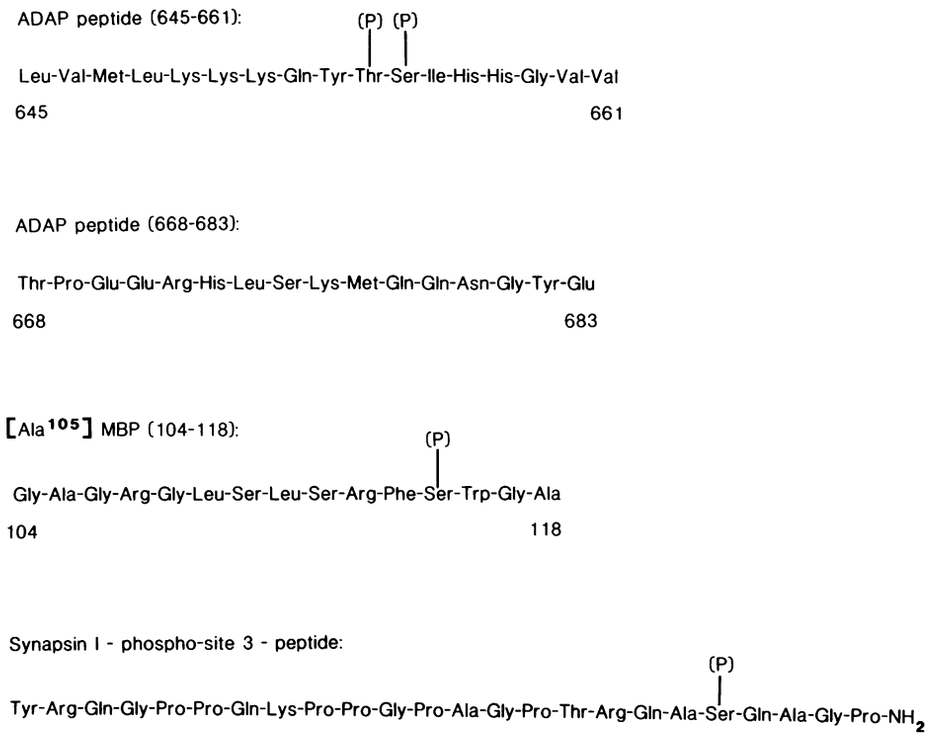

FIG. 1. Amino acid sequence of synthetic protein kinase substrate peptides derived from ADAP, myelin basic protein (MBP), and bovine synapsin I.

Rat cerebral cortex was homogenized in a buffer (1:5, wt/vol) containing 20 mM Tris·HCl (pH 7.4), 100 mM NaCl, 10 μg of leupeptin per ml, 10 μg of antipain per ml, 10 μg of aprotinin per ml, and 100 μM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 5000 × *g* for 5 min. The supernatant was frozen and stored under liquid nitrogen until use. Assays performed with rat cortical preparations contained 300 μM Na_3VO_4, 15 mM $MgCl_2$, 1.5 mM EGTA, 15 mM 2-mercaptoethanol, and either ADAP peptide(645–661) (15–150 μM), synapsin I (1.8 μM), or the 87-kDa protein (0.25 μM). In some experiments, 2 mM $CaCl_2$ and either 50 μg of CaM per ml or 0.5 μg of phosphatidylserine per ml plus 0.02 μg of diolein per ml were also present during the phosphorylation assay.

Reactions were initiated by addition of [γ-^{32}P]ATP (final concentration, 200 μM, 1800–2200 cpm/pmol) and terminated by addition of 0.2 vol of a solution containing 200 mM EDTA and 10 mM ATP. Aliquots of the reaction mixture were subjected to $NaDodSO_4$/PAGE with 15% acrylamide gels. ^{32}P-labeled phosphopeptides and phosphoproteins were localized by autoradiography of the dried gels. Phosphorylation of peptides and proteins was quantitated by liquid scintillation spectrometry of excised gel pieces. Recovery of ^{32}P-labeled ADAP peptide(645–661) was 90.3 ± 1.6% and was not affected by removal of free [^{32}P]ATP by HPLC prior to electrophoresis. Kinetic parameters were obtained from double-reciprocal plots by least-squares linear regression analysis of the data points.

Analysis of phosphorylated amino acids was performed as described (16).

RESULTS

ADAP peptide(645–661) was rapidly phosphorylated by protein kinase C and by Ca^{2+}/CaM kinase II (Table 1). Protein kinase C catalyzed phosphorylation almost exclusively on Ser-655 (Fig. 2). Ca^{2+}/CaM kinase II catalyzed phosphorylation on both Thr-654 and Ser-655 in a ratio of 1.6:1 in favor of Thr-654 (Fig. 3). Prolonged incubation (18 hr) resulted in incorporation of 1.03 mol of phosphate per mol of peptide in the reaction catalyzed by protein kinase C and 0.7 mol of phosphate per mol of peptide in that catalyzed by Ca^{2+}/CaM kinase II. The observed k_{cat} values were 9.6 and 1.1 s^{-1}, respectively, while the apparent K_m values were 126–138 μM and 17–31 μM. The catalytic efficiency (k_{cat}/K_m) for these reactions was on the order of 0.04–0.08 $\mu M^{-1} \cdot s^{-1}$. cAMP-dependent protein kinase, cGMP-dependent protein kinase (Table 1), casein kinase II, and insulin receptor protein kinase (data not shown) failed to phosphorylate this peptide significantly.

The 87-kDa protein (12) and myelin basic protein (11) are excellent substrates for protein kinase C. Therefore, we compared the kinetic constants for the phosphorylation of ADAP peptide(645–661), the 87-kDa protein, and a model peptide containing the sequence surrounding the phosphorylation site of myelin basic protein (Table 2). The k_{cat} value for the phosphorylation of ADAP peptide(645–661) by protein kinase C compared favorably with the k_{cat} values for this enzyme using either the 87-kDa holoprotein or the myelin basic protein peptide as substrate. However, the apparent K_m

Table 1. Kinetics of phosphorylation of ADAP peptide(645–661) by various protein kinases

Protein kinase	k_{cat}, s^{-1}	K_m, μM	Catalytic efficiency, $\mu M^{-1} \cdot s^{-1}$
Protein kinase C			
Experiment 1	9.7	138	0.070
Experiment 2	9.6	126	0.076
Ca^{2+}/CaM kinase II			
Experiment 1	1.2	31	0.039
Experiment 2	1.1	17	0.065
cAMP-dependent protein kinase			
Experiment 1	0.01	90	0.0001
Experiment 2	0.01	160	0.00006
cGMP-dependent protein kinase	<0.01	—	—

Catalytic efficiency, k_{cat}/K_m.

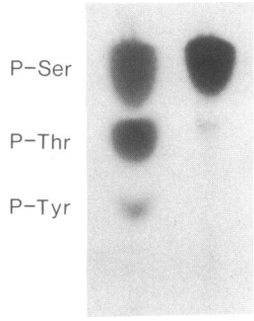

FIG. 2. Protein kinase C phosphorylation of ADAP peptide(645–661) on Ser-655. The ^{32}P-labeled phosphorylated ADAP peptide(645–661) was subjected to acid hydrolysis. The resultant phosphorylated amino acids were separated by electrophoresis (right lane) and compared with phosphorylated amino acid standards (ninhydrin stain) (left lane) as described (16).

for the phosphorylation of ADAP peptide(645–661) by protein kinase C was much higher than that observed for the other substrates, resulting in a diminished catalytic efficiency.

Synapsin I is an excellent substrate for Ca^{2+}/CaM kinase II (5). Therefore, we compared the kinetic constants for the phosphorylation of ADAP peptide(645–661), synapsin I, and a model peptide containing the sequence surrounding phosphorylation site 3 of bovine synapsin I (Table 3). The k_{cat} value for phosphorylation of ADAP peptide(645–661) by Ca^{2+}/CaM kinase II was similar to that for the phosphorylation of synapsin I, but <1/10th that for the phosphorylation of the synapsin I phosphorylation site 3 peptide. The apparent K_m for the phosphorylation of ADAP peptide(645–661) by this protein kinase was similar to the value obtained for the reaction catalyzed by this enzyme using the synapsin I phosphorylation site 3 peptide as substrate. The apparent K_m value for the phosphorylation of synapsin I was considerably lower than that observed for either its derivative peptide or ADAP peptide(645–661).

In experiments with homogenates of rat cerebral cortex used as the source of protein kinase activity, the addition of calcium, phosphatidylserine, and diolein stimulated the phosphorylation of ADAP peptide(645–661) to a level 4.6 (±0.6)-fold above basal levels. A 4.8-fold increase in the phosphorylation of the 87-kDa protein was observed in parallel assays. Ca^{2+}/CaM stimulated the phosphorylation of ADAP peptide(645–661) by rat cortical homogenates by 3.5 (±0.6)-fold, compared with a 6.3-fold increase observed in phosphorylation of synapsin I under identical conditions. The apparent K_m values for these interactions were similar to those observed for the peptide using purified protein kinase C and Ca^{2+}/CaM kinase II, respectively. Therefore, these

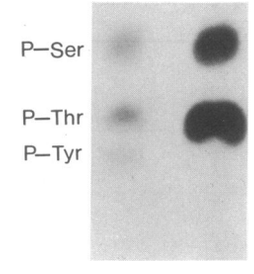

FIG. 3. Ca^{2+}/CaM kinase II phosphorylation of ADAP peptide(645–661) on Thr-654 and Ser-655. The ^{32}P-labeled phosphorylated ADAP peptide(645–661) was subjected to acid hydrolysis. The resultant phosphorylated amino acids were separated by electrophoresis (right lane) and compared with phosphorylated amino acid standards (ninhydrin stain) (left lane) as described (16).

Table 2. Comparison of kinetic constants for phosphorylation by protein kinase C

Substrate	k_{cat}, s^{-1}	K_m, μM	Catalytic efficiency, $\mu M^{-1} \cdot s^{-1}$
ADAP peptide(645–661)			
Experiment 1	9.7	138	0.070
Experiment 2	9.6	126	0.076
87-kDa protein			
Experiment 1	7.2	0.8	9.0
Experiment 2	7.6	0.4	19.0
[Ala^{105}]MBP(104–118)			
Experiment 1	1.4	2.2	0.64
Experiment 2	2.3	4.1	0.56
ADAP peptide(668–683)	—	>1000	—

Catalytic efficiency, k_{cat}/K_m; MBP, myelin basic protein.

enzymes are probably responsible for most of the phosphorylation of ADAP peptide(645–661) observed in experiments with homogenates of rat cerebral cortex, although contributions by other protein kinases cannot be excluded.

Protein kinase C (Table 2) and the catalytic subunit of cAMP-dependent protein kinase (data not shown) each catalyzed a very slow phosphorylation of ADAP peptide-(668–683). The other protein kinases tested (cGMP-dependent, Ca^{2+}/CaM kinase II, casein kinase II, insulin receptor) failed to catalyze detectable phosphorylation of ADAP peptide(668–683) during a 30-min incubation period.

Antipeptide antisera were prepared against thyroglobulin-conjugated synthetic peptides corresponding to residues 597–624 (extracellular domain) or residues 668–683 (intracellular domain) of ADAP. Both of these antisera identified a protein of ≈135 kDa on immunoblots of rat cerebral cortical homogenates. Using rat cerebral cortex synaptosomes prelabeled with $^{32}P_i$ (24), a ^{32}P-labeled phosphoprotein of ≈135 kDa was immunoprecipitated by anti-ADAP peptide(597–624) antiserum, consistent with the possibility that the holoform of ADAP in rat brain is a phosphoprotein (data not shown).

DISCUSSION

The residues surrounding the sites phosphorylated in ADAP peptide(645–661) are not typical consensus sequences for phosphorylation by protein kinase C or Ca^{2+}/CaM kinase II. Nevertheless, the kinetics of phosphorylation of ADAP peptide(645–661) by protein kinase C and by Ca^{2+}/CaM kinase II, relative to the kinetics of phosphorylation for various standard substrates, suggest that phosphorylation of ADAP by either of these kinases might occur under physiological conditions. For example, the catalytic efficiency provides a lower limit estimate of the second-order rate constant for enzyme–substrate interactions. For macromolecules, second-order rate constants are in the range of 10^6 $M^{-1} \cdot s^{-1}$ (17). Thus, catalytic efficiency values in the range

Table 3. Comparison of kinetic constants for phosphorylation by Ca^{2+}/CaM kinase II

Substrate	k_{cat}, s^{-1}	K_m, μM	Catalytic efficiency, $\mu M^{-1} \cdot s^{-1}$
ADAP peptide(645–661)			
Experiment 1	1.2	31	0.039
Experiment 2	1.1	17	0.065
Synapsin I	2.6	0.4	6.5
Synapsin I phosphorylation site 3 peptide			
Experiment 1	16.6	40	0.41
Experiment 2	14.1	34	0.41

Catalytic efficiency, k_{cat}/K_m.

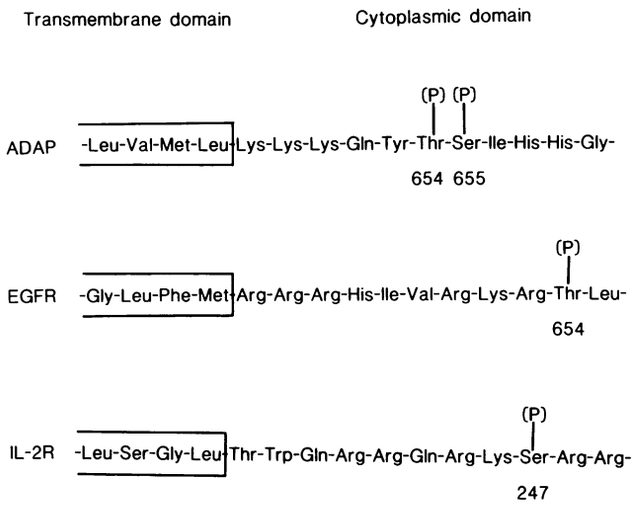

FIG. 4. Location of phosphorylatable residues in the putative cytoplasmic domain of ADAP, epidermal growth factor receptor (EGFR), and interleukin 2 receptor (IL-2R). In each case, the phosphorylation site is within 10 residues of the plasma membrane.

of 10^4–10^5 $M^{-1}\cdot s^{-1}$ (0.01–0.1 $\mu M^{-1}\cdot s^{-1}$) obtained for the phosphorylation of ADAP peptide(645–661) by protein kinase C and Ca^{2+}/CaM kinase II are consistent with the possibility that ADAP is phosphorylated *in vivo*.

Peptides modeled after holoprotein sequences can offer insights regarding the interactions of protein kinases with their holoprotein substrates. The relatively high apparent K_m values observed for the ADAP peptide(645–661) and synapsin I phosphorylation site 3 peptide may be attributable to these peptides lacking some features of higher order structure that contribute to the interaction between the holoprotein and the protein kinase. The apparent K_m values observed for synthetic peptides can be considerably higher than those observed for their parent holoproteins (e.g., see Table 3 and refs. 6 and 18). Therefore, an analysis of the phosphorylation of the ADAP holoprotein is needed to assess reliably the likelihood that it is phosphorylated *in vivo* by protein kinase C and/or by Ca^{2+}/CaM kinase II.

It is of interest to compare the structural properties of the ADAP holoprotein (1) with those of the epidermal growth factor (EGF) receptor and the interleukin 2 (IL-2) receptor (19–22) (Fig. 4). Protein kinase C phosphorylates Thr-654 of the EGF receptor (19) and Ser-247 of the IL-2 receptor (20). In each case, the phosphorylated residue is located in the putative cytoplasmic domain, within 10 residues of the plasma membrane. The serine residue we have found to be rapidly phosphorylated by protein kinase C in ADAP peptide(645–661) corresponds to Ser-655 of ADAP, which is also located in the cytoplasmic domain within 10 residues of the plasma membrane (1). In addition, Ser-655 as well as Thr-654 of ADAP peptide(645–661) can be phosphorylated by Ca^{2+}/CaM kinase II. Of interest in relation to the present study, phosphorylation of Thr-654 of the EGF receptor by protein kinase C stimulates receptor internalization (21, 22). Similarly, phosphorylation of Ser-247 of the IL-2 receptor by protein kinase C has been suggested to regulate its internalization (20, 23). It is worth speculating that, in an analogous fashion, the state of phosphorylation of ADAP on Thr-654 or Ser-655 might regulate its rate of internalization and metabolic disposition. Since internalization is likely to be required for degradation, pharmacologic manipulation of the state of phosphorylation of ADAP might modify the cerebral amyloidosis associated with Alzheimer disease.

We thank Drs. Peter Davies, Richard Huganir, and Angus C. Nairn for helpful discussions and Dr. Davies for the gift of some of the ADAP peptide(645–661) used in preliminary experiments. This research was supported by a Clinical Investigator Development Award NS 01095-02 (S.G.), and U.S. Public Health Service Grants NS-21550 and NS-22789 (P.G.). The amino acid analyses and synthesis of some peptides were performed at The Rockefeller University Protein Sequencing Facility, which is supported in part by funds provided by the U.S. Army Research Office for the purchase of equipment.

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