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The 93 kDa protein gephyrin and tubulin associated with the inhibitory glycine receptor are phosphorylated by an endogenous protein kinase

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The 93 kDa protein gephyrin is a tubulin binding peripheral membrane protein that is associated with the inhibitory glycine receptor and has been implicated in its anchoring at central synapses. Here, we demonstrate that gephyrin as well as co-purifying tubulin are phosphorylated by a kinase activity which is endogenous to highly purified glycine receptor preparations. This kinase phosphorylates serine and threonine residues and utilizes ATP, but not GTP, as phosphate donor. Its activity is not affected by various activators and/or inhibitors of cyclic nucleotide-dependent kinases, calcium/calmodulin-dependent kinases, or protein kinase C. A five-fold stimulation of kinase activity was, however, observed in the presence of poly-lysine. Phosphorylation of gephyrin and/or tubulin might regulate receptor/cytoskeleton interactions at postsynaptic membrane specializations.

Glycine receptor; Tubulin; Protein phosphorylation

1. INTRODUCTION

A 93 kDa peripheral membrane protein called gephyrin (P. Prior et al., submitted) copurifies with the inhibitory glycine receptor (GlyR), which is a ligand-gated chloride channel protein of the CNS composed of 48 kDa (α) and 58 kDa (β) membrane spanning subunits [1–3]. By immunoelectron microscopy, the 93 kDa protein has been shown to decorate the cytoplasmic face of the glycinergic postsynaptic membrane [4,5]. Recently, tubulin has been detected in highly purified GlyR preparations. The co-purifying tubulin has been demonstrated to coelute with 93 kDa protein upon differential elution of the receptor polypeptides from a strychnine agarose column used for affinity-purification [6]. Moreover, tubulin overlay and co-sedimentation experiments revealed cooperative high affinity binding of 93 kDa protein to tubulin. Hence, the 93 kDa protein has been proposed to represent a novel microtubule-binding protein that links the GlyR to subsynaptic tubulin [6].

Here, we describe a kinase activity endogenous to purified GlyR preparations, which phosphorylates the 93 kDa protein and co-purifying tubulin. This enzyme may be implicated in the regulation by phosphorylation of receptor–cytoskeleton interactions at glycinergic synapses.

Abbreviations: GlyR, glycine receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *GlyR purification*

Affinity purification of the GlyR was performed as described [7]; however, the concentration of Triton X-100 in the buffers used for washing and elution was reduced to 0.3% (w/v). Separation of the 93 kDa protein and tubulin from the GlyR bound to the affinity matrix by elution with a buffer containing 1% (w/v) sodium cholate has been described [6].

2.2. *Phosphorylation assay*

Prior to phosphorylation, affinity-purified GlyR was dialyzed for 4 h at 4°C against 1000 vols. of 50 mM Tris-Cl, pH 7.4, 200 mM NaCl, 0.3% (w/v) Triton X-100, 5 mM $MgCl_2$, 5 mM ethyleneglycol-bis-(β-aminoethyl ether)tetraacetic acid, 2 mM β-mercaptoethanol and protease inhibitors as in [6]. For experiments requiring the presence of Ca^{2+}, ethyleneglycol-bis-(β-aminoethyl ether)tetraacetic acid was omitted. Dialyzed GlyR aliquots (≈0.5 μg protein) were incubated at 37°C with [γ-^{32}P]ATP (Amersham) at concentrations and specific activities specified in the figure legends. Phosphorylation reactions were terminated by methanol precipitation [8]. For determination of phosphorylation kinetics and substrate specificity, phosphorylation was performed either with GlyR (≈2.5 μg) alone or in the presence of 25 μg of casein, histone f_2b, myelin basic protein, or myosin light chain (all from Sigma); aliquots of equal volume were removed after incubation for 2, 5, 10, 25, and 60 min and precipitated with methanol.

2.3. *Gel electrophoresis*

SDS-PAGE of phosphorylated proteins was carried out according to Laemmli [9]. Gels were silver-stained [10], dried and exposed to Kodak X-Omat AR film using intensifying screens.

2.4. *Quantitation of proteins and phosphate incorporation*

The protein content of individual bands on silver-stained SDS-polyacrylamide gels was determined by densitometry with reference to defined amounts of molecular weight standards (Sigma) run in separate lanes using a Hirschmann Elscript 400 densitometer. Subsequently, individual protein bands were excised from the dried gels, and the contents of incorporated phosphate determined by measuring Cerenkov radiation with reference to external [γ-^{32}P]ATP standards. Relative extents of phosphorylation were also determined by densitometry of autoradiograms. It was found that the amount of phosphate detected by Cerenkov counting of gel slices decreased by ≈40% after silver staining; corresponding corrections were made for all phosphate incorporation values reported.

2.5. *Phosphoamino acid analysis*

For phosphoamino acid analysis, published procedures were followed [11].

2.6. *Western blotting*

After SDS-PAGE, proteins were transferred to nitrocellulose and probed with the following antibodies: a monoclonal antibody specific for the 93 kDa protein, mAb GlyR 7a ([12], 1:50); a monoclonal antibody specific for tubulin, DM1A (Sigma; 1:3000); and a polyclonal antiserum specific for the GlyR β subunit (I. Pribilla, unpublished; 1:5000). Bound antibodies were visualized using a secondary antibody coupled to alkaline phosphatase and nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate as chromogenic substrate. Alternatively, horseradish peroxidase-coupled secondary antibody was used in combination with a chemiluminescence system (ECL-System, Amersham).

3. RESULTS

3.1. *A kinase activity is associated with GlyR polypeptides*

GlyR protein was purified from detergent extracts of rat spinal cord membranes by affinity chromatography on strychnine-agarose and subsequent elution with 200 mM glycine. This results in the isolation of polypeptides of 48 kDa and 58 kDa (GlyR α and β subunits) as well as of 93 kDa protein (Fig. 1a; [7]). Upon incubation of the preparation with [γ-^{32}P]ATP, radioactivity was incorporated in the 93 kDa and, to a lower extent, 58 kDa bands (Fig. 1d). To test whether the kinase activity specifically co-purified with GlyR polypeptides, we added casein to phosphorylation assays either containing eluate from a mock-purification performed in the presence of 200 mM glycine, or to purified GlyR. Only negligible casein phosphorylation occurred with the mock-eluate (Fig. 1b) as compared to that seen in the presence of GlyR (Fig. 1c). Thus, the kinase activity appears to be associated with GlyR polypeptides.

The ATP concentration yielding optimal phosphate incorporation into 93 kDa polypeptide was roughly 20 μM, no phosphorylation was detected using [γ-^{32}P]GTP in concentrations between 2 μM and 2 mM (not shown). The presence of several established activators or inhibitors of protein kinases had no significant effect on phosphorylation of the 93 kDa and 58 kDa polypeptides (Fig. 1e–m): Ca^{2+}/phosphatidylserine, Ca^{2+}/calmodulin, cyclic AMP, cyclic GMP, protein kinase inhibitor (an inhibitor of cyclic AMP dependent protein kinase A; [13]), trifluoperazine (an inhibitor of calcium-dependent protein kinases; [14]); H7 (an inhibitor of protein kinase C and cyclic nucleotide-dependent kinases; [15]) or heparin (an inhibitor of casein kinase II; [16]).

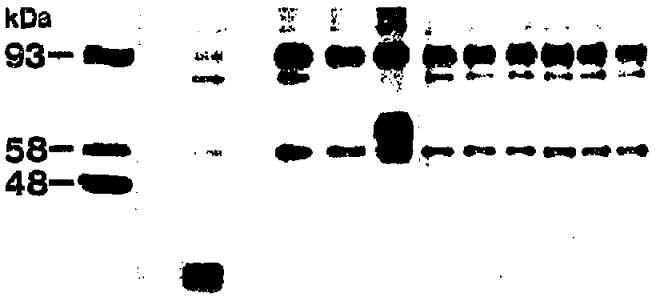

Fig. 1. Phosphorylation of 93 kDa and 58 kDa polypeptides by endogenous kinase. Affinity-purified GlyR was incubated with 200 nM [γ-^{32}P]ATP (100 Ci/mmol) at 37°C for 30 min. Phosphorylated polypeptides were separated by SDS-PAGE and visualized by autoradiography. (Lane a) Silver stained 10% SDS-polyacrylamide gel of purified GlyR. (Lanes b–m) Autoradiograms of radioactively labeled polypeptides. (Lane b) Negligible phosphorylation of casein (5 μg) added to a phosphorylation reaction containing eluate from a mock-purification performed in the presence of 200 mM glycine; (lane c) phosphorylation of casein added to a phosphorylation reaction containing purified GlyR; (lane d) phosphorylation of purified GlyR polypeptides; (lane e) as in lane d, but plus 1 mM $CaCl_2$ and 50 μg/ml phosphatidylserine; (lane f) plus 1 mM $CaCl_2$ and 10 μg/ml calmodulin; (lane g) plus 1 mM cyclic AMP; (lane h) plus 1 mM cyclic GMP; (lane i) plus 5 μg/ml protein kinase inhibitor; (lane k) plus 0.1 mM trifluoperazine; (lane l) plus 0.03 mM H 7; (lane m) plus 100 μg/ml heparin (all from Sigma).

A phosphorylated band at 80 kDa was not seen in the presence of Ca^{2+} (Fig. 1e,f) and another one at 63 kDa was phosphorylated in the presence of Ca^{2+}/calmodulin

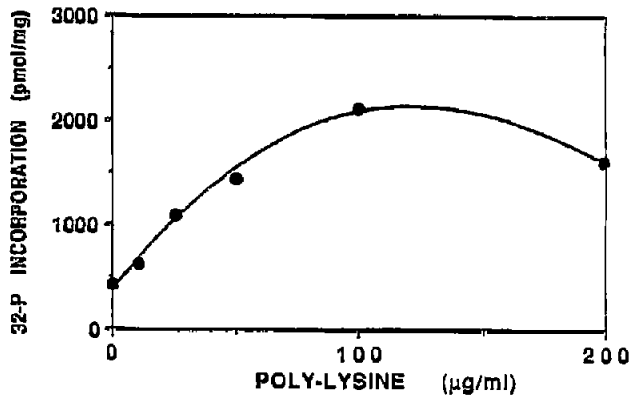

Fig. 2. Stimulation of endogenous kinase activity by poly-lysine. Purified GlyR was incubated with 20 μM [γ-^{32}P]ATP (3 Ci/mmol) in the presence of the indicated concentrations of poly-lysine (Sigma) for 60 min at 37°C. Phosphorylated polypeptides were separated by SDS-PAGE and visualized by autoradiography. Phosphate incorporation was calculated based upon determination of protein and phosphate content as described in Materials and Methods.

(Fig. 1f). These bands are attributed to trace amounts of Ca^{2+} inhibitable or Ca^{2+}/calmodulin-dependent kinases, respectively. As, under these conditions, phosphorylation of the 93 kDa and 58 kDa polypeptides remained unaffected, these observations were not pursued further in this study.

Addition of poly-lysine (100 μg/ml) stimulated phosphorylation of the 93 kDa protein about five-fold; at a higher poly-lysine concentration (200 μg/ml), the extent of phosphorylation decreased again (Fig. 2). Likewise, phosphate incorporation into the 58 kDa polypeptide appeared enhanced (not shown).

The stoichiometry of phosphate incorporation calculated from the radioactivity of electrophoretically separated polypeptide was 0.035 ± 0.013 (mean±SD, n=6) mol of phosphate per mol of 93 kDa polypeptide. (For corresponding data on the phosphorylated 58 kDa protein, see below.) This value increased to 0.19 phosphates per 93 kDa molecule in the presence of 100 μg/ml poly-lysine.

Fig. 3 shows how various purified proteins added to the assay compare to the 93 kDa protein in their efficiency as substrates for the endogenous kinase. The following rank order was obtained: myelin basic protein > casein > histone f_2b > 93 kDa protein > myosin light chain.

3.2. *The endogenous kinase activity phosphorylates co-purifying tubulin*

Previously, we have demonstrated that a significant amount of tubulin co-purifies with GlyR polypeptides upon affinity chromatography and co-migrates with the GlyR β subunit in SDS-PAGE [6]. Fig. 4a shows the silver stain of 93 kDa and tubulin polypeptides which have been partially extracted with sodium cholate from GlyR bound to the affinity-matrix. GlyR polypeptides subsequently eluted with glycine are shown in Fig. 4b.

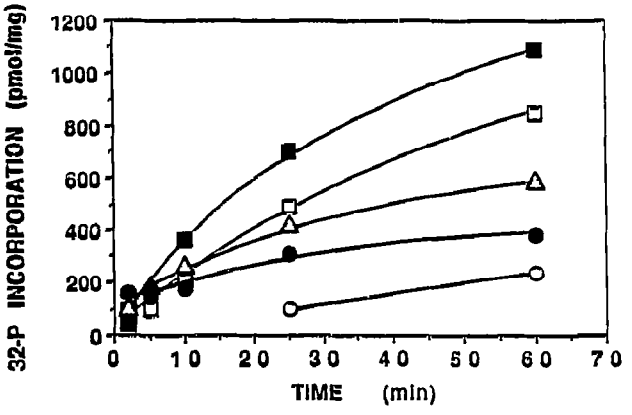

Fig. 3. Time-course of phosphorylation of the 93 kDa protein and of exogenous substrate proteins: (●) 93 kDa protein; (■) myelin basic protein; (□) casein; (△) histone f_2b; (○) myosin light chain. Phosphorylation was performed by incubating purified GlyR with or without the indicated substrate proteins in the presence of 20 μM [γ-^{32}P]ATP (1 Ci/mmol) for the times indicated.

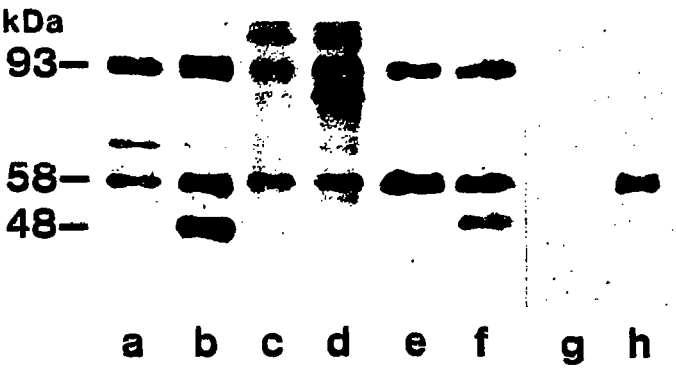

Fig. 4. Identification of tubulin as phosphorylated polypeptide species. GlyR bound to the affinity matrix was washed with buffer containing 1% (w/v) sodium cholate to partially remove 93 kDa protein and tubulin and subsequently eluted with glycine buffer. Both fractions were incubated with 200 nM [γ-^{32}P]ATP (100 Ci/mmol) at 37°C for 30 min. Lanes a, c, e, g correspond to the cholate fraction, and lanes b, d, f, h to the glycine eluate. Lanes: (a and b) silver-stained 10% SDS-polyacrylamide gel; (c and d) audioradiogram showing phosphorylated bands; (e and f) Western blot with a mixture of antibodies mAb7a and DM1A which recognize 93 kDa protein and tubulin, respectively (the band at 48 kDa in lane f arises from crossreactivity of mAb7a with the GlyR α subunit; see [12]); (g and h) Western blot with an antiserum specific for the GlyR β subunit. The absence of the β subunit from the cholate fraction indicates that the phosphorylated 58 kDa band in this fraction corresponds to tubulin.

Upon incubation with [γ-^{32}P]ATP, the polypeptides of 93 kDa and 58 kDa were phosphorylated in both fractions (Fig. 4c,d) although phosphate incorporation appeared to be diminished in the presence of sodium cholate. By Western blot analysis, both fractions were shown to contain tubulin and 93 kDa protein (Fig. 4e,f). As, however, the GlyR β subunit was immuno-detected only in the glycine eluate (Fig. 4g,h), the phosphorylated species of 58 kDa in the cholate fraction (Fig. 4c) is inferred to represent endogenous tubulin (the phosphorylated 58 kDa protein in the glycine eluate (Fig. 4d) may contain GlyR β subunit in addition). The stoichiometry of phosphate incorporation into tubulin was estimated, based upon the tubulin contents of cholate extracts, to be ca. 0.02 mol of phosphate per mol of tubulin monomer, with some variability between different preparations.

3.3 *Phosphorylation occurs on serine and threonine residues*

Phosphoamino acid analysis of phosphorylated 93 kDa and 58 kDa polypeptides isolated by SDS-PAGE revealed that phosphorylation occurred mainly on serine and, to a lesser extent, on threonine residues (Fig. 5). This indicates that the endogenous protein kinase belongs to the serine/threonine class of kinases.

4. DISCUSSION

In this study, we show that a kinase activity endogenous to affinity-purified GlyR preparations phos-

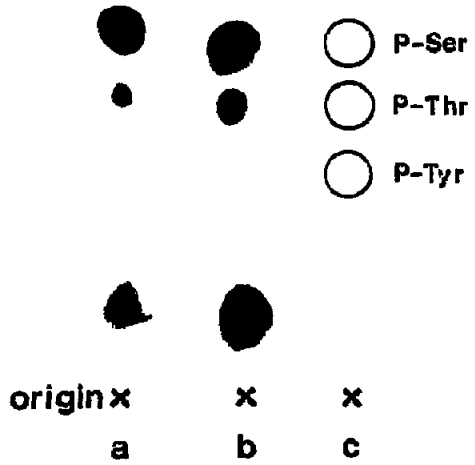

Fig. 5. Phosphoamino acid analysis. After incubation of purified GlyR with 200 nM [γ-^{32}P]ATP (500 Ci/mmol) at 37°C for 60 min, polypeptides were electrophoretically separated and hydrolysed. The phosphate-labelled amino acids were separated by paper electrophoresis and visualized by autoradiography. Lanes: (a) phosphoamino acids from 93 kDa band; (b) phosphoamino acids from 58 kDa band; (c) positions of the internal markers phosphoserine, phosphothreonine, and phosphotyrosine as detected with ninhydrin. (Radioactive spots above the origin correspond to partially hydrolysed phosphopeptides.)

phorylates the associated tubulin-binding 93 kDa protein as well as co-purifying tubulin. Moreover, the kinase activity co-fractionated with the 93 kDa protein and tubulin which were separated from the GlyR.

The 93 kDa protein itself appears unlikely to have kinase activity, as no consensus sequences for ATP binding are found within the amino acid sequence of the 93 kDa protein predicted from cDNAs (P. Prior and B. Schmitt, personal communication). Hence, the kinase activity in our GlyR preparation might reflect substoichiometric binding of a kinase to GlyR associated 93 kDa protein and/or tubulin. The results of Ruiz-Gomez et al. [17], who did not detect endogenous kinase activity in purified GlyR preparations devoid of 93 kDa protein, are consistent with this interpretation.

Several brain-derived kinases have been described which phosphorylate neuronal tubulin: calcium/calmodulin-dependent protein kinase [18]; a casein kinase II associated with microtubular proteins, which utilizes ATP and GTP and is inhibited by heparin [19–21] and a microtubule-associated heparin-insensitive casein kinase I [21]. The endogenous kinase studied here was not regulated by various activators and/or inhibitors of cyclic nucleotide-dependent kinases, calcium/calmodulin-dependent kinases, protein kinase C, or casein kinase II, nor did it accept GTP as a phosphate donor. Further, it phosphorylates myelin basic protein and histone, a property not found for casein kinases [22]. Our kinase activity can therefore not readily be classified. Interestingly, its activity was stimulated by poly- lysine; this behaviour may indicate that this kinase is subject to regulation by a basic protein in vivo [23]. The relatively low levels of phosphate incorporation found here may be due to low amounts and/or activity of the kinase present. In addition, the 93 kDa protein and tubulin in our preparations may already contain high levels of bound phosphate. Indeed, tubulin purified from brain has been shown to be phosphorylated [24], a condition which severely limits the extent of phosphate incorporation in vitro [18]. A similar situation may exist for the 93 kDa protein; its increased mobility in SDS-PAGE after phosphatase treatment supports this interpretation (D.L., unpublished). Attempts to phosphorylate phosphatase-treated 93 kDa protein have, however, so far been unsuccessful.

At present, we can only speculate about the functional significance of 93 kDa protein and tubulin phosphorylation. For the microtubule-associated protein 2 (MAP 2), it has been shown that phosphorylation by a co-purifying cyclic AMP-independent kinase reduces its affinity to microtubules [25]. Conversely, phosphorylation by calmodulin-dependent kinase reversibly renders tubulin incapable of assembly and binding to microtubule-associated proteins [18]. By analogy, phosphorylation of the 93 kDa protein gephyrin in vivo might control its binding to microtubule and thus affect its proposed function as a linker between the GlyR and subsynaptic tubulin.

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