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Fyn kinase contributes to tyrosine phosphorylation of the $GABA_A$ receptor γ2 subunit

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ABSTRACT

Phosphorylation of $GABA_A$ receptors is an important mechanism for dynamically modulating inhibitory synaptic function in the mammalian brain. In particular, phosphorylation of tyrosine residues 365 and 367 (Y365/7) within the $GABA_A$ receptor γ2 subunit negatively regulates the endocytosis of $GABA_A$ receptors and enhances synaptic inhibition. Here we show that Fyn, a Src family kinase (SFK), interacts with the γ2 subunit in a phosphorylation-dependent manner. Furthermore, we demonstrate that Fyn binds within a region of the γ2 intracellular domain that is centered on residues Y365/7, with the phosphorylation of Y367 being particularly important for mediating this interaction. Tyrosine phosphorylation of the γ2 subunit is significantly reduced in the hippocampus of Fyn knockout mice, suggesting that Fyn is an important kinase that contributes to the phosphorylation of this subunit *in vivo*. Tyrosine phosphorylation of the γ2 subunit is not completely abolished in Fyn kinase mice, suggesting that other SFKs, such as Src, also contribute to maintaining and regulating the endogenous phosphorylation level of γ2-containing GABA(A) receptors. In summary, we demonstrate Fyn as one of the SFKs that binds to and phosphorylates the γ2 subunit of the $GABA_A$ receptor. This has important implications for the regulation of synaptic $GABA_A$ receptors via signaling pathways that lead to the activation of Fyn kinase.

Introduction

$GABA_A$ receptors are heteropentameric chloride-selective ligand-gated ion channels that mediate fast synaptic inhibition in the adult central nervous system (Jacob et al., 2008). They are also the therapeutic sites of action for many important classes of drugs, including benzodiazepines, barbiturates and general anesthetics (Rudolph and Mohler, 2004). Multiple subtypes of $GABA_A$ receptors exist, however, the majority of synaptic $GABA_A$ receptors in adult brain are composed of 2α, 2β and 1γ subunit (Olsen and Sieghart, 2009).

Mechanisms that regulate $GABA_A$ receptor function are of critical importance in modulating overall synaptic inhibition. Phosphorylation is one important way in which $GABA_A$ receptor function can be dynamically regulated (Jacob et al., 2008). $GABA_A$ receptor subunits have been shown to be phosphorylated by a number of serine/threonine protein kinases, including protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase G (PKG) (Brandon et al., 2000; Houston et al., 2009; Vithlani and Moss, 2009), in addition to protein tyrosine kinases such as Src (Brandon et al., 2001; Moss et al., 1995; Valenzuela et al., 1995). Phosphorylation sites for these kinases have been mapped to the major intracellular domains (ICD) between transmembrane region (TM) 3 and TM4 of β1–3 and γ2 subunits (Brandon et al., 2000).

We have recently demonstrated an important role for tyrosine phosphorylation of the γ2 subunit in regulating the efficacy of synaptic inhibition and in hippocampal-dependent learning (Tretter et al., 2009). This study was performed using a knock-in mouse in which the two major tyrosine phosphorylation sites within the γ2 subunit (residues Y365 and Y367) were mutated to phenylalanines. Mice heterozygous for this mutation exhibited profound alterations in the membrane trafficking of $GABA_A$ receptors (Tretter et al., 2009). This is consistent with previous *in vitro* studies demonstrating that residues Y365/7 are part of a classical tyrosine-based (Yxxϕ) binding motif for the clathrin adaptor protein 2 (AP2) complex (Kittler et al., 2008). Phosphorylation of these residues negatively regulates clathrin-dependent endocytosis of $GABA_A$ receptors (Kittler et al., 2008).

In our current study, we aimed to identify novel γ2 subunit-interacting proteins whose interaction is positively regulated by phosphorylation. Using a mass spectroscopy-based analysis we identified a protein tyrosine kinase, Fyn, that interacts directly with the ICD of the γ2 subunit in a phosphorylation-dependent manner. Furthermore, we demonstrate that Fyn is an important mediator of tyrosine phosphorylation of the $GABA_A$ receptor γ2 subunit in the hippocampus.

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Results

Fyn kinase interacts with the intracellular domain of the γ2 subunit in a phosphorylation-dependent manner

Since tyrosine phosphorylation of the $GABA_A$ receptor critically affects receptor trafficking and function (Kittler et al., 2008), we were interested in identifying proteins that interact with the receptor in a tyrosine phosphorylation-dependent manner. The γ2 subunit is found in the majority of synaptic $GABA_A$ receptors and it is known to be tyrosine phosphorylated at residues Y365 and Y367 (Moss et al., 1995). These residues are contained within the ICD of the γ2 subunit. Thus, in order to screen for proteins binding to this subunit in a phosphorylation-dependent manner, we synthesized peptides encoding the intracellular region around tyrosine residues 365 and 367 and immobilized them to sepharose beads. One set of peptides was chemically di-phosphorylated on residues Y365 and Y367, while another set of peptides remained unphosphorylated. We then tested the binding of these tyrosine motif peptides in the di-phosphorylated and unphosphorylated forms to proteins found in brain lysates by performing an *in vitro* binding assay, followed by SDS-PAGE and Coomassie blue staining. Numerous proteins were observed to bind to both unphosphorylated and phosphorylated versions of the peptide encoding the region surrounding the tyrosine residues (Fig. 1A). Of greatest interest to us for this study were proteins that bound to the phosphorylated peptide but not the unphosphorylated peptide. In this regard, one major band interacting only with the phospho-peptide was observed at 55 kDa (Fig. 1A). This band was excised and subjected to Maldi-TOF mass spectrometry. The sequence of the protein led to its identification as Fyn kinase.

To confirm that Fyn kinase interacts with the intracellular region of the γ2 subunit in a phosphorylation-dependent manner, we repeated the *in vitro* binding assay. After overnight incubation of the immobilized unphosphorylated and phosphorylated peptides with hippocampal lysates, we performed SDS-PAGE on the bound proteins followed by Western blot analysis using an antibody specific to Fyn kinase (Fig. 1B). This confirmed that Fyn did indeed bind to the phosphorylated γ2 peptide but not to the unphosphorylated peptide or to beads alone (Fig. 1B, lane 3 versus lanes 1 and 2).

To identify whether one or both of the tyrosine residues within the γ2 subunit were important for mediating the binding of Fyn, we designed peptides that were chemically phosphorylated on only one of the two residues (i.e. Y365 or Y367). When we repeated the *in vitro* binding assay using these singularly phosphorylated peptides, we saw that there was a significant reduction in the binding of Fyn to the peptide phosphorylated on residue Y365 compared to the peptide phosphorylated at both Y365 and Y367 (Fig. 1B, lane 4 versus lane 3). No reduction in binding was observed to the peptide singularly phosphorylated on Y367 (Fig. 1B, lane 5). Thus, these results demonstrate that Fyn kinase associates with the ICD of the γ2 subunit in a tyrosine phosphorylation-dependent manner, and furthermore, that phosphorylation at tyrosine residue Y367 within the γ2 subunit is the critical factor in mediating this interaction.

Tyrosine phosphorylation of the γ2 subunit is mediated by Fyn kinase

Tyrosine phosphorylation of the γ2 subunit has been observed in both cultured neurons and rodent brain (Brandon et al., 2001), however the identity of the tyrosine kinases responsible for this phosphorylation remains unknown. Having established that Fyn kinase binds to the intracellular region of the γ2 subunit in a tyrosine phosphorylation-dependent manner, we next set-out to determine whether this kinase is responsible for phosphorylating the γ2 subunit. To do this, we performed experiments using cultured hippocampal neurons that had been pre-treated for 30 min prior to lysis with the tyrosine phosphatase inhibitor, sodium pervanadate (NV). As

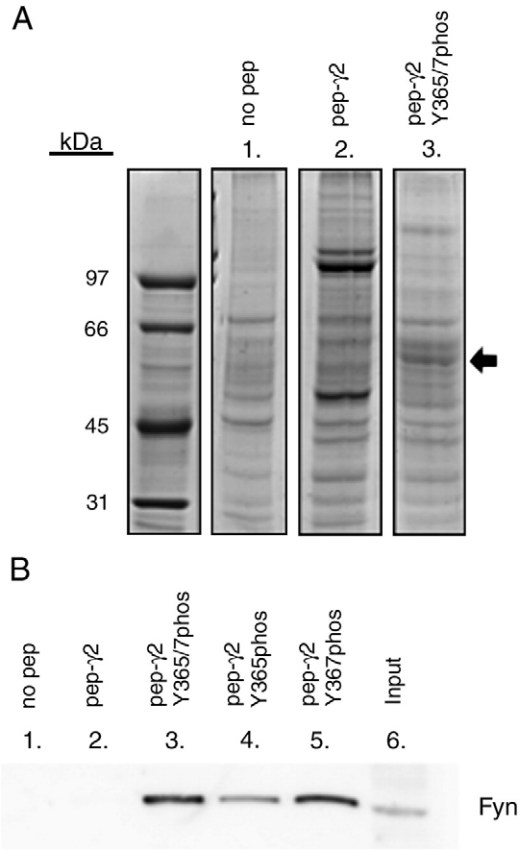

Fig. 1. Phosphorylation-dependent binding of Fyn to the YECL peptide of the γ2 subunit is critically dependent on tyrosine residue 367 within γ2. A. Coomassie blue staining of hippocampal proteins copurifying with the unphosphorylated (lane 2) and di-phosphorylated (lane 3) YECL peptide of the γ2 subunit. An arrow indicates the presence of a protein of 55 kDa that binds to the peptide only when it is phosphorylated (lane 3) but not in the absence of phosphorylation (lane 2) or to beads alone (lane 1). Mass spectroscopy analysis of this band identified the protein as Fyn kinase. B. Western blot analysis using anti-Fyn antibodies reveals a copurification of Fyn from hippocampal lysates with the YECL peptide when it is phosphorylated on Y365/7 (lane 3) but not in the absence of phosphorylation (lane 2) or to beads alone (lane 1). A clear reduction in Fyn binding is observed for the peptide phosphorylated on Y365 (lane 4), whereas phosphorylation of Y367 (lane 5) results in similar binding levels as to the phosphorylated Y365/7 peptide (lane 3).

had been previously established (Brandon et al., 2001), this treatment was necessary to observe robust levels of tyrosine phosphorylation of the γ2 subunit by Western blot analysis (Fig. 2A, lane 2 versus lane 1). Fyn kinase is a member of the Src family kinase (SFK) family and we observed a dramatic reduction in the level of tyrosine phosphorylation of the γ2 subunit upon pre-treatment of cultured neurons with NV and PP2, a pharmacological inhibitor of SFKs, compared to NV treatment alone (Fig. 2A, lane 3 versus 2, and Fig. 2B). Pre-treatment with NV and PP3 (an inactive analog of PP2) resulted in similar levels to NV treatment alone (Fig. 2A, lane 4 versus 2, and Fig. 2B). Thus, this result demonstrates that SFKs are responsible for tyrosine phosphorylation of the γ2 subunit.

SFKs comprise a number of kinases. Five members of this family, namely Src, Fyn, Yes, Lck and Abl, are known to be expressed within the forebrain (Kalia and Salter, 2003; Salter and Kalia, 2004; Sugrue et al., 1990; Yagi et al., 1993). Pharmacological inhibition of the individual kinases within this family is currently not possible. Thus, to unequivocally demonstrate an involvement of Fyn kinase in the phosphorylation of the γ2 subunit, we decided to assess the level of tyrosine phosphorylation within hippocampal lysates derived from Fyn knockout mice. These mice have been widely characterized in previous studies (Grant et al., 1992; Stein et al., 1992). Hippocampal slices were prepared from homozygous Fyn mice and pre-treated