Biochimica et Biophysica Acta 1833 (2013) 110–121

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Phosphorylation and nitration of tyrosine residues affect functional properties of Synaptophysin and Dynamin I, two proteins involved in exo-endocytosis of synaptic vesicles

Cinzia Mallozzi *, Carmen D'Amore, Serena Camerini, Gianfranco Macchia, Marco Crescenzi, Tamara Corinna Petrucci, Anna Maria Michela Di Stasi *

Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, (Italy)

ARTICLE INFO

Article history:
Received 26 July 2012
Received in revised form 8 October 2012
Accepted 21 October 2012
Available online 25 October 2012

Keywords:
Peroxynitrite
Phosphotyrosine
Nitrotyrosine
Src tyrosine kinase
Redox signaling
Synaptic vesicle

ABSTRACT

Phosphorylation and nitration of protein tyrosine residues are thought to play a role in signaling pathways at the nerve terminal and to affect functional properties of proteins involved in the synaptic vesicle (SV) exo-endocytotic cycle. We previously demonstrated that the tyrosine residues in the C-terminal domain of the SV protein Synaptophysin (SYP) are targets of peroxynitrite (PN). Here, we have characterized the association between SYP and *c-src* tyrosine kinase demonstrating that phosphorylation of Tyr_{273} in the C-terminal domain of SYP is crucial in mediating SYP binding to and activation of *c-src*. SYP forms a complex with Dynamin I (DynI), a GTPase required for SV endocytosis, which may be regulated by tyrosine phosphorylation of SYP. We here report that, in rat brain synaptosomes treated with PN, the formation of SYP/DynI complex was impaired. Noteworthy, we found that DynI was also modified by PN. DynI tyrosine phosphorylation was down-regulated in a dose-dependent manner, while DynI tyrosine nitration increased. Using mass spectrometry analysis, we identified Tyr_{354} as one nitration site in DynI. In addition, we tested DynI self-assembly and GTPase activity, which are enhanced by *c-src*-dependent tyrosine phosphorylation of DynI, and found that both were inhibited by PN. Our results suggest that the site-specific tyrosine residue modifications may modulate the association properties of SV proteins and serve as a regulator of DynI function via control of self-assembly, thus influencing the physiology of the exo-endocytotic cycle.

1. Introduction

Neurotransmitter release is triggered by Ca^{2+}-dependent influx at the nerve terminal and relies on specific protein interactions between synaptic vesicles (SVs)[1] and presynaptic terminals resulting in the fusion process. After fusion, SV membrane proteins and lipids in turn are recycled at the periactive zone that surrounds the release site to restore functional SV pools [1]. To work appropriately, the synaptic proteins associate transiently in functional complexes with membrane promoting the correct steps from exo- to endocytosis.

Synaptophysin (SYP) is one of the most abundant integral proteins of SV membrane [2] involved in several steps of synaptic function including exocytosis, synapse formation, biogenesis, and endocytosis of SVs [3–5]. SYP interacts with other synaptic proteins, including Vesicle-Associated Membrane Protein 2 (VAMP2), also known as Synaptobrevin 2 [6], the vesicular proton pump V-ATPase [7], the adaptor protein AP1 [8] and Dynamin I (DynI) [5,9]. However, how the multiple associations of SYP are regulated is still to be clarified.

SYP shares with its homologous Synaptoporin and Pantophysin a common transmembrane topology, with four membrane-spanning domains and a short N- and a long C-terminal tail, both exposed on the cytoplasmic side [10]. SYP C-terminal cytoplasmic tail contains nine pentapeptide repeats, each starting with a tyrosine residue (YG(P/Q)QC) that may be the target of tyrosine kinases within the nerve terminal. Indeed, SYP is phosphorylated *in vitro* by the non-receptor tyrosine kinase *src* [11], but the functional role of its phosphorylation is still unknown [12]. It has been suggested that tyrosine phosphorylation of SYP may regulate its interaction with DynI, a GTPase required for SV endocytosis, since the tyrosine-based pentapeptide motifs are confined to DynI binding sites [5,9]. Actually, SYP forms a complex with DynI *in vivo* in a Ca^{2+}-dependent manner [9] and, recently, optical imaging and electrophysiological studies have provided evidence that the C-terminal cytoplasmic tail of SYP plays a role in facilitating rapid and efficient SV endocytosis [13].

* Corresponding authors. Tel.: +39 0649903674; fax: +39 0649902040.
E-mail addresses: cinzia.mallozzi@iss.it (C. Mallozzi), michela.distasi@iss.it (A.M.M. Di Stasi).

[1] Abbreviations used: PN, peroxynitrite; SYP, Synaptophysin; DynI, Dynamin I; SV, Synaptic Vesicles; VAMP2, Vesicle-Associated Membrane Protein 2; SNARE, Soluble N-ethylmaleimide sensitive fusion proteins Attachment Receptor; pTyr, Phosphotyrosine; NO_2Tyr, Nitrotyrosine; GST, Glutathione-S-Transferase; SH, *Src* Homology; PRD, Proline-Rich-Domain; AD, Alzheimer's Disease; SDS-PAGE, Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis; LC–MS/MS, Liquid Chromatography tandem Mass Spectrometry; PMSF, PhenylMethylSulfonylFluoride; WB, Western Blot.

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http://dx.doi.org/10.1016/j.bbamcr.2012.10.022

These findings indicate that SYP recruits DynI to the release site at nerve terminal and suggest that SYP/DynI complexes are involved in exo-endocytosis (for a review on DynI see [14]).

We previously demonstrated that in rat brain synaptosomes treated with peroxynitrite (PN), a potent oxidizing and nitrating species formed in a diffusion-limited radical–radical reaction between nitric oxide and superoxide anion [15], the tyrosine residues in the C-terminal cytoplasmic tail of SYP were either phosphorylated or nitrated [16]. The formation of 3-nitrotyrosine (NO_2Tyr) in proteins is an *in vivo* post-translational modification induced by nitric oxide-derived oxidants, such as PN [17]. PN-mediated nitration is a selective process where only a small amount of proteins is found nitrated and one or a few tyrosine residues are modified [17]. Because NO_2Tyr are poor substrates for tyrosine kinases, the nitration of a tyrosine residue may down-regulate phosphotyrosine (pTyr)-dependent signaling [18–20]. On the other hand, several *in vitro* studies have shown that PN may promote rather than inhibit tyrosine phosphorylation [21–26]. In the nervous system, protein tyrosine nitration is a major component of PN-dependent cytotoxicity in the course of neurodegenerative disorders and structural neuronal proteins have been found to be key targets for tyrosine nitration. For instance, α-synuclein undergoes oligomerization upon PN-dependent nitration, and oligomerized α-synuclein forms Lewis bodies, the hallmark of Parkinson's disease [27]. In addition, the presence of amyloid β nitrated at Tyr_{10} has been detected in Alzheimer's disease (AD) brain, as well as in brains from mouse models of AD [28]. Protein nitration may also contribute to amyotrophic lateral sclerosis pathogenesis by interfering with the function of specific proteins, protein degradation pathways and phosphorylation cascade [29].

In a previous work, we showed that in rat brain synaptosomes tyrosine-phosphorylated, but not tyrosine-nitrated SYP, binds to *c-src* tyrosine kinase and enhances its catalytic activity. Moreover, we identified by mass spectrometry analysis one nitration site at Tyr_{250} and two phosphorylation sites at Tyr_{263} and Tyr_{273} in the SYP C-terminal cytoplasmic tail [16]. To get additional insights into the role of PN-dependent tyrosine modifications of SYP C-terminal cytoplasmic tail, we have further characterized the functional effects of SYP tyrosine phosphorylation and nitration on *c-src* recruitment and activity. Moreover, we have analyzed the formation of SYP/DynI complex in synaptosomes treated with PN and we have identified a site-specific nitration in DynI at Tyr_{354} by mass spectrometry analysis. Since *c-src*-dependent tyrosine phosphorylation of DynI induces its self-assembly and increases its GTPase activity, we have also investigated the effect of PN on both GTPase activity and self-assembly and found that these functions were impaired. Collectively, our findings advance our understanding of the critical role that PN-induced post-translational modifications (phosphorylation and nitration) play at the nerve terminal in modulating mutual affinities of SYP with two binding partners, DynI and *c-src*, both involved in the exo-endocytotic cycle of SVs.

2. Materials and methods

2.1. Materials

Antibodies were obtained from the following sources: monoclonal anti-phosphotyrosine (pTyr) (clone 4G10) and anti-3-nitrotyrosine (NO_2Tyr) (monoclonal and polyclonal) from Millipore Bioscience Research Reagent (Billerica, MA, USA); anti-SYP (monoclonal and polyclonal) and monoclonal anti-Syntaxin 1 from Synaptic System (Göttingen, Germany); monoclonal anti-*v-src* (Ab-1, clone 327) from Calbiochem (EDM Chemical, Merck, Darmstadt, Germany); monoclonal anti-DynI from BD Biosciences (Franklin Lakes, NJ, USA); monoclonal anti-*lyn* (clone sc-15) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); peroxidase-conjugated goat anti-mouse and goat anti-rabbit from Bio-Rad (Hercules, CA, USA). The antibody against GST was in house-made [30]. Recombinant purified human *src* ($pp60^{src}$) was purchased from Millipore Bioscience Research Reagent. Immunopure-Protein G and Protein A immobilized to Trysacryl were purchased from Thermo Scientific (Rockford, IL, USA). Nitrocellulose paper from Shleicher and Schuell Bioscience Inc. (Dassel, Germany). [γ^{32}P] ATP (>3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA, USA) and Chelex 100 from Bio-Rad. SYP C-terminal-derived peptides containing tyrosine 250 (aa sequence 274–260), tyrosine 263 (aa sequence 261–271) and tyrosine 273 (aa sequence 272–288) were synthesized by NeoMPS PolyPeptide Laboratories (Strasbourg, France). Complete™ protease inhibitor cocktail, EDTA-free, was obtained from Roche Diagnostics (Basel, Switzerland). Commercial PN was purchased from Alexis (Enzo Life Sciences Inc., Farmingdale, NY, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Synaptosomes preparation

Communities Council Directive (86/609/EEC) was formally guided by the Italian Ministry of Health. Synaptosomes were prepared as described by Carlin et al. [31] with minor modifications. Briefly, brain tissue was homogenized in 10 vol (w/v) of Buffer A (0.32 M sucrose, 0.5 mM EGTA, 4 mM Hepes-NaOH, pH 7.4, 10 mM NaF, 1 mM Na_3VO_4 and Complete™) using a Teflon-glass grinder. The homogenate was centrifuged at 1000 ×*g* for 5 min at 4 °C to remove nuclei and debris and the supernatant centrifuged at 13,800 ×*g* for 10 min at 4 °C. The resulting pellet was suspended in Buffer A, stratified on a discontinuous sucrose gradient (0.85, 1 and 1.2 M v/v in Hepes buffered sucrose) and centrifuged at 85,000 ×*g* for 2 h at 4 °C. The layer between 1.0 and 1.2 M sucrose (synaptosomal fraction) was collected and centrifuged at 140,000 ×*g* for 20 min. Protein content was determined by Bicinchoninic Acid Assay (BCA kit, Thermo Scientific, Waltham, MA, USA).

2.2.2. Peroxynitrite treatment

PN was synthesized as described by Radi et al. [32]. The concentration of PN was determined by absorbance at 302 nm in 1.5 M NaOH ($\varepsilon_{302} = 1700\ M^{-1}\ cm^{-1}$). Diluted stock solutions were freshly prepared in 0.1 M NaOH. To avoid metal-catalyzed oxidation of PN, the phosphate buffer was treated extensively with Chelex100 and all samples contained 0.1 mM diethylenetriaminepenta-acetic acid. PN was added as a single bolus directly to synaptosomes (1 mg/ml) in 150 mM phosphate buffer (pH 7.5). After 5 min at room temperature, synaptosomes were washed once with phosphate buffer (pH 7.5). Control experiments were performed with decomposed PN to exclude the participation of contamination products. Decomposed PN was obtained by adding PN to the phosphate buffer for 10 min at room temperature before the addition to synaptosomes. Treatment with 0.5 and 1 mM PN was performed in the presence of 25 mM sodium bicarbonate (at pH 7.5, CO_2 is 1.3 mM) to enable NO_2Tyr formation [33].

2.2.3. Immunoprecipitation and in vitro kinase assay

Immunoprecipitation and *in vitro* kinase assay were performed as previously described [34]. Synaptosomes were solubilized by incubation for 30 min at 0 °C with an equal volume of 4× RIPA buffer (100 mM Tris–HCl, pH 7.5, 0.6 M NaCl, 4% (w/v) Triton X-100, 4% (v/v) Na-deoxycholate, 0.4% (v/v) SDS, 0.4 mM Na_3VO_4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 4 mM PMSF), diluted twice with TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) and then centrifuged at 12,000 ×*g* for 10 min at 4 °C. After preclearing with 20 μl of Protein A/G beads prepared as a 50% (v/v) suspension for 1 h at 4 °C, supernatants were incubated with the appropriate antibodies and the immunocomplexes were precipitated by addition of Protein A or Protein G bead suspensions (50% v/v). The immunoprecipitates were collected by centrifugation and washed twice with RIPA buffer and twice with TBS. Bound proteins were eluted with 4× SDS loading buffer, resolved on SDS-PAGE and