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Ex vivo identification of protein–protein interactions involving the dopamine transporter

Gregory C. Hadlock[a,*], Chad C. Nelson[b], Anthony J. Baucum II.[a], Glen R. Hanson[a], Annette E. Fleckenstein[a]

[a] Department of Pharmacology and Toxicology, University of Utah, Skaggs Hall, 30 South 2000 East, Room 201, Salt Lake City, UT 84121, USA
[b] Mass Spectrometry and Proteomics Core Facility, University of Utah, 20 South 2030 East, Room 470, Salt Lake City, UT, USA

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ABSTRACT

The dopamine (DA) transporter (DAT) is a key regulator of dopaminergic signaling as it mediates the reuptake of extrasynaptic DA and thereby terminates dopaminergic signaling. Emerging evidence indicates that DAT function is influenced through interactions with other proteins. The current report describes a method to identify such interactions following DAT immunoprecipitation from a rat striatal synaptosomal preparation. This subcellular fraction was selected since DAT function is often determined ex vivo by measuring DA uptake in this preparation and few reports investigating DAT–protein interactions have utilized this preparation. Following SDS-PAGE and colloidal Coomassie staining, selected protein bands from a DAT-immunoprecipitate were excised, digested with trypsin, extracted, and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS). From the analysis of the tryptic peptides, several proteins were identified including DAT, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) β, CaMKII δ, protein kinase C (PKC) β, and PKC γ. Co-immunoprecipitation of PKC, CaMKII, and protein interacting with C kinase-1 with DAT was confirmed by Western blotting. Thus, the present study highlights a method to immunoprecipitate DAT and to identify co-immunoprecipitating proteins using LC/MS/MS and Western blotting. This method can be utilized to evaluate DAT protein–protein interactions but also to assess interactions involving other synaptic proteins. Ex vivo identification of protein–protein interactions will provide new insight into the function and regulation of a variety of synaptic, membrane-associated proteins, including DAT.

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1. Introduction

Protein–protein interactions (PPIs) are involved in virtually every cellular process. Within synapses, PPIs facilitate complex and coordinated processes including neurotransmitter release (Sudhof, 1995), signaling complex organization (Huber, 2001), and receptor trafficking (Sheng, 2001). Not surprisingly, membrane-associated proteins, including neurotransmitter receptors and transporters, have many PPIs. For example, multi-protein complexes have been identified for the N-methyl-D-aspartate receptor (Husi et al., 2000), the metabotropic glutamate receptor 5 (Farr et al., 2004), and the β2 subunit of the nicotinic acetylcholine receptor (Kabbani et al., 2007). Identifying the constitutive PPIs of synaptic membrane proteins will provide important insight into the function and regulation of those proteins.

Within the human protein interaction network, there are an estimated 650,000 PPIs (Stumpf et al., 2008), many of which remain to be identified. There are multiple methods to identify PPIs including yeast two-hybrid (Y2H), affinity purification, and co-immunoprecipitation (for review, see Torres and Caron, 2005). While these methods have yielded important information, they are limited in that they often utilize over-expressed, modified, or truncated target proteins which may not represent the protein as it occurs in vivo. Thus, there remains a need to develop additional methodologies to identify PPIs under physiological and pathophysiological conditions.

The present report describes a novel method to identify PPIs involving the dopamine (DA) transporter (DAT). The DAT is a transmembrane protein that transports extracellular DA from the synaptic cleft into the neuron, thereby terminating and regulating dopaminergic signaling. A rat striatal synaptosomal, subcellular fraction was selected for study because DAT function is often determined ex vivo by measuring DA uptake in synaptosomal preparations. A number of DAT-interacting proteins have been identified including protein phosphatase 2A (Bauman et al., 2000), α-synuclein (Lee et al., 2001), protein interacting with C kinase-1

* Corresponding author. Tel.: +1 801 587 9203.
E-mail address: gregory.hadlock@hsc.utah.edu (G.C. Hadlock).

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(PICK1; Torres et al., 2001), Hic-5 (Carneiro et al., 2002), syntaxin 1A (Lee et al., 2004), receptor for activated C kinase 1 (Lee et al., 2004), protein kinase C (PKC; Johnson et al., 2005), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Fog et al., 2006), D2 receptor (Lee et al., 2007), G protein-coupled receptor 37 (Marazziti et al., 2007), and synaptogyrin-3 (Egana et al., 2009). These interactions contribute to the function and regulation of the DAT (for review, see Torres, 2006; Eriksen et al., 2010). The present study highlights a novel method to identify DAT-interacting proteins ex vivo and confirms some of these previously reported interactions with DAT from a synaptosomal preparation. The methodology described herein can be readily adapted to assess interactions involving other synaptic proteins, and thereby provide novel insights into the function and regulation of a variety of synaptic membrane-associated proteins, including DAT.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–450 g; Charles River Laboratories, Raleigh, NC) were maintained under controlled lighting and temperature conditions, with food and water provided ad libitum. Rats were sacrificed by decapitation, and striata were dissected and quickly placed in ice-cold 10 mM HEPES, 0.32 M sucrose, 10 mM N-ethylmaleimide (NEM), pH 7.4 until processing. All procedures were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Utah Institutional Animal Care and Use Committee.

2.2. Immunoprecipitation

Both striata from one rat were homogenized in 2 ml ice-cold 10 mM HEPES, 0.32 M sucrose, 10 mM NEM, pH 7.4 and centrifuged ($800 \times g$; 12 min; 4 °C). Since the DAT contains multiple cysteine residues, a free-sulfhydryl alkylating agent, NEM, was added to this homogenization buffer to prevent spurious disulfide bond formation. The supernatant was then centrifuged ($22{,}000 \times g$; 15 min; 4 °C) to yield a crude synaptosome pellet. The resultant pellet was resuspended at 100 mg/ml original wet weight in 10 mM HEPES, 300 mM NaCl, 1% Triton-X 100, 1 mM PMSF, pH 7.4, and 1:200 protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Following a 1 h incubation at 4 °C with repeated inversion, the mixture was centrifuged ($22{,}000 \times g$; 15 min; 4 °C). Protein A- and G-linked magnetic beads (Invitrogen, Carlsbad, CA) were pre-washed with 10 mM HEPES, 300 mM NaCl, 1% Triton-X 100, pH 7.4. To pre-clear the supernatant of endogenous immunoglobulins, 50 μl protein A- and 50 μl protein G-linked magnetic beads were added per 1 ml of the supernatant. After incubating with repeated inversion (45 min; 4 °C), the protein A- and G-linked magnetic beads were pelleted using a magnet (Invitrogen), and the pre-cleared supernatant was removed and aliquoted. For the DAT immunoprecipitation experiments, 6 μg of a polyclonal rabbit anti-DAT antibody (produced by Sigma–Aldrich using the N-terminally derived amino acid sequence LTNSTLINPPQTPVEAQE (amino acids 42–59)) per 1 mg total protein, or an equal volume of rabbit pre-immune serum (Sigma–Aldrich), was added to the aliquots. Following an overnight incubation at 4 °C with repeated inversion, 50 μl of protein A- and 50 μl of protein G-linked magnetic beads were added to each aliquot and incubated with repeated inversion (45 min; 4 °C). The magnetic beads were then pelleted using a magnet (Invitrogen) and the beads were washed 3–5 times with a buffer containing 10 mM HEPES, 300 mM NaCl, 1% Triton-X 100, 1.0 mM PMSF, pH 7.4. To elute the immunoprecipitated proteins, the magnetic beads were resuspended in 4.5% sodium dodecyl sulfate (SDS), 36% glycerol, 360 mM Tris, pH 6.8, bromophenol blue and 3% (v/v) beta-mercaptoethanol, and heated at 60 °C (10 min). For the Westerns showing co-immunoprecipitation of CaMKII and PICK1 with DAT (Fig. 2), no beta-mercaptoethanol was added to the IP elution buffer because non-specific immunoreactivity from the heavy-chain of the DAT antibody obscures the specific CaMKII or PICK1 immunoreactivity when beta-mercaptoethanol is used. The magnetic beads were pelleted using a magnet (Invitrogen) and the supernatant was frozen at −80 °C until SDS-polyacrylamide gel electrophoresis (PAGE) was performed.

2.3. Western blotting

Equal volumes of the immunoprecipitates and the corresponding controls were loaded into a 4–16% SDS-polyacrylamide gel and electrophoresed using a Hoefer SE 660 gel apparatus (Amersham Biosciences, Piscataway, NJ). Samples were then transferred overnight to a polyvinylidene difluoride (PVDF) hybridization transfer membrane (Perkin-Elmer Life Sciences, Waltham, MA). Each PVDF membrane was blocked for 30 min with Starting Block Blocking Buffer (Pierce Biotechnology, Rockford, IL), and the PVDF membrane was then incubated for 1 h at room temperature or overnight at 4 °C with the primary antibody. Following the incubation, the PVDF membrane was washed 5 times in Tris-buffered saline with Tween (TBST; 250 mM NaCl, 50 mM Tris, pH 7.4, and 0.05% Tween 20). The PVDF membrane was incubated for 1 h with an appropriate horseradish peroxidase conjugated secondary antibody. Following 5 washes in TBST, immunoreactivity was detected using Western Lightning Chemiluminescence Reagents Plus (Perkin-Elmer Life Sciences, Waltham, MA) and the FluorChem SP Imaging System (Alpha Innotech Corporation, San Leandro, CA). The following antibodies were used for Western blotting: PKC (sc17804, Santa Cruz Biotechnology, Santa Cruz, CA), CaMKII (sc5306, Santa Cruz Biotechnology), DAT (sc1433, Santa Cruz Biotechnology), and PICK1 (75-040, NeuroMab, Davis, CA).

2.4. Protein staining

Proteins were visualized, following SDS-PAGE (described above), using a mass spectrometry-compatible silver stain, ProteoSilver Plus (Sigma–Aldrich), or a colloidal Coomassie blue stain, Colloidal Blue (Invitrogen), according to the manufacturer's specifications.

2.5. Protein digest and identification by mass spectrometry

Following SDS-PAGE, selected gel sections were excised, destained in methanol/H_2O, and digested in-gel with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-modified trypsin (Promega; Madison, WI). Trypsin was added (in 50 mM ammonium bicarbonate) in an approximately 1 to 25 ratio (enzyme to protein), and in-gel digestion was allowed to continue overnight (37 °C). Peptides were extracted from the gel slices into a 50% acetonitrile solution. Individual liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analyses of the various tryptic peptides isolated from the selected gel sections were performed using a LTQ-FT hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with nano-liquid chromatography (nanoLC; Eksigent Inc., Dublin, DA) and nano-electrospray (Thermo Fisher Scientific). NanoLC was performed using a C18 nanobore column made in-house (75 μm ID × 10 cm; Atlantis C18, 3 μm particle (Waters Corp., Milford, MA)) at a flow rate of 350 nl/min. 5 μl aliquots of protein digests (corresponding to approximately 10 femtomoles of peptides) were injected on-column and peptides were eluted using a 50 min