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

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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, Ca2+/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.

Keywords

dopamine transporter; protein-protein interaction; immunoprecipitation; mass spectrometry; synaptosome; synapse

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 (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), Ca2+/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.

Materials and Methods

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.

Immunoprecipitation

Both striata from one rat were homogenized in 2ml ice-cold 10 mM HEPES, 0.32 M sucrose, 10 mM NEM, pH 7.4 and centrifuged (800 * 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 * g; 15 min; 4° C) to yield a crude synaptosome pellet. The resultant pellet was resuspended at 100 mg/ml original wet weight in 10mM 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 * 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 (Figure 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-polyacrilamide gel electrophoresis (PAGE) was performed.

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).

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.

Protein Digest and Identification by Mass Spectrometry

Following SDS-PAGE, selected gel sections were excised, destained in methanol/H20, 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 x 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 linear gradient from 5% acetonitrile (with 0.1% formic acid) to 60% acetonitrile (with 1% formic acid). Primary mass spectra of peptides were obtained by Fourier transform ion cyclotron resonance, and peptide molecular masses were measured with mass errors typically less than 2 ppm. Amino-acid sequencing of peptides was performed by MS/MS on peptide molecular ions by low-energy collision-induced dissociation in the linear ion trap of this hybrid instrument, yielding fragment ions with mass errors typically less than 0.2 Da.

MASCOT Database Searching

Proteins were identified using the Mascot search engine (version 2.2.1, Matrix Science, Inc., Boston, MA) based on the combined information of the accurate peptide molecular weight and the corresponding MS/MS sequence information. Protein searches were conducted using the National Center for Biotechnology Information (NCBI) non-redundant database in which predicted “tryptic” peptides were specifically searched. The “MS/MS” Mascot search option was used, including the following parameters: mass data peak lists for Mascot searching were generated using Sequest in Qual Browser software (Excalibur, ThermoElectron Corp.); 5 ppm mass error tolerance for peptide parent mass; 0.5 Da mass error tolerance for peptide fragment ion masses; Mascot threshold cutoffs for acceptable identified peptides typically had MASCOT scores >20, mass errors <3 ppm, and expect values less than 1. However, these cutoffs only generally apply and other criteria can be used to determine if a peptide assignment is correct. The MASCOT scores were determined using a probability-based scoring algorithm (see Perkins et al., 1999 for a thorough description). Briefly, LC/MS/MS-identified tryptic peptide fragment ion masses were searched against the NCBI protein sequence database. If a database sequence entry matched the observed mass, the absolute probability that the match occurred by chance alone was calculated. The score of the identified peptide was calculated as $-10\mathrm{Log}_{10}(P)$ where P is the probability of the match occurring by chance. Higher scores indicated a lower probability that the match was a random event. Total protein scores were defined as the sum of the scores of the unique identified tryptic peptides from an individual protein. Although numerous proteins were identified by LC/MS/MS, only proteins whose co-immunoprecipitation with DAT was confirmed by Western blotting from multiple experiments were reported in Table 1.

Results

Results presented in Fig. 1A demonstrate the immunoprecipitation of DAT from rat striatal synaptosomes. Multiple potential DAT-interacting proteins also co-immunoprecipitated with the DAT as indicated by discrete silver-stained protein bands present in the immunoprecipitation lane and not in the pre-immune lane (Fig. 1B).

Following SDS-PAGE and colloidal Coomassie staining of the gel, discrete bands present in the DAT-immunoprecipitation lane and not in the pre-immune lane were excised and separately analyzed by LC/MS/MS (indicated by lines labeled “Band 1” and “Band 2” in

Fig. 1B). Table 1 lists proteins identified including DAT, PKC β, and PKC γ from Band 1 and CaMKII β CaMKII δ from Band 2. Table 1 also list the respective NCBI accession numbers for the identified proteins, the number of unique tryptic peptides identified for each protein, and the corresponding MASCOT scores (see METHODS for description of MASCOT scoring).

Results presented in Fig. 2 demonstrate the co-immunoprecipitation of proteins reported previously to interact with DAT including PICK1 (Torres et al., 2001), CaMKII (Fog et al., 2006), and PKC (Johnson et al., 2005).

Discussion

Immunoprecipitation and mass spectrometry based approaches are increasingly utilized to identify central nervous system PPIs ex vivo. For example, interacting proteins of the N-type calcium channel CaV2.2 were identified from a purified synaptosomal fraction prepared from whole rat brains (Khanna et al., 2007). Also, a number of potential DAT-interacting proteins were identified from mouse striatal lysates (Maiya et al. 2007), although no previously reported DAT-interacting proteins were detected. However, many proteins, including DAT, are not uniformly expressed throughout the central nervous system, and thus additional methods are necessary to identify PPIs that may be unique to a particular brain region or even subcellular localization. Accordingly, the present study describes a novel method to immunoprecipitate DAT and DAT-interacting proteins from rat striatal synaptosomes followed by identification of DAT-interacting proteins by LC/MS/MS and Western blotting.

Utilization of a rat striatal synaptosomal fraction to identify potential DAT-interacting proteins is an important aspect of this study. This fraction was chosen for a number of reasons. First, DAT is highly expressed in the striatum (Ciliax et al., 1999). Second, synaptosomes preserve many of the main structural features of intact nerve terminals (Whittaker et al., 1964). Third, measuring DA uptake in synaptosomal preparations is a common, well-established technique utilized to assess DAT function. Fourth, the crude synaptosomal fraction likely retains some endogenous post-translational modifications that may be necessary for some DAT PPIs. Fifth, immunoprecipitating from a synaptosomal fraction may reduce the potential for some non-specific interactions (e.g., with non-synaptic proteins removed in the initial centrifugation steps). However, one caveat to this latter point is that artifactual PPIs may be created during the processes involved in the isolation of this fraction. The utility of the present approach to co-immunoprecipitate DAT-interacting proteins was verified by the identification of the previously identified DAT-interacting proteins PICK1 (Torres et al., 2001), PKC (Johnson et al., 2005), and CaMKII (Fog et al., 2006). In the present study, the antibodies used for Western blotting to identify PKC and CaMKII (Santa Cruz Biotechnology; sc17804 and sc5306 respectively) detect all isoforms of their respective target proteins, and the interaction of DAT with particular isoforms was therefore not specifically confirmed. The utility of the LC/MS/MS technique following excision of bands unique to the DAT immunoprecipitation was verified by the detection of multiple peptides including from DAT, PKC β, PKC γ, CaMKII β, and CaMKII γ. Of the proteins identified by LC/MS/MS, only proteins whose co-immunoprecipitation was confirmed by Western blotting from multiple experiments were included in Table 1.

The identification of PPIs using immunoprecipitation-based approaches represents a powerful tool to investigate the function and regulation of various proteins. The LC/MS/MS methods used here permits the highly accurate identification of proteins at extremely low levels (i.e., attomole to femtomole amounts injected onto the column) allowing for the identification of immunoprecipitated proteins with low abundance. Limitations to this

strategy include detection of false-positives arising from the formation of non-specific PPIs formed by the mixing different neuronal compartments while isolating synaptosomes and/or the detergent-based extraction of the DAT-protein complexes from synaptosomes. Further, immunoprecipitation-based identification of PPIs requires suitable antibodies for the target protein and does not discriminate between direct and indirect protein interactions. Another limitation is that this approach only detects stable interactions. However, chemical crosslinking strategies have been used to detect labile or transient PPIs (for review, see Sinz, 2010) and could potentially be used in synaptosomes. In identifying PPIs, multiple approaches, including co-localization, reciprocal immunoprecipitation, etc., are necessary to confirm identified PPIs. Functional studies can then be performed to assess the functional relevance of the identified PPIs and their role in the regulation of those proteins.

In conclusion, we have developed a novel and straightforward method to immunoprecipitate DAT from rat striatal synaptosomes and to identify DAT-interacting proteins by LC/MS/MS and Western blotting. The validity of this model was confirmed by replicating previous reports of specific DAT-protein interactions. This method could also be used to identify PPIs of other membrane-associated and synaptosomal proteins or to confirm PPIs identified using other techniques. Identifying additional PPIs will provide insights into mechanisms underlying DAT function and regulation and could represent new avenues of research and novel therapeutic targets for DA-related disorders.

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RESEARCH HIGHLIGHTS

- description of a method to immunoprecipitate DAT and identify potential DAT protein-protein interactions ex vivo by Western blot and LC/MS/MS
- DAT can be immunoprecipitated from striatal synaptosomes and numerous DAT co-immunoprecipitating proteins are detected by silver-stain
- CaMKII, PKC, and PICK1, co-immunoprecipitate with DAT
- DAT, CaMKII, and PKC are identified from the immunoprecipitate by LC/MS/MS

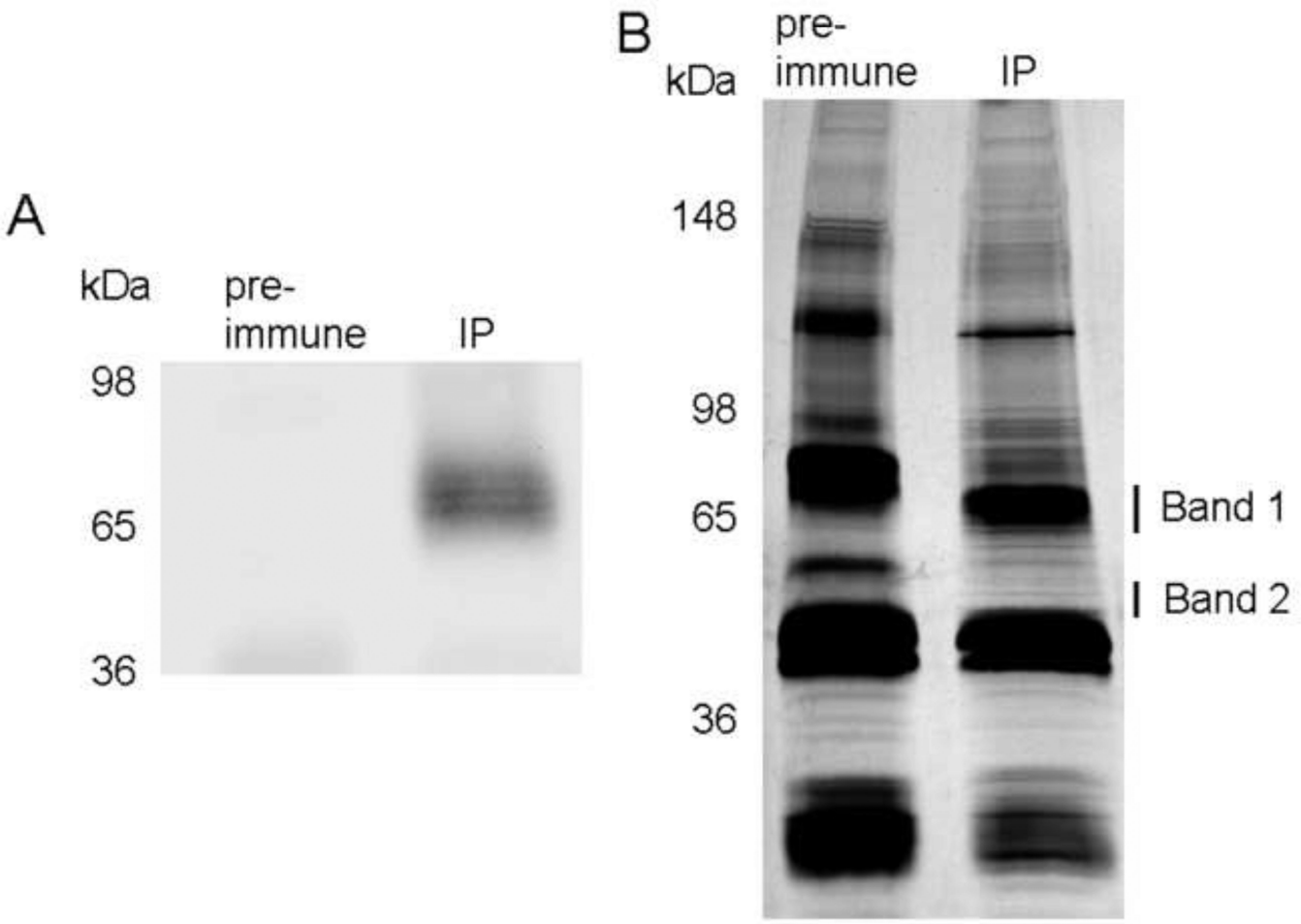

Figure 1.

Immunoprecipitation of DAT and DAT-interacting proteins from rat striatal synaptosomes. DAT was immunoprecipitated from rat striatal synaptosomes. Following SDS-PAGE, the immunoprecipitates were analyzed by Western blotting for DAT (A) or silver-stained for total protein levels (B). A, DAT immunoreactivity was detected in the DAT-immunoprecipitation (IP) lane and was not detected in the immunoprecipitation lane produced with rabbit pre-immune serum (pre-immune). B, numerous discrete protein bands are present in the DAT-IP lane that are not present in the IP lane produced with rabbit pre-immune serum (pre-immune).

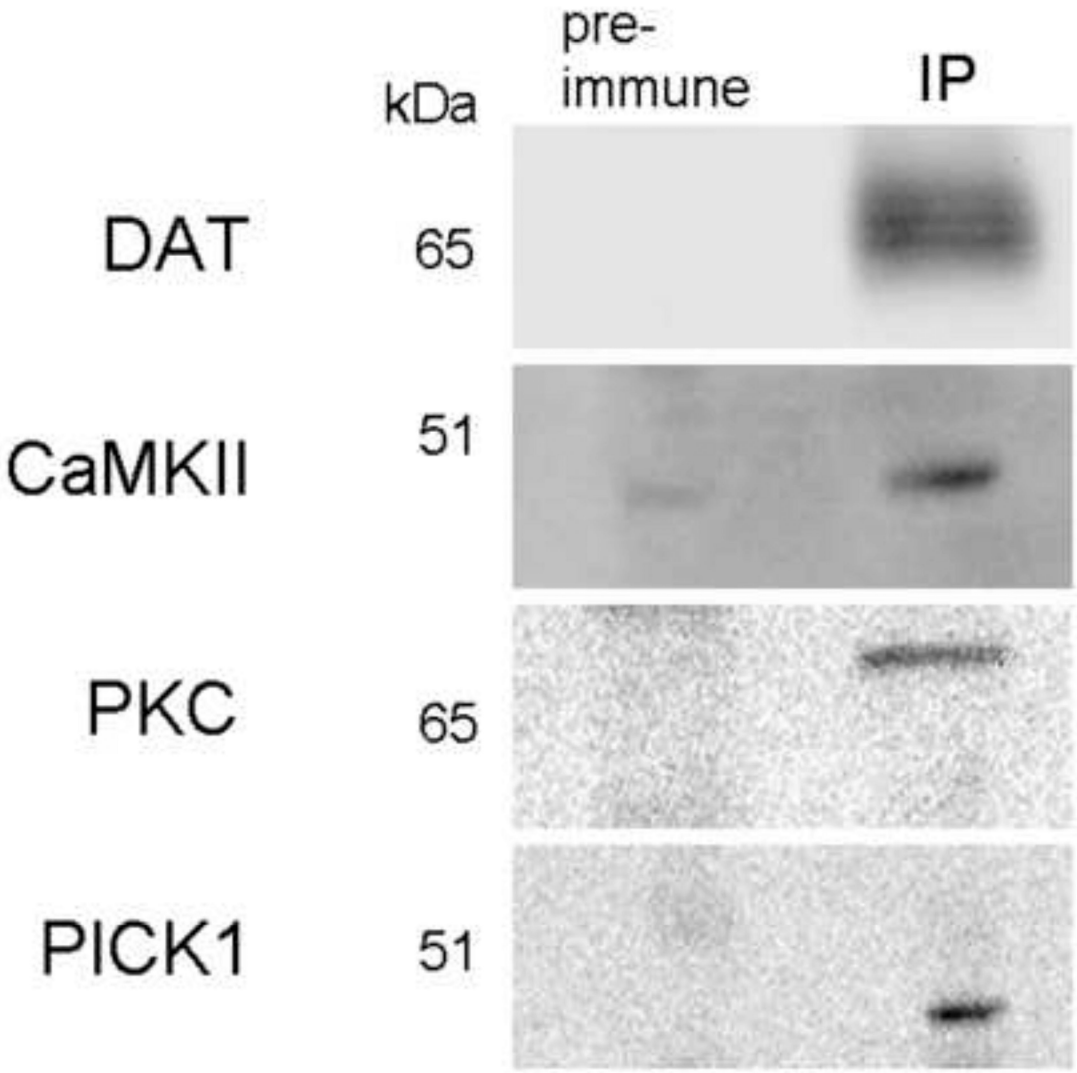

Figure 2.

Co-immunoprecipitation of potential DAT-interacting proteins. DAT was immunoprecipitated from rat striatal synaptosomes. Co-immunoprecipitation of PICK1, CaMKII, and PKC was verified by immunoreactivity present in the DAT-IP lane and not detected in the IP lane produced with rabbit pre-immune serum (pre-immune).

Table 1

DAT and several potential DAT-interacting proteins were identified by LC/MS/MS following immunoprecipitation of DAT from rat striatal synaptosomes.

NCBI accession number	protein	No. of unique peptides	MASCOT SCORE
gi|7106413	dopamine transporter (1)	8	217
gi|76880457 gi|206189	protein kinase C β (1) protein kinase C II (1)	13	370
gi|6755080	protein kinase C γ (1)	8	237
gi|125287	Ca2+/calmodulin dependent protein kinase II-β (2)	5	153
gi|6978595	Ca2+/calmodulin dependent protein kinase II-δ (2)	2	128

Following immunoprecipitation of DAT and SDS-PAGE, the immunoprecipitates were stained with Colloidal Coomassie, and the gel sections (indicated by lines adjacent to the IP lane in Figure 1B) were excised, digested in-gel with trypsin, and analyzed by LC/MS/MS. Table 1 lists the identified proteins, their NCBI accession numbers, the number of unique peptides identified for each protein, and the corresponding MASCOT scores (see METHODS for description of MASCOT scoring). The excised gel section from which the tryptic peptides were identified is indicated in parentheses next to the protein name.