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Protein synthesis in synaptosomes: a proteomics analysis

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Abstract

A proteomics approach was used to identify the translation products of a unique synaptic model system, squid optic lobe synaptosomes. Unlike its vertebrate counterparts, this preparation is largely free of perikaryal cell fragments and consists predominantly of pre-synaptic terminals derived from retinal photoreceptor neurones. We metabolically labelled synaptosomes with [^{35}S]methionine and applied two-dimensional gel electrophoresis to resolve newly synthesized proteins at high resolution. Autoradiographs of blotted two-dimensional gels revealed *de novo* synthesis of about 80 different proteins, 18 of which could be matched to silver-stained gels that were run in parallel. In-gel digestion of the matched spots and mass spectrometric analyses revealed the identities of various cytosolic enzymes, cytoskeletal proteins, molecular chaperones and nuclear-encoded mitochondrial proteins. A number of novel proteins (i.e. not matching with database sequences) were also detected. *In situ* hybridization was employed to confirm the presence of mRNA and rRNA in synaptosomes. Together, our data show that pre-synaptic endings of squid photoreceptor neurones actively synthesize a wide variety of proteins involved in synaptic functioning, such as transmitter recycling, energy supply and synaptic architecture.

Keywords: local protein synthesis, mass-spectrometry, pre-synaptic, proteomics, squid, synaptosomes.

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In neurones, translocation of somatic mRNA and subsequent translation in dendrites and nerve terminals is considered crucial for neurotransmission (Martin *et al*. 2000). The evidence for this hypothesis comes from two main experiments. First, using hippocampal slices, it was demonstrated that neurotrophin-evoked potentiation of synaptic transmission is dependent on local protein synthesis (Kang and Schuman 1996). Second, serotonin-induced long-term facilitation in cultured sensory neurones of *Aplysia* was shown to be dependent on pre-synaptic protein synthesis (Martin *et al*. 1997). These studies build on earlier experiments showing that a selective population of mRNA together with the co-translational machinery, including ribosomes, is targeted to axons (van Minnen 1994; Koenig and Giuditta 1999) and dendrites (Steward and Worley 2001). Additional studies have led to the identification of synaptic proteins that are locally synthesized (Crispino *et al*. 1993a; Gioio *et al*. 2001), and have made it clear that extrasomatic mRNAs can be locally translated (Crino and Eberwine 1996; van Minnen *et al*. 1997). However, the identities of most proteins that are synthesized in synaptic domains remain elusive; therefore, their contribution to synaptic function and plasticity has not been established.

Synaptosomes from the mammalian brain seem to be suitable for the design of experiments aimed at analysing synaptic protein synthesis. These synaptosomes consist of pre-synaptic elements with attached post-synaptic structures, that retain their cytoplasmic contents, including polysomes and mRNAs (Rao and Steward 1991; Weiler *et al*. 1997). However, a major objection to the use of mammalian synaptosomes is that they cannot be purified to such an extent that they are free of contamination with fragments of

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Abbreviations used: 2DE, two-dimensional gel electrophorsis; DTT, dithiothreitol; HSP, heat shock protein; MS, mass spectrometry; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SSC, sodium–saline citrate buffer.

neuronal cell bodies and glial processes. Such contamination is evident by the presence in synaptosomal preparations of the glial cell-specific mRNA encoding glial fibrillary acid protein (for review see Steward and Worley 2001).

By contrast, synaptosomal preparations from squid optic lobes do not suffer from these experimental confounds. The purity of this preparation has been exhaustively demonstrated previously using biochemical, molecular biological and electron microscopic techniques (Crispino *et al*. 1993b, 1997; Gioio *et al*. 2001). Furthermore, these synaptosomes can easily be obtained in sufficient quantities for large-scale protein screening (Crispino *et al*. 1993b). For these reasons we decided to use squid synaptosomal preparations as a suitable model for a proteomics analysis of synaptic protein synthesis. Briefly, we metabolically labelled synaptosomes and applied two-dimensional gel electrophoresis (2DE) to detect *de novo* synthesized proteins at high resolution. We next applied mass spectrometry (MS) to identify the proteins. To corroborate our findings we used molecular biological techniques to analyse synaptosomal mRNA, and *in situ* hybridization to confirm its synaptosomal location.

Among the synaptosomal translation products we identified cytoskeletal proteins, such as α- and β-tubulin, β- and γ-actin, various enzymes, nuclear-encoded mitochondrial proteins, a molecular chaperone and several novel proteins. Our results expand previous observations (Crispino *et al*. 1997; Martin *et al*. 1998) and show that many proteins are synthesized in squid pre-synaptic endings. In addition, they demonstrate that a proteomics approach is a powerful strategy to investigate protein synthesis in synaptosomes.

Experimental procedures

Animals and tissue fractionation

Optic lobes of adult squid (*Loligo vulgaris* or *Loligo pealii*) were dissected and washed in ice-cold filtered seawater and treated as described (Crispino *et al*. 1997). Briefly, a 10% tissue homogenate in 0.7 M sucrose, 20 mM Tris–HCl (pH 7.4), was cleared of nuclei and large particles by centrifugation in rotor JA-20 of a J2–21 M Beckman centrifuge (700 *g*, 11 min at 4°C). The supernatant was subsequently centrifuged in the same rotor at 11 000 *g*, 30 min at 4°C. This yielded a sedimented pellet (mitochondrial fraction), a floating particulate layer (synaptosomal fraction) and an opaque supernatant that contained microsomes and cytosol (microsomal fraction). The synaptosomal fraction was collected by decantation, washed several times with homogenizing medium, resuspended in the same medium and used for metabolic labelling, and polysome and RNA preparations.

The microsomal fraction from squid optic lobes was prepared from a 10% tissue homogenate in a buffer designed to preserve the structure of polysomes (polysomal buffer). The buffer contained 0.32 M sucrose, 25 mM Tris–HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 10 U/mL RNasin (Promega, Madison, WI, USA). Following centrifugation of the homogenate (see above), the opaque supernatant containing microsomes and cytosol was centrifuged at 2°C in the SW41 rotor of a Beckman ultracentrifuge (200 000 *g*, 2 h) to pellet the microsomal fraction.

Total RNA was isolated from the synaptosomal and microsomal fractions by resuspension in TRISOL reagent (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

Metabolic labelling and sample preparation

Three synaptosomal fractions were obtained from different samples of optic lobes (1 g each). A suitable aliquot of each fraction was incubated at a protein concentration of 50 μg/mL in 4 mL artificial seawater (460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 11 mM $CaCl_2$, 0.5 mM $KHCO_3$, 10 mM Tris–HCl, pH 7.8) containing 25 μCi/mL [^{35}S]methionine (NEN Life Science Products, Boston, MA, USA). At the end of the incubation period (90 min at 18–20°C), synaptosomes were collected by centrifugation (4°C; 30 min at 150 000 *g* in rotor SW55 of a Beckman ultracentrifuge), gently washed in cold ASW to remove the radiolabelled precursor, and stored at −80°C until analysis. The remaining unlabelled synaptosomes were also frozen.

Two-dimensional gel electrophoresis and electroblotting

For 2DE, labelled and unlabelled synaptosome pellets were resuspended in 2DE solubilization buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, 20 mM Tris, 65 mM DTT and 0.5% ampholytes (3–10 NL, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The samples were further solubilized with 10 strokes in a glass-glass homogenizer, followed by brief sonication (1 × 10 s). A modified Bradford procedure was used to determine the protein concentration of the samples. Next, 100 000 counts of each labelled synaptosome sample was mixed with unlabelled synaptosomes yielding ∼300 μg protein per sample, and submitted to 2DE and autoradiography ($n = 3$). For silver staining, an equal amount of unlabelled synaptosomes (∼300 μg protein) was subjected to 2DE ($n = 3$).

Immobilized pH gradient gels (IPG strip, Immobiline DryStrip, pH range 3–10-non-linear, Amersham Pharmacia Biotech) were rehydrated with 370 μL of solubilized synaptosomes and focussed overnight (65 kVh, 20°C) using an IPG-Phor (Amersham Pharmacia Biotech). Prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), IPG strips were equilibrated in 6 M urea, 2% SDS, 65 mM DTT, 50 mM Tris (pH 8.4), 30% glycerol for 15 min, followed by equilibration in 6 M urea, 2% SDS, 140 mM iodoacetamide, 50 mM Tris (pH 8.4), 30% glycerol for 15 min. The second dimension separation was carried out using the Isodalt System (Amersham Pharmacia Biotech) in 1.5 mm 10% Laemmli gels (Duracryl, Genomic Solutions, Ann Arbor, MI, USA), run at 25 mA per gel for 16 h at 15°C. Immediately after electrophoresis, gels were fixed in 5% acetic acid, 50% methanol for 1 h, and then stained with a silver staining procedure that is compatible with mass spectrometric analysis (Shevchenko *et al*. 1996). All the silver-stained gels were scanned using a CCD camera (FluorS, Biorad, Hemel Hamstead, UK). For transblotting followed by autoradiography, gels were incubated in about five volumes of transblot buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 15% methanol), and electroblotted to nitrocellulose paper at 400 mA for 5 h using the Isodalt System containing the transblot buffer. After transblotting, the nitrocellulose papers were air dried and then exposed in a Phospho-Imager (Bio-Rad Laboratories, Hercules, CA, USA) to

visualize the radiolabelled proteins. Protein patterns in the gels and the blots were compared manually by overlaying blow-ups of different gel areas using landmark spots that were clearly identifiable by spot shape and/or pattern.

Protein identification by mass spectrometry

Protein spots were manually excised from the gel, washed, and in-gel digested by trypsin (at 12.5 ng/μL, overnight at 37°C) as described (Shevchenko *et al*. 1996), with some minor modifications. Tryptic peptides were extracted from the gel pieces with two volumes of water followed by two extractions with two volumes of 5% formic acid, 50% acetonitrile. The three extraction volumes were combined in the same tube and partially lyophilized to about 20 μL. Subsequently, the extract was loaded into a homemade poros microtip extraction column, and the bound tryptic peptides were eluted with 10 μL 60% methanol, 5% formic acid. The eluent was loaded into a nano-electrospray capillary, which was pulled from borosilicate glass capillary GC 100F-10 with a microcapillary puller. All the measurements were performed in an electrospray (ESI) Q-TOF tandem mass spectrometer (Micromass Inc., Manchester, UK) as described previously (Nagle *et al*. 2001). In brief, an internal wire electrode inserted inside the capillary was used for the mass spectrometric measurement. For single-stage mass spectrometry (MS), the quadrupole was operated in the rf-only mode and mass analysis was performed using the TOF analyser. For tandem MS, precursor ions were selected using the quadrupole, fragmented in the collision chamber using energies between 20 and 30 eV and argon as the collision gas, and the daughter ions were detected by the TOF analyser.

Isolation and fractionation of synaptosomal polysomes

Polysomes were isolated as described previously (Giuditta *et al*. 1991). Briefly, the synaptosomal fraction was homogenized in polysomal buffer (see the first subsection), the homogenate was adjusted to 0.5% (v/v) for both Triton X-100 and sodium deoxycholate and was clarified by centrifugation in an Eppendorf centrifuge (11 000 *g*, 10 min, 4°C). Polysomes were purified from the supernatant fraction by sedimentation through a 2-M sucrose layer (0.1 mL) prepared in 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 10 mM DTT (TKMD buffer). Centrifugation was in a SW 50.1 rotor of a Beckman ultracentrifuge (120 000 *g* for 4 h at 4°C). The polysome pellet was resuspended in TKMD buffer, layered on a linear 15–45% (w/v) sucrose gradient (5.0 mL) in TKMD buffer, and centrifuged in rotor SW 50.1 (120 000 *g*, 1 h, 4°C). The gradient was divided into monosome and polysome fractions based on UV absorbance (254 nm) using an ISCO gradient fractionator. RNA was extracted from the monosome and polysome fractions using TRISOL reagent (Sigma, St Louis, MO, USA).

Under these experimental conditions, greater than 99.9% of the free mRNA present in the tissue samples are eliminated from the polysome fraction (Kaplan and Gioio 1986). The integrity and biological activity of the polysome preparation has also been established by *in vitro* radiolabelling experiments (Giuditta *et al*. 1991), as well as cell-free translation analysis (Crispino *et al*. 1997).

RT-PCR analyses

cDNA was reverse transcribed from 0.5 μg purified RNA samples using gene-specific primers for the squid sodium channel (Gioio *et al*. 1994) or heat-shock protein 70 (HSP70) (Gioio *et al*. 2001). The samples were loaded onto a 2% agarose gel and visualized with ethidium bromide.

In situ hybridization

Pelleted synaptosomes were fixed in 4% paraformaldehyde, 460 mM NaCl, 10 mM KCl, and 20 mM Tris–HCl (pH 7.4), dehydrated in a graded series of ethanol and embedded in paraffin. Five-micrometer sections were dewaxed and processed for *in situ* hybridization. Digoxigenin-labelled sense and antisense RNA, complementary to the coding region of HSP70 (Gioio *et al*. 2001) and 28S rRNA (Bauman and Bentvelzen 1988) was synthesized from linearized pGem-t easy (HSP70) or pBluescript phagemids (van Kesteren *et al*. 1996), respectively, using the appropriate RNA polymerases and digoxigenin-UTP labelling mixture (both from Boehringer Mannheim, Mannheim, Germany). The *in situ* hybridization procedure is described in detail elsewhere (van Minnen *et al*. 1997). Briefly, sections were hybridized at 55°C in a hybridization mixture containing 60% formamide, 4 × sodium–saline citrate buffer (SSC), 1 × Denhardt's solution, 0.25 mg/mL denatured salmon sperm DNA, 1 mM phosphate buffer, pH 7.4, 100 mg/mL dextran sulfate and 2 ng/μL digoxigenin-labelled cRNA probe. After 16 h of hybridization, the sections were washed stringently (up to 2 × SSC containing 50% formamide at 50°C), and treated with RNase A to remove non-specifically bound probe-RNA. Hybrids were visualized using alkaline phosphatase-conjugated antidigoxigenin antiserum (Boehringer Mannheim) according to the manufacturer's instructions.

Results

Purity of the squid synaptosome preparation

To determine the degree of contamination of the squid synaptosomal fraction with microsomes, we analysed synaptosomes for the presence of a mRNA present in the cell bodies of the squid giant axon, but absent from the axon compartment. This mRNA encoded a squid Na^+ channel, and was shown to be expressed abundantly in the cell bodies of the giant axon, but absent from the giant axon (Gioio *et al*. 1994; Chun *et al*. 1996). As shown in Fig. 1, amplicons generated from Na^+ channel primers are readily detected in the microsomal fraction, which is derived from the optic lobe perikarya, but are largely absent from the synaptosomal fraction after 35 cycles of PCR amplification (Fig. 1, lane 3). The trace levels of Na^+ channel amplicons in the synaptosomal reactions establish that the synaptosomal fraction is relatively free of microsomal contamination. In this regard, it bears mention that these trace levels of mRNA contamination do not contribute to the protein synthetic activity of the synaptosome fraction as subsequent labelling experiments were conducted in artificial seawater, a media which lacks on energy generating system, tRNAs, and the appropriate translation factors (Crispino *et al*. 1993b).

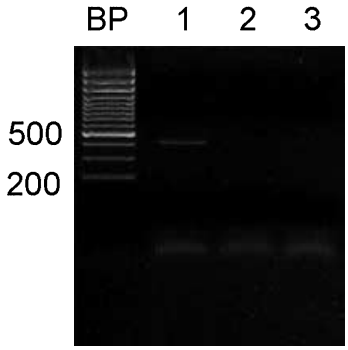

Fig. 1 RT-PCR analysis using squid sodium channel primers. Amplicons are readily detected in the microsome fraction only, but are barely discernable in synaptosomes. BP: base-pair marker; Lane 1: RNA isolated from squid microsomes; Lane 2: RNA isolated from the monosome fraction of purified synaptosomal polysomes; Lane 3: RNA isolated from the polysome fraction of purified synaptosomal polysomes.

Identification of *de novo* synthesized proteins in synaptosomes

To analyse protein synthesis in squid synaptosomes, we incubated purified synaptosomes for 90 min in artificial seawater containing [^{35}S]methionine and processed the samples for 2DE. After autoradiographic exposure for 7 days in a phospho-imager, about 80 spots were visible on the autoradiograms of synaptosome preparations obtained from three different preparations (a representative example is shown in Fig. 2a). These results reveal the complexity of the extrasomal translation products and correspond well with previous studies demonstrating that synaptosomes sustain endogenous protein synthesis (Crispino *et al*. 1997). The profile of *de novo* synthesized proteins was very similar in the three synaptosomal preparations, as only minor individual differences were observed for a few spots.

Using silver-staining, synaptosome 2DE gels were found to contain about 300 spots (Fig. 2b). All gels were run in a single batch, and the protein profiles were highly similar, thus readily permitting intergel spot correlation. Comparison of the autoradiograms with the protein profiles of silver-stained gels revealed partly overlapping patterns (Fig. 2a,b). Eighteen radiolabelled spots could reliably be matched to their corresponding spots on silver-stained gels, while the remainder were below the detection level of the silver-stain procedure and were not considered further. The matched silver-stained spots were excised, in-gel digested with trypsin, and the resulting peptide mixtures were analysed by ESI-(tandem) MS and database searching. The results are summarized in Table 1.

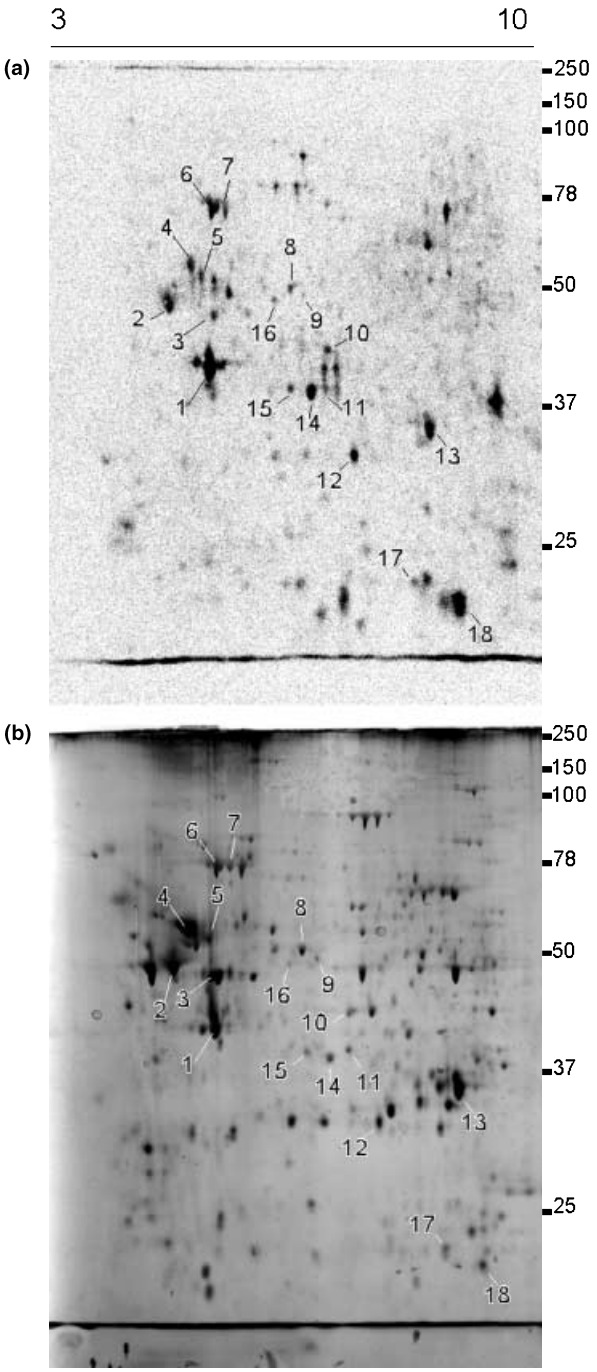

Fig. 2 Synaptosome proteins from squid resolved by 2DE. Autoradiogram (a) and silver-stained (b) two-dimensional gels of a synaptosome preparation from one squid optic lobe. (a) Metabolic labelling with [^{35}S]methionine of squid synaptosomes *in vitro* reveals about 80 newly synthesized proteins. (b) The silver stain protein pattern of squid synaptosomes reveals about 300 spots. The numbered spots present on both the autoradiograms and silver-stained gels indicate spots that were excised from the silver stained gel for in-gel digestion and mass spectrometric analysis. A total of 300 μg of protein was loaded on each gel. In the horizontal dimension, proteins were focussed to 65 kVh in a non-linear immobilized pH 3–10 gradient gel. In the vertical dimension proteins were separated in a 10% SDS–PAGE gel. The location of the molecular weight markers (kDa) is indicated along the *y*-axis of each gel.

Table 1 Peptide mapping and MS/MS sequencing of [^{35}S]methionine-labelled synaptosomes

Spot no.	Protein name	Acc. No.	No. peptides matched	Theoretical Mw/pI	Observed Mw/pI1	MS/MS sequences
1	β/γ Actin	P30169	6 of 10	42.0/5.2	42/5.2	n.d.
2	β-Tubulin	BAA22381	9 of 16	49.8/4.7	48/4.5	n.d.
3	Oxireductase short chain	AAK44298	n.a.	32.4/8.1	48/5.2	LANVMFTYE LANLLFTYE matched
	Oxireductase WW domain	NP_062519	n.a.	46.5/6.5	48/5.2	BVVLLTGANSGLGFETA KVVLVTGANSGIGFETA matched EGGNDVLLA unmatched
4	Hypothetical	AE001382	n.a.	57/9.8	55/4.9	BLNFSLLTVR KLNFSILTTR matched
	α-Tubulin	PO6604	3 of 14	49.8/4.9	55/4.9	Confirmed
	60 kDa chaperonin	M31918	n.a.	58/5.3	55/4.9	EEGLVAGGEV EEGLVAGGEV matched VAAQPAVDSDPDVLK No conclusive match
5	α-Tubulin	A56635	5 of 12	49.8/4.9	54/5.0	n.d.
6	HSP70	NP006588	13 of 33	71.2/5.5	70/5.2	BDAGTIAGLNVLR KDAGTIAGLNVLR matched BTVTNAVVTVPAYFNDSQR KTVTNAVVTVPAYFNDSQR matched
7	No MS signal	–	–	–	70/5.3	n.d.
8	Aldehyde dehydrogenase	P27463	n.a.	55/7.5	51/6.0	ELGEYGLQEYTEVK ELGEYGLQEYTEVK matched
9	Methylmalonate semialdehyde dehydrogenase	T13418	n.a.	56.5/7.5	50/6.1	ALSFVGSDQAGT AVSFVGSDQAGT matched BTLLDAEGDVLR KTLADAEGDVLR matched
10	Ornithine aminotransferase	P29758	n.a.	48.4/6.2	45/6.4	BLVFADGNFWGR KIVFADGNFWGR matched
	Citrate synthase	P34575	n.a.	51.5/7.7	45/6.4	ALVTETSVLDAEE ALVTETSVLDADE matched
11	?	–	n.a.	–	37/6.4	BFPNLS unmatched
12	Hypothetical protein C18D11	Q9XTZ6	n.a.	22.7/5.9	32/6.6	LQMALNMADA LQAALNMAAA matched QEFNGEL QEFDGQL matched LYMAEVL unmatched
13	Arginine kinase	Q9NKV4	n.a.	39.1/6.3	33/7.5	BTFLCWVNQQDHLR KTFLCWVNEEDHLR matched LTADHFLFND LTADHFLFND matched
14	Glutamine synthetase	JN0716	n.a.	40.7/6.2	36/6.2	BVAEDFGLVVSLDPK RVAEDFDVVVTLDPK matched VLCETYK VLCETYK matched
15	Glutamine synthetase	JN0716	n.a.	40.7/6.2	37/6.1	n.d.
16	No MS signal	–	–	–	49/6.0	n.d.
17	Glutathione peroxidase	CAC38779	n.a.	24/6.3	20/7.6	BLSLLYPATTGR BLSLLYPATTGR matched PLLSDK PIIADK matched
18	?	–	n.a.	–	19/7.8	[EL]FSEW[NV]PK unmatched

Acc. no., accession number of protein in public databases; ?, Unidentified/Novel; Matched, sequence in public databases to which peptide is matched; n.a., not applicable/no match MS-Fit search; n.d., not determined; B, Lys or Arg; L, Leu or Ile.

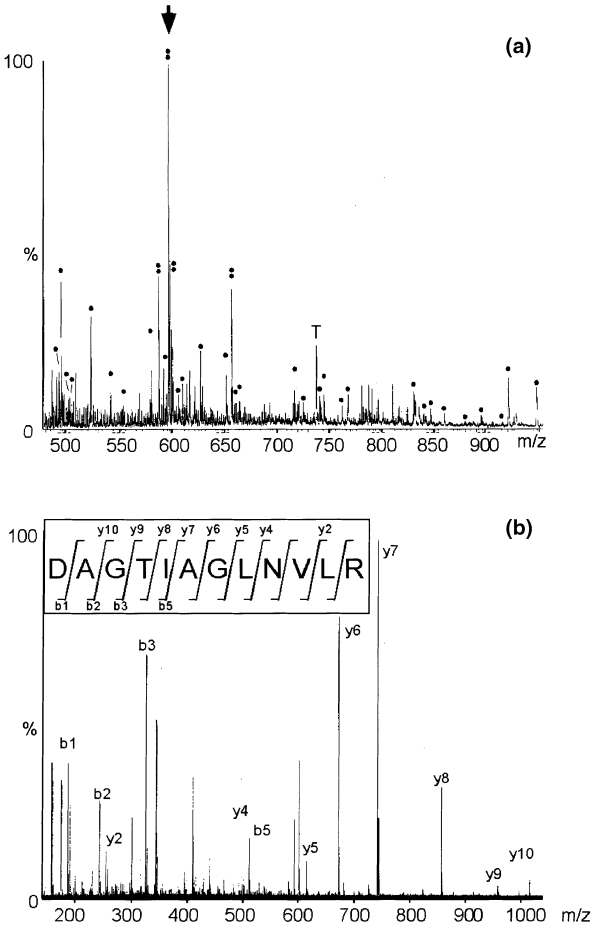

Fig. 3 Protein identification of spot 6 of the 2DE gel in Fig. 2 using tryptic peptide mass mapping and MS/MS sequencing. (a) Nano ESI-MS spectrum of the tryptic peptide mixture obtained after in-gel digestion of spot 6. Dots indicate ions that were used for database searching; arrow indicates ion at m/z 600.38 used for MS/MS sequencing; T, trypsin derived peptide. (b) MS/MS spectrum of the ion at m/z 600.38. A complete series of y ions is indicated and reveals the sequence DAGTIAGLNVLR confirming the protein identity of spot 6 as HSP70. *x*-Axis, m/z, mass-to-charge. *y*-axis, relative ion intensity in percentage.

Using the database program MS-fit, spots 1, 2, 5 and 6 were identified as β- and γ-actin, β-tubulin, α-tubulin and HSP70, respectively, which could be confirmed by tandem MS. As an example, the in gel-tryptic digestion of spot six generated 33-peptide fragments (Fig. 3a), 13 of which could be matched to cognate HSP70 in various species. This protein hit was confirmed by tandem MS sequencing of the peptide at m/z 600.38 (Fig. 3b), which revealed the sequence DAGTIAGLNVLR. This appears to be a highly conserved sequence in cognate HSP70, which can be found in mollusks (Gourdon *et al*. 2000), as well as mammals (Dworniczak and Mirault 1987). For the remainder of the proteins, tryptic mass-mapping using MS-fit did not yield any conclusive hits. Using MS/MS-sequencing and *de novo* sequence interpretation, we were able to further identify spots 8, 9, 13 and 14 as aldehyde dehydrogenase, methylmalonate semialdehyde-dehydrogenase, arginine kinase and glutamine synthetase, respectively (see Table 1 for sequences). Spot 15 showed a similar Maldi MS spectrum as spot 14, but showed a slightly higher molecular weight and lower pI, which may indicate that the protein in spot 15 is an isoform of the protein in 14, or that the protein is phosphorylated, which could result in the observed changes in molecular weight and pI. Spot 12 yielded peptides that are matched to a hypothetical protein (i.e. a protein predicted in the genomic databases). The identities of spots 4 and 10 are ambiguous because both contained multiple peptides that could be matched to multiple proteins. This suggests that spots 4 and 10 are not homogenous and contain more than one protein. The remainder of the spots could not be identified because either they contained peptides that could not be matched to any protein in the NCBI database (spots 11 and 18), or the levels of the tryptic peptides were lower than the detection limit of the mass spectrometer (spot 16). Together, the identified proteins belong to different classes, and include cytoskeletal, cytosolic and nuclear-encoded mitochondrial proteins.

Synaptosomal polysomes contain HSP70 mRNA

To further support the postulate that squid synaptosomes have the ability to synthesize the proteins that were identified by the proteomics approach, we set out to determine whether synaptosomal polysomes contained mRNA encoding these proteins. The presence of polysomes has previously been shown to be indicative for protein synthesis (Giuditta *et al*. 1991). To this aim, we selected the HSP70 transcript since partial sequence information of the squid HSP70 gene was available (Gioio *et al*. 2001). To investigate whether polysomes purified from squid synaptosomes contain HSP70 mRNA, we isolated polysomes from the synaptosomal fraction by sedimentation through 2.0 M sucrose and displayed the polysomes on a linear 15–45% sucrose gradient. Two fractions were collected, i.e. the monosome fraction, which contains ribonucleoprotein particles and single ribosomes, and the polysome fraction, which consists of mRNA associated with ribosomes (Fig. 4a). These fractions were analysed by RT-PCR using a squid HSP70 primer set. As shown in Fig. 4(b), HSP70 mRNA is exclusively detected in the polysomal fraction, thus supporting the conclusion that this protein is synthesized in synaptosomes, as indicated by the proteomics data.

In situ hybridization of squid synaptosomes

As a further independent method to localize the HSP70 transcript and the translational machinery (i.e. ribosomes) to synaptosomes, we used hybridization cytochemistry. Probes complementary to HSP70 mRNA and 28S rRNA (Bauman and Bentvelzen 1988) were hybridized to paraffin sections of synaptosomal pellets. Figure 5 demonstrates that both RNAs

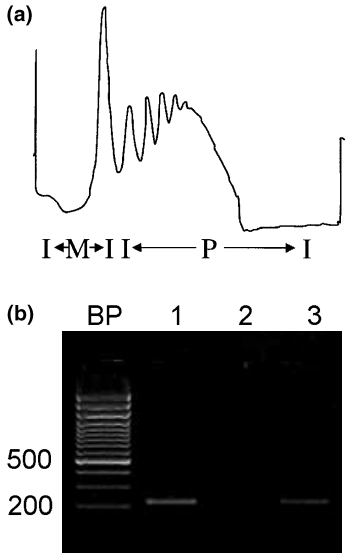

Fig. 4 Analysis of synaptosomal polysomes. (a) Sedimentation profile of polysomes prepared from a synaptosomal fraction and displayed on a 15–45% sucrose gradient, UV absorbance 254 nm. The gradient was divided in a monosome (M) and polysome (P) fraction. (b) Lane 1–3: RT-PCR analysis using the HSP70 primers. HSP70 amplicons are only detected in synaptosomes and purified synaptosomal polysomes fraction (lanes 1 and 3, respectively), the latter being indicative for synthesis of the HSP70 molecule in synaptosomes. BP, base pair marker; Lane 1: Total RNA isolate from squid synaptosomes; Lane 2: RNA isolate from monosome fraction of purified synaptosomal polysomes; Lane 3: RNA isolate from polysome fraction of purified synaptosomal polysomes.

are present in synaptosomes. Based on the incubation time required to generate a hybridization signal (rRNA, 30 min; HSP70, 24 h), we conclude that rRNA is much more abundant than the HSP70 transcript. The data on the presence of synaptosomal rRNA extend previous studies in which ribosomes and polyribosomes were identified in synaptosomes with electron spectroscopic imaging techniques (Martin *et al*. 1998).

Discussion

Squid synaptosomes are able to synthesize a large variety of proteins

Dendritic, axonal and pre-synaptic protein synthesis is thought to play an important role in neuronal development and activity-dependent synaptic plasticity (Martin *et al*. 1997; Schuman 1999). However, an overall characterization of the translation products of synaptic protein synthesis has yet to be performed, mainly because previous approaches toward protein identification were extremely labour-intensive. In addition, *de novo* synthesized synaptosomal proteins could not be reliably attributed to pre-synaptic, dendritic, or glial structures, in view of the considerable heterogeneity of the mammalian synaptosomal preparations. To address this issue, we turned to squid optic lobe synaptosomes, that consist largely of nerve terminals from retinal photoreceptor neurones (Crispino *et al*. 1997). Furthermore, at variance with conventional approaches that have led to the identification of single, newly synthesized pre-synaptic proteins, such as neurofilament proteins (Crispino *et al*. 1993a) and nuclear-encoded mitochondrial proteins (Gioio *et al*. 2001), we applied a proteomics strategy that is able to analyse large sets of proteins. This approach combines 2DE of metabolically labelled synaptosomes, tandem-MS and database searching to identify newly synthesized proteins.

The proteomics strategy is presently, from an analytical point of view, the most powerful methodology for the detection of large numbers of *de novo* synthesized proteins (Andersen and Mann 2000). However, this method does not cover the total spectrum of proteins, because very acidic, very basic and hydrophobic proteins cannot be resolved by 2DE. Thus, for the moment, we ignore whether synaptosomes synthesize these classes of proteins. Moreover, 2DE is unable to detect proteins that are present at low copy numbers. Indeed, only 18 out of the 80 protein spots that were *de novo* synthesized in squid synaptosomes were above the detection level of silver-staining in the corresponding gel (Fig. 2) and could be further analysed. Future experiments using a narrow pH range immobiline strips, and strips with acidic and basic pH gradient should enable us to resolve and characterize a larger number of *de novo* synthesized synaptosomal proteins.

From the 18 *de novo* synthesized proteins that matched silver-stained spots in the 2DE gels (see Fig. 2), tryptic peptide mass-mapping and MS/MS sequencing in conjunction with database searching revealed 12 known proteins and six novel proteins (Table 1). For some of these, synaptosomes were previously shown to contain the encoding mRNAs, such as α-actin and α-tubulin (Kaplan *et al*. 1992) and HSP70 (Gioio *et al*. 2001). For the remainder of the proteins we demonstrate their synaptosomal synthesis for the first time. Two spots apparently contained multiple proteins, because they yielded peptides that could be matched to more than one protein. Spot 4 contained peptides that could be matched to a hypothetical protein, α-tubulin, and a 60-kDa chaperonin. It is however, not clear which of the proteins present in the spot is responsible for the signal on the autoradiogram, hence we cannot conclude which protein is synthesized in synaptosomes. The same holds for spot 10. However, in this case, the two proteins, ornithine aminotransferase and citrate synthetase, belong to the same group of nuclear-encoded mitochondrial proteins, which indicates that either one or both can be synthesized by synaptosomes. That synaptosomes contain several mRNAs coding for mitochondrial proteins has recently been demonstrated (Gioio *et al*. 2001).

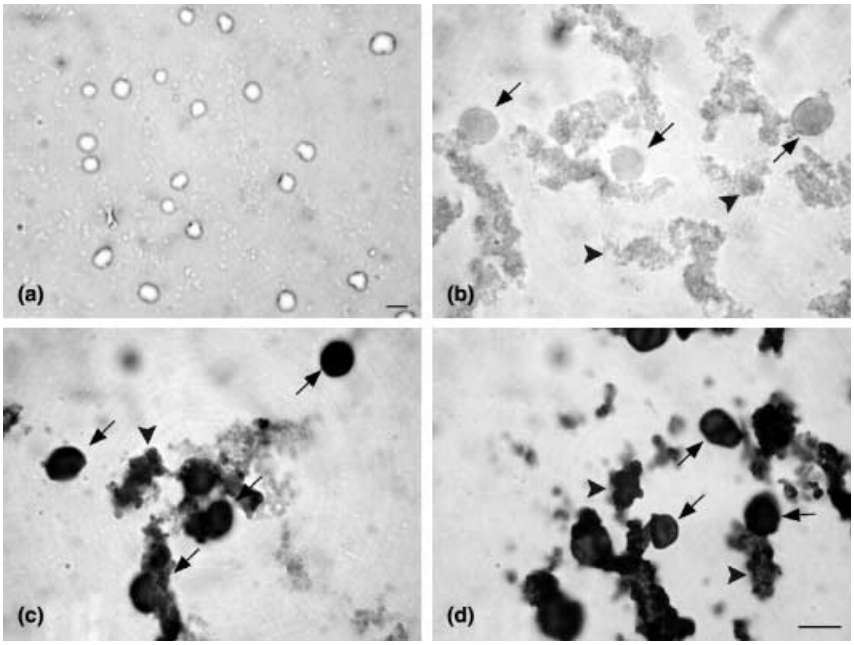

Fig. 5 *In situ* hybridization of synaptosomes. (a) Phase-contrast image of a suspension of optic lobe synaptosomes after fixation in paraformaldehyde, but before embedding in paraffin. The suspension consists of spheres of about 5 μm. Bar is 5 μm. (b) *In situ* hybridization with the sense HSP70 probe. Synaptosomes do not show a hybridization signal. Note that the embedding and *in situ* hybridization procedure has had a deleterious effect on the morphology of the synaptosomes, resulting in many collapsed synaptosomes. (c,d) *In situ* hybridization using the antisense probe for HSP70 (c) and 28S rRNA (d). Synaptosomes including collapsed synaptosomes show a strong hybridization signal. (b,c,d) Arrows indicate intact synaptosomes, arrowheads collapsed synaptosomes. Bar is 5 μm.

In addition to the identified proteins, six newly synthesized proteins could not be matched to any protein in the available databases (Table 1). Therefore, they may represent novel proteins. Alternatively, the tryptic peptides sequenced by tandem-MS may derive from the less conserved regions of the proteins and therefore could not be matched. To further characterize these proteins, the partial amino acid sequence information generated by MS/MS sequencing will be used to derive degenerate oligonucleotides for cDNA cloning studies, and elucidate the predicted precursor protein structure.

A prerequisite for protein synthesis is the presence of mRNA associated with the cellular organelles (i.e. ribosomes) involved in protein synthesis. To substantiate our identification of *de novo* synthesized synaptosomal proteins, we demonstrated that one of the corresponding transcripts is present in synaptosomes. We selected the HSP70 protein as an example, and by PCR analyses showed that HSP70 mRNA is associated with synaptosomal polysomes (Fig. 4). Furthermore, using *in situ* hybridization methods, we showed that HSP70 mRNA and ribosomes are present within synaptosomes (Fig. 5). This finding confirms the previous detection of ribosomes in squid synaptosomes by electron spectroscopic imaging and conventional electron microscopy (Crispino *et al*. 1997; van Minnen and Syed 2001).

In conclusion, as the squid synaptosomal preparation consists mainly of the nerve terminals of photoreceptor neurones, our data strongly support the presence of an active system of protein synthesis in the pre-synaptic nerve terminal (Crispino *et al*. 1993a, 1993b, 1997; Martin *et al*. 1998; Gioio *et al*. 2001). The presence of a protein synthetic machinery has been reported in mammalian post-synaptic elements (Schuman 1999).

Functional implications of *de novo* synthesized proteins

Our data provide strong additional evidence that the plasticity of the pre-synaptic terminal may rely on local protein synthesis (Koenig and Giuditta 1999; Alvarez *et al*. 2000). A similar view is widely accepted for dendrites and post-synaptic structures (Schuman 1999; Steward and Worley 2001). The present data demonstrate that synaptosomes synthesize different classes of proteins, including cytoskeletal proteins, enzymes, molecular chaperones and nuclear-encoded mitochondrial enzymes. Because the experiments were not aimed to elucidate the function of *de novo* synthesized synaptosomal proteins, we may only speculate on their role. For instance, the synthesis of cytoskeletal proteins may be reflecting the need for maintenance and/or structural modification of the pre-synaptic

terminal, as it may occur during activity-induced plasticity. Furthermore, one of the enzymes synthesized by squid synaptosomes (glutamine synthetase) may be involved in the metabolism of released glutamate. In mammals, this enzyme catalyses the conversion of released glutamate into glutamine (Rao and Murthy 1993). Because glutamate is released from optic lobe synaptosomes (A. D'Aniello, personal communication), glutamate metabolism may be dependent on synaptically synthesized enzymes. In addition, the observation that nuclear-encoded mitochondrial proteins are synthesized in squid synaptosomes points to a further important function of extrasomatic protein synthesis, which concerns the maintenance of mitochondria and hence the supply of energy in distal neuronal domains. The latter data are in agreement with the demonstration that several mRNAs specifying nuclear-encoded mitochondrial enzymes are present in synaptosomes isolated from squid optic lobe and are being locally translated (Gioio *et al*. 2001).

In summary, we have demonstrated that: (i) squid synaptosomes synthesize a large number of proteins; and (ii) the newly synthesized synaptosomal proteins include cytoskeletal proteins, enzymes implicated in glutamate metabolism, and nuclear-encoded mitochondrial proteins. Taken together, these findings support the notion that local protein synthesis plays an important role in pre-synaptic functions, notably in the supply of cytoskeletal proteins, in transmitter recycling and in the maintenance of mitochondrial activity.

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