

## REGULAR ARTICLE

# A subcellular prefractionation protocol for minute amounts of mammalian cell cultures and tissue

Isabelle Guillemin\*, Michael Becker\*, Kornelia Ociepka, Eckhard Friauf and Hans Gerd Nothwang

Abteilung Tierphysiologie, Technische Universität Kaiserslautern, Kaiserslautern, Germany

Subcellular localization represents an essential, albeit often neglected, aspect of proteome analysis. Generally, the subcellular location of proteins determines the function of cells and tissues. Here we present a robust and versatile prefractionation protocol for mammalian cells and tissues which is appropriate for minute sample amounts. The protocol yields three fractions: a nuclear, a cytoplasmic, and a combined membrane and organelle fraction. The subcellular specificity and the composition of the fractions were demonstrated by immunoblot analysis of five marker proteins and analysis of 43 proteins by two-dimensional gel electrophoresis and mass spectrometry. To cover all protein species, both conventional two-dimensional and benzyldimethyl-*n*-hexadecyl ammonium chloride-sodium dodecyl sulfate (16-BAC-SDS) gel electrophoresis were performed. Integral membrane proteins and strongly basic nuclear histones were detected only in the 16-BAC-SDS gel electrophoresis system, confirming its usefulness for proteome analysis. All but one protein complied to the respective subcellular composition of the analyzed fractions. Taken together, the data make our subcellular prefractionation protocol an attractive alternative to other prefractionation methods which are based on less physiological protein properties.

Received: March 30, 2004

Revised: May 6, 2004

Accepted: May 25, 2004

**Keywords:**

Benzyldimethyl-*n*-hexadecylammonium chloride-sodium dodecyl sulfate / Mass spectrometry / Subcellular fractionation / Two-dimensional gel electrophoresis

## 1 Introduction

Proteomic approaches allow the analysis of hundreds of proteins within days or weeks and thus provide unprecedented insight into the molecular repertoire of cells or tissues [1, 2]. This information is important to identify proteins responsible for cell-specific or stage-specific functions. Additionally, protein profiling is useful to characterize physiological and pathophysiological conditions of cells or tissues on a molecular level and to define diagnostic markers [3, 4]. For in-depth understanding of biological systems, many studies

aim at the identification of the entire proteome. However, low- to medium-abundant proteins, and membrane proteins in particular, which often confer cell and tissue specificity, are frequently underrepresented or remain undetected in proteome studies so far [5, 6]. Much effort is therefore directed at detecting those interesting, though elusive, protein species. To enrich for these proteins by reducing sample complexity, several prefractionation protocols have been developed [5, 7–10]. Furthermore, multidimensional chromatography methods have been introduced to increase coverage [11–13]. A drawback of many of these approaches is the loss of information concerning the subcellular location of the proteins analyzed. They either apply biochemical and biophysical separation criteria or aim at the entire cellular proteome. Although the function of a tissue is often governed by the presence or absence of specific protein isoforms, a change in the subcellular location of a given protein can

**Correspondence:** Dr. Hans Gerd Nothwang, TU Kaiserslautern, FB Biologie, Tierphysiologie, Postfach 3049, 67653 Kaiserslautern, Germany

**E-mail:** nothwang@rhrk.uni-kl.de

**Fax:** +49-631-205-4684

**Abbreviation:** 16-BAC, Benzyldimethyl-*n*-hexadecylammonium chloride

\* Both authors contributed equally.

likewise have a major impact. Small changes in the ratio between intracellularly located and plasma membrane-bound ion channels can profoundly alter the physiology of a neuron, as these proteins have transport rates of up to  $10^8$  ions/sec [14]. Another example is glucose uptake from blood which is mainly regulated by translocation of a glucose transport protein from intracellular storage vesicles to the plasma membrane [15]. Profiling proteomes without information on the subcellular location of identified proteins will hence lack important physiological data [16].

The widespread utilization of separation criteria, such as *pI* or solubility in detergents [5, 7–10] to reduce sample complexity, is likely due to the fact that subcellular fractionation protocols are long and tedious, and most often they have to be adapted to the cell type or tissue analyzed. Furthermore, classical subcellular fractionation protocols have not kept pace with the increased sensitivity in protein analysis. Modern MS can identify proteins at fMol concentrations. In theory, this allows proteome analysis to be conducted on minute amounts of biological samples, such as biopsy material or narrowly defined tissue areas of specific function, for example brain nuclei. In contrast, most subcellular fractionation protocols require large amounts of starting material. Therefore, protocols are required that can be applied to different samples, such as cultured cells or tissues, with minimal changes, and which can easily be scaled down.

Towards this goal, we first evaluated a subcellular pre-fractionation protocol for cultured cells [17] and subsequently adopted it to both freshly prepared and frozen tissue. The protein content of the fractions was evaluated by immunoblot analysis of marker proteins, 2-DE and MS. We show that the protocol is applicable to all mammalian samples tested. Moreover, it yields a pure cytosolic fraction, a highly enriched nuclear fraction, and a combined membrane and organelle fraction.

## 2 Materials and method

### 2.1 Materials

IPG strips and carrier ampholytes were purchased from Amersham Biosciences (Freiburg, Germany). Acrylamide and other reagents for polyacrylamide gel electrophoresis as well as most other chemicals were from Roth (Karlsruhe, Germany). SB3-10 was supplied by Sigma (Munich, Germany) and the protease inhibitor cocktail by Roche (Mannheim, Germany). The antibodies anti-GM130, which targets a 130 kD protein in the Golgi matrix, and anti-Calnexin, against a 90 kD protein of the ER membrane, were purchased from BD Bioscience (Heidelberg, Germany). Anti-PDI, against protein disulfide isomerase also located in the ER, was purchased from MBL International Corporation (Woburn, MA, USA).  $\alpha 5$ , against the plasma membrane bound  $\text{Na}^+/\text{K}^+$ -ATPase, was obtained from the Developmental Studies Hybridoma Bank of the University of Iowa

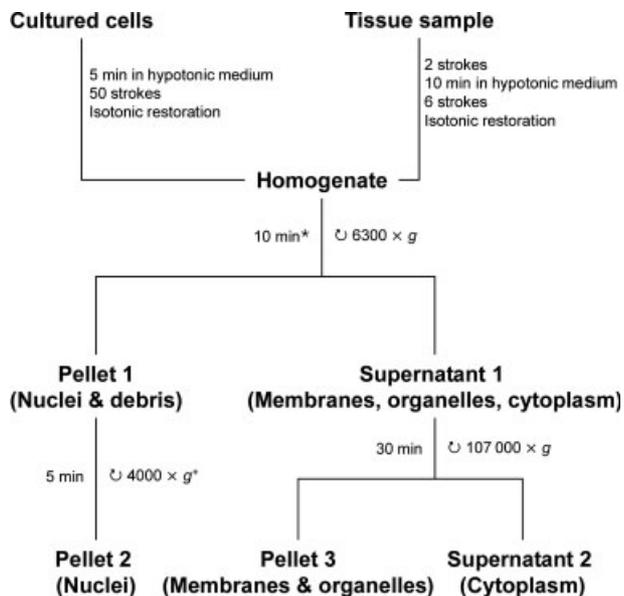
(Iowa, IA, USA), H516, which targets the nuclear histone H3, was kindly provided by Dr. G. Schlenstedt (Saarbrücken, Germany). COX IV, directed against the mitochondrial subunit IV of the cytochrome C oxidase, was obtained from Molecular Probes (Eugene, OR, USA).

### 2.2 Animals

Sprague-Dawley rats (8–9 weeks old of both gender) were deeply anaesthetized by a peritoneal injection of 700 mg/kg chloral hydrate and sacrificed by decapitation. All protocols complied with the current German Animal Protection Law and were approved by the local animal care and use committee (Landesuntersuchungsamt, Koblenz, Germany). The cerebellum was separated from the underlying brainstem by cutting horizontally through its pedunculi. Tissue samples were either immediately frozen in liquid nitrogen or directly used for sample preparation.

### 2.3 Sample preparation

Monolayer cultures (75 cm<sup>2</sup>) of both COS-7 and HEK293 cells ( $\sim 8 \times 10^6$  cells) were harvested by trypsination and resuspended for 5 min on ice in 500  $\mu\text{L}$  CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM  $\text{KH}_2\text{PO}_4$ , 5 mM  $\text{NaHCO}_3$ , 5 mM EDTA, 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ ) [17]. Homogenization was performed by applying 50 strokes with a motorized homogenizer (teflon/glass) at 250 rpm. Thereafter, 50  $\mu\text{L}$  of 2.5 M sucrose was added to restore isotonic conditions. One-hundred microliters of this suspension (crude cell homogenate) were collected and kept at  $-20^\circ\text{C}$  until further analysis; the remainder was subjected to differential centrifugation at  $4^\circ\text{C}$  (Fig. 1). The first round of centrifugation was performed at  $6300 \times g$  for 5 min in a tabletop centrifuge (5417R; Eppendorf, Hamburg, Germany). The pellet was resuspended in 1 mL TSE buffer (10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% IGEPAL-CA 630 v/v, pH 7.5) and homogenized with 30 strokes of a motorized Teflon potter at 250 rpm. This suspension was then centrifuged at  $4000 \times g$  for 5 min, the resulting supernatant discarded, and the pellet washed with TSE buffer until the supernatant was clear. The resulting pellet was resuspended in 100  $\mu\text{L}$  of TSE buffer and kept at  $-20^\circ\text{C}$ . The supernatant from the first round of differential centrifugation was sedimented at  $107\,000 \times g$  for 30 min in a Beckman JA21 rotor (Beckman Coulter, Krefeld, Germany) or for 150 min at  $14\,000$  rpm in a tabletop centrifuge. The resulting pellet was resuspended in 80  $\mu\text{L}$  PBS (130 mM NaCl, 7 mM  $\text{Na}_2\text{HPO}_4$ , 3 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.4). The supernatant was used without further treatments. Protocols for the subcellular fractionation of cerebellar tissue were similar to those for cell cultures, with the following modifications: 500 mg of fresh or frozen (stored at  $-80^\circ\text{C}$ ) tissue were transferred to 1 mL CLB buffer, and pre-homogenized by applying two strokes in a glass homogenizer. The suspension was incubated on ice for 10 min,



**Figure 1.** Schematic illustration of the subcellular fractionation protocol. Differential centrifugation was performed. Two rounds of centrifugation with increasing centrifugal force yielded three final fractions. Pellet 2 was enriched for nuclear proteins, whereas pellet 3 predominantly contained proteins from plasma membrane and organelles. Supernatant 2, obtained after the second round of centrifugation, represented the highly purified cytoplasmic fraction. The protocol was used for frozen cerebellar tissue; for freshly prepared cerebellar tissue; and for cultured COS-7 and HEK293 cells; minor modifications were made between the different samples: \* for cultured cells, the first round of centrifugation lasted only 5 min; + for freshly prepared brain tissue, the nuclei were pelleted at only  $1000 \times g$  to prevent cosedimentation with other subcellular compartments.

followed by six strokes of a motorized homogenizer at 250 rpm. After restoration with 0.1 volume 2.5 M sucrose, differential centrifugation was performed with a prolonged initial centrifugation step at  $6300 \times g$  for 10 min (Fig. 1). When fresh tissue was used, pellet 1 was centrifuged after its homogenization at only  $1000 \times g$  (Fig. 1). Membrane and nuclear fractions (pellets 2 and 3, respectively) were cleaned up by precipitation using the 2-D clean up kit (Amersham Biosciences), and dissolved in rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10 and 40 mM Tris) prior to 2-DE. The protein concentration of the cytoplasmic fraction was determined using a micro BCA assay (Pierce, Bonn, Germany). The protein contents of the nuclear and membrane fractions (resuspended in a thiourea-containing buffer) were quantified by comparing an aliquot with a dilution series of cytosolic proteins previously quantified with the micro BCA assay after separation by 12% 1-D SDS-PAGE. After adding 1.7  $\mu$ L IPG buffer and 4.1  $\mu$ L DeStreak™ (Amersham Biosciences), 300  $\mu$ g protein were applied to 18 cm long IPG strips (Immobiline™ Dry strip, pH 3–10; Amersham Biosciences) and IEF was performed (33 000 Vh) with an IPGphor IEF unit (Amersham Biosciences). After

equilibration, the gel strips were transferred to a 12% SDS-PAGE gel for the second dimension, using a Protean II xi cell (25 mA for 6 h; Bio-Rad, Munich, Germany). Gels were stained with colloidal Coomassie blue G250 (17% ammonium sulfate, 34% methanol, 0.5% acetic acid, 0.1% Coomassie blue G250) for 5 days. 16-BAC-SDS gel electrophoresis was performed as described previously [18]. In brief, proteins from the nuclear and membrane fractions were separated by molecular mass twice, using different detergents. In the first dimension, benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) was used as a detergent and the separation was performed under acidic conditions at 10 mA until the dye had migrated to the lower end of the gel. In the second dimension, separation under alkaline conditions was performed with SDS as the detergent (25 mA for 6 h). Due to the different effects of the detergents on proteins with similar mass, this method allows the separation of such proteins which is impossible in one-dimensional gels.

## 2.4 Immunoblot analysis

Immunoblot analysis was conducted as described previously [19]. Briefly, 40  $\mu$ g protein of each fraction were resolved by SDS-PAGE using either an 8% or a 12% Laemmli system. Proteins were electrophoretically transferred to PVDF membranes (Roth) for 2 h at 1 mA/cm<sup>2</sup> using a semi-dry blotter (Pierce, Erlangen, Germany). PVDF-bound proteins were visualized by staining with Ponceau S. Membranes were cut and only a part of the respective membrane was probed with a given antibody in order to save sample material and antibody. Membranes were then blocked in TTBS/milk (20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20, 5% nonfat dry milk) for 2 h and subsequently incubated in the same buffer with the respective antiserum overnight at 4°C. After four washes in TTBS, the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:5000; Amersham Biosciences) was applied for 1 h. After four washes in TTBS, bound antibody was detected using an enhanced chemiluminescence assay (Amersham Biosciences) and a VersaDoc 3000 documentation system (Bio-Rad).

## 2.5 Protein identification by MS

Protein spots were manually excised from the gel, washed, and in-gel digested using the Montage in-gel Digest96 kit (Millipore, Eschborn, Germany) and following the manufacturer's instructions. The sample was desalted using  $\mu$ C18-Ziptips (Millipore), and the eluent was loaded into a nano-electrospray capillary (Waters, Eschborn, Germany). All measurements were performed on a tandem mass spectrometer (Q-ToF 2; Waters) equipped with an electrospray source. To determine peptide sequences, MS/MS was performed by selecting precursor ions using the quadrupole. Peptides were fragmented in the collision chamber using energies between 20 and 40 eV and argon as the collision gas.

Peptide fragments were detected by the TOF analyzer. Data acquisition and processing were performed using the MassLynx 4.0 software package (Waters). Resultant peptide sequences were submitted to a BLAST search for short and nearly exact matches using NCBI's nonredundant database [20]. Both the  $M_r$  and the  $pI$  of the intact protein, as judged from the 2-DE results, were used as further criteria for protein identification. For most of the identified proteins, 2–4 tryptic peptides were matched by mass and sequence to the top candidates retrieved by the searches. Second-pass searches were routinely performed but did not identify any less abundant proteins comigrating with the major proteins. To classify identified proteins, functional information was retrieved mainly from the Gene Ontology database [21] or from the Swiss-Prot database [22].

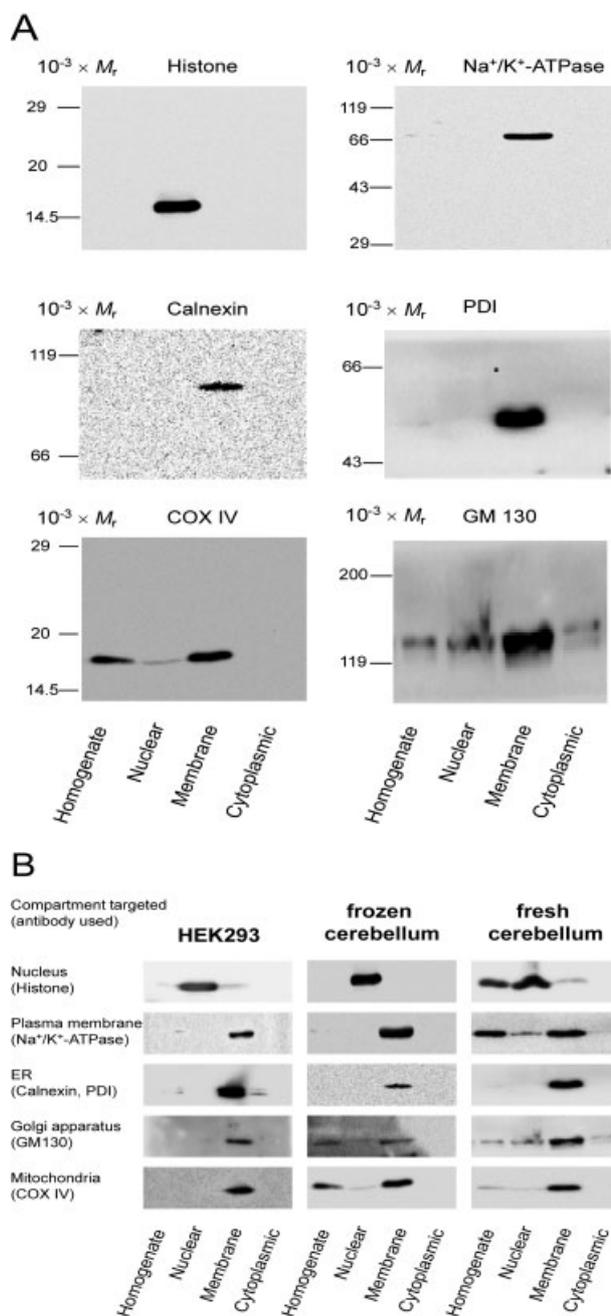
### 3 Results

#### 3.1 Subcellular fractionation of cultured HEK293 and COS-7 cells

To establish a general protocol for subcellular fractionation, we first analyzed a protocol for cell culture which was reported to yield pure nuclei and cytoplasm, and a so-called membrane fraction that contains the remainder of cellular components, most importantly proteins of the plasma membrane and the organelles [17]. This protocol is based on incubation in hypotonic buffer, subsequent mechanic shearing in a potter, and immediate restoration of isotonic conditions thereafter. Using HEK293 cells as a model system, the three fractions were assessed by immunoblot analysis employing antibodies against five marker proteins of various subcellular compartments, namely nuclei, mitochondria, ER, Golgi apparatus, and plasma membrane (Fig. 2A). This analysis confirmed the presence of histone H3, a nuclear marker protein, exclusively in the nuclear fraction, whereas the mitochondrial marker protein (COX IV) and the marker proteins for the biosynthetic-secretory pathway (Calnexin, GM130,  $\text{Na}^+/\text{K}^+$ -ATPase) were all present in the membrane fraction (Fig. 2B). None of the marker proteins were found in supernatant 2, indicating that it mainly contained cytoplasmic proteins. Identical results were obtained for various sample amounts (from  $4 \times 10^6$  to  $30 \times 10^6$  cells) and also for COS-7 cells (data not shown). This demonstrates the general utility of the protocol for cell culture. The absence of immunoblot signals in the crude homogenate extracts was due to the high complexity of this sample and demonstrates an enrichment of the marker proteins in the respective subcellular fractions.

#### 3.2 Subcellular fractionation of fresh and frozen brain tissue

To test whether the protocol used on cell cultures can also be applied to brain tissue samples, freshly prepared as well as frozen rat cerebelli were used as model systems. In order to



**Figure 2.** Immunoblot assessment of the subcellular fractionation. (A) To demonstrate the specificity of the antibodies used, they were probed on fractionated fresh cerebellum. Each antibody detected a single specific protein band in the  $M_r$  range analyzed. (B) Protein fractions derived from cultured HEK293 cells, frozen cerebellar tissue, and freshly prepared cerebellar tissue were analyzed by immunoblotting with marker antibodies for five different subcellular compartments. The compartments and antibodies are listed on the left of the figure. The three fractions (nuclear, membrane, cytoplasmic) were compared to the whole homogenate. For all samples, a substantial enrichment of nuclear marker protein can be seen in the nuclear fraction. Marker proteins for the biosynthetic-secretory pathway (ER and Golgi apparatus) and the plasma membrane are almost exclusively detected in the membrane fraction.

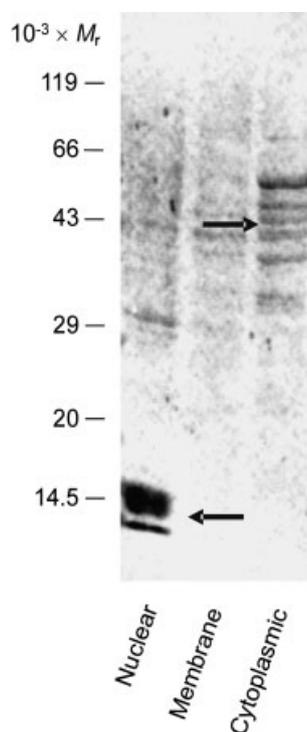
adapt and optimize the protocol to these samples, minor modifications were introduced. To maximize the exposure of cells to the hypotonic buffer, cerebellar tissue was pre-homogenized with two strokes in a potter. After 10 min of incubation in hypotonic CLB buffer to render the cells fragile, only six strokes (instead of 50 strokes) were applied as cells in tissue contain less cytoskeleton than cultured cells. Finally, the first round of centrifugation was prolonged from 5 to 10 min which led to a better enrichment of the nuclear marker in pellet 1 (data not shown). Immunoblot analysis revealed that these modifications were sufficient to obtain subcellular fractions from frozen cerebellar tissue that were of similar quality as those derived from cultured cells (Fig. 2B). In pellet 2, a positive signal was seen only for the nuclear marker protein, whereas the marker proteins for mitochondria and the biosynthetic-secretory pathway were restricted to the membrane fraction. The cytosolic fraction was negative for all marker proteins tested. Subcellular fractions from freshly prepared cerebelli were not as clean, as COX IV, GM130, and the  $\text{Na}^+/\text{K}^+$ -ATPase were not restricted to the membrane fraction but were also found in pellet 1 (data not shown). To prevent transfer of these contaminations to the nuclear fraction, pellet 1 (containing nuclei and cell debris), was sedimented with reduced centrifugal force ( $1000 \times g$ ) after resuspension. This modification prevented the membranous components from cosedimenting and resulted in a almost pure nuclear fraction in pellet 2 (Fig. 2B).

During the establishment of the protocol, we typically applied 500 mg brain tissue to the first round of centrifugation. Using such a minute amount of tissue material, our protocol routinely yielded enough sample material to run 8–10 preparative 2-D gels of each fraction with 300  $\mu\text{g}$  protein being loaded on a gel. This represents an improvement over older protocols, which often used several grams of starting material or whole organs [23, 24]. Furthermore, all protocol steps can be performed in 1.5 mL reaction tubes. Thus, the protocol can be applied to minute amounts of cell cultures and tissue.

### 3.3 Analysis of subcellular fractions by conventional 1-DE and 2-DE

To study the protein composition of the fractions in more detail, we performed 1-DE and 2-DE experiments. Analysis of the three subcellular fractions from cultured COS-7 cells on a 1-D SDS-PAGE showed clear differences in the protein pattern (Fig. 3). In the nuclear fraction, protein bands of low  $M_r$  (10 000–15 000) were observed, which likely correspond to histones. These bands were not visible in the membrane or cytosolic fractions. In contrast, the cytosolic fraction contained several protein bands of  $M_r$  40 000–60 000 that were not present, or less prominent, in the other fractions (Fig. 3).

To corroborate these data, we also performed conventional 2-DE with samples extracted from cerebellar tissue and identified randomly selected gel spots by MS/MS (Figs. 4 and 5). Information on the subcellular location of identified

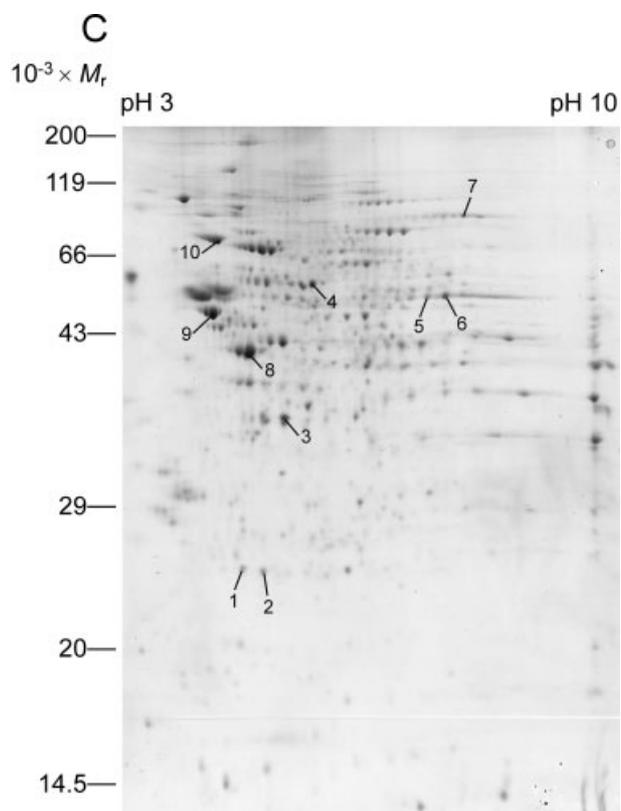
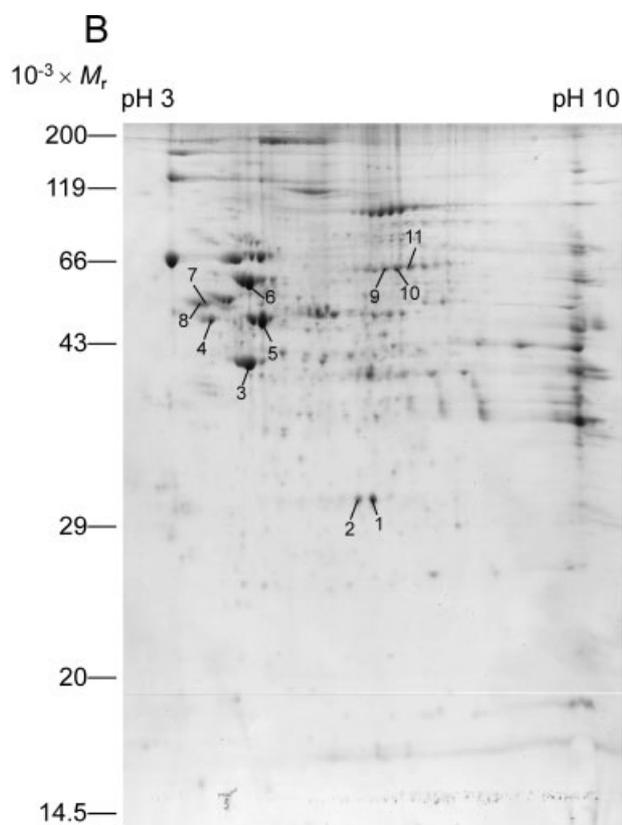
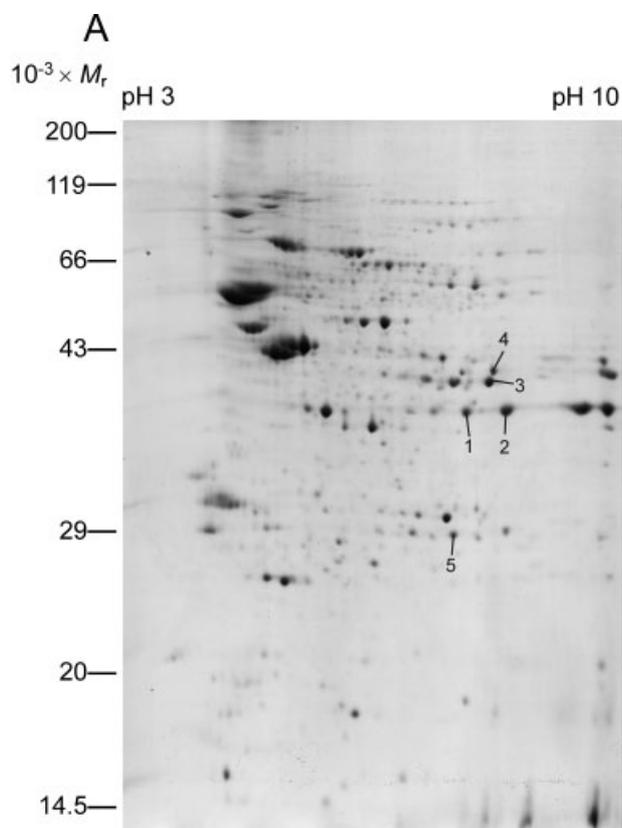


**Figure 3.** SDS-PAGE analysis of subcellular fractions obtained from COS-7 cells. Cells were fractionated by differential centrifugation and 40  $\mu\text{g}$  protein from each resulting fraction (nuclear, membrane, and cytoplasmic, as depicted below the gel) were loaded on a 12% SDS-gel. After electrophoresis and staining with colloidal Coomassie blue G250, clear differences were obvious between the fractions (arrows). Several bands in the low  $M_r$  range ( $\sim 14.5$  kDa) were only visible in the nuclear fraction, whereas proteins in the 40–60 kDa range were enriched in the cytoplasmic fraction.

proteins was subsequently derived from data bank entries or previous studies (Tables 1–3). In terms of the cytoplasmic fraction, all five spots analyzed were identified as cytosolic housekeeping enzymes (Table 1), thus confirming the purity of this fraction implied earlier by the immunoblot results (Fig. 2). Ten out of 11 protein spots from the nuclear fraction were either nuclear proteins or represented cytoskeleton proteins (Table 2, spot nos. 1–10). The latter are likely attached to the nuclear envelope and were thus copurified with the nuclei. Only the mitochondrial ATP synthase beta chain protein was not compatible with the nuclear fraction. All 10 protein spots analyzed from the membrane fraction (Table 3, spot no. 1–10) represented proteins that are either found in organelles, such as lysosomes, ER or mitochondria, or associated with membranes, such as cytoskeleton proteins.

### 3.4 Analysis of subcellular fractions by 16-BAC-SDS gel electrophoresis

Nuclear and membrane fractions are well-known to contain proteins that are difficult to display by conventional 2-DE, for example, the strongly basic histones ( $pI > 10$ ) or the hydrophobic integral membrane proteins. To analyze these problematic protein species, we prepared 16-BAC-SDS gels, which were reported to resolve them [18, 25]. From the nuclear fraction, seven spots were identified by MS. All were compatible with either a nuclear location or a cytoskeletal one possibly associated with the nuclear envelope (Table 2, spots 12–18). Two of the identified proteins represented histones, demonstrating the potential of 16-BAC-SDS 2-DE to

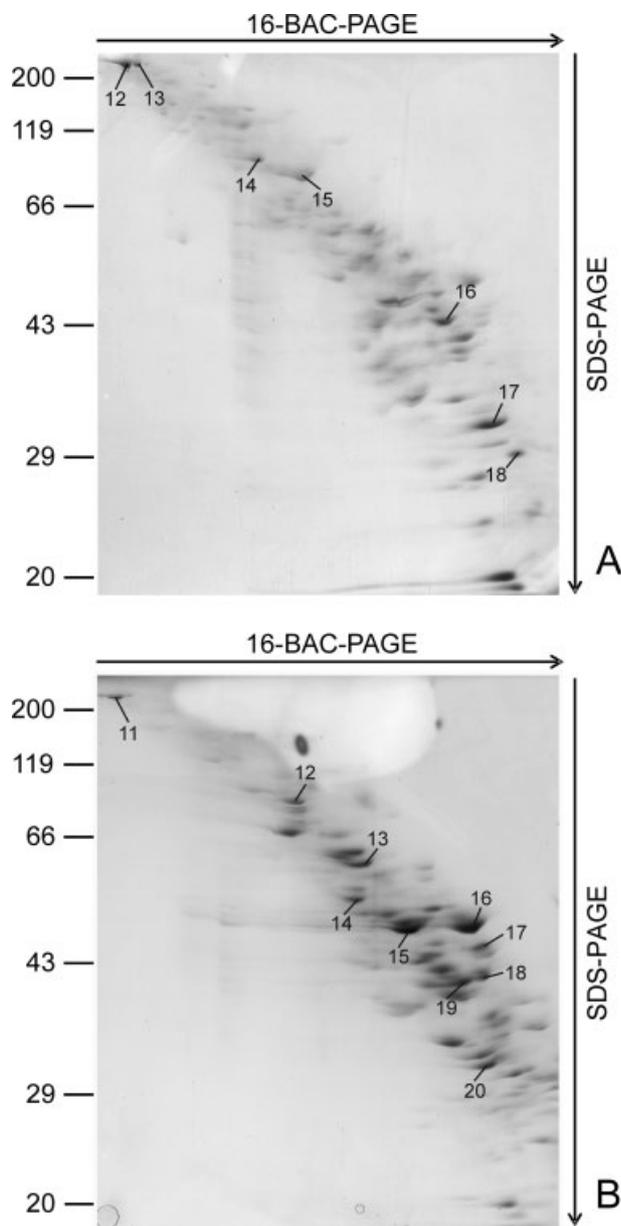


**Figure 4.** Conventional 2-DE of subcellular protein fractions obtained from frozen cerebellar tissue. To compare the protein content of the different fractions, 300  $\mu$ g protein from each fraction were separated by 2-D SDS-PAGE and stained with colloidal Coomassie blue G250. (A) cytoplasmic fraction; (B) nuclear fraction; (C) membrane fraction. Numbered spots were excised from the gel, in-gel digested with trypsin, and identified by MS/MS. The results are listed in Tables 1–3.

display strongly basic proteins. From the membrane fraction, ten spots were analyzed by MS (Table 3, spots 11–20). This resulted in the identification of eight proteins of organellar or plasma membranous origin, with one of those being an integral membrane protein (Table 3, spot 20). One spot represented a cytoskeletal protein (Table 3, spot 11), and one protein was identified as a cytoplasmic enzyme previously localized in the postsynaptic density of brain tissue (Table 3, spot 19) [26]. Taken together, the combination of MS with two varieties of 2-DE demonstrated convincingly the enrichment of compartment-specific proteins by our subcellular prefractionation protocol. Only one (ATP synthase beta chain) out of 43 analyzed proteins did not comply with the analyzed fraction (Fig. 6).

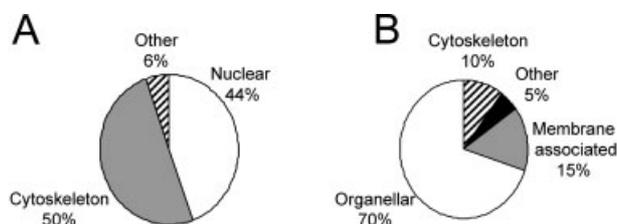
#### 4 Discussion

Over the past years, proteome analysis has become a widespread and common approach to gain insight into the protein repertoire of biological systems. To fully exploit the



**Figure 5.** 16-BAC-SDS gel electrophoresis of subcellular protein fractions obtained from frozen cerebellar tissue. To further characterize the protein content of the nuclear (A) and the membrane (B) fraction, 300  $\mu$ g of total protein from each fraction were separated by 16-BAC-SDS gel electrophoresis, which facilitates the display of strongly basic and membrane proteins. Numbered spots were excised from the gel, in-gel digested with trypsin, and identified by MS/MS. The results are listed in Tables 2 and 3.

potential of proteomics, efficient protocols have to be provided that identify all proteins, their PTM, their subcellular location, and their interacting partners, as these are the key features for protein function [27]. Towards this goal, we present a rapid and robust protocol for sample prefractionation by differential centrifugation which retains the information on subcellular location. This protocol has three major



**Figure 6.** Subcellular localization of proteins identified in the nuclear and membrane fractions. The pie charts summarize the results presented in Figs. 4–5 and in Tables 2–3. All identified protein spots from the nuclear and membrane fractions were classified according to their subcellular location. Spots from the nuclear fraction (A, Figs. 4B and 5A) include a high percentage of proteins of nuclear and cytoskeletal origin. The membrane fraction (B, Figs. 4C and 5B) predominantly contains proteins located in organelles or associated with the plasma membrane.

advantages over protocols published so far. First, it yields three clearly separated fractions, one containing nuclei and the associated cytoskeleton, the second one representing cytosolic proteins, and the third one being enriched with proteins from membranes and organelles other than the nucleus (*e.g.* mitochondria, ER and Golgi apparatus). This latter fraction may further be separated by density gradient centrifugation or immunoprecipitation. Cross contamination in the three fractions was highly reduced, as was demonstrated by immunoblot analysis and identification of proteins by MS. Therefore, the protocol presented here represents a major improvement compared to a commonly used subcellular prefractionation protocol that does not sharply separate the cellular compartments [19, 28, 29]. Second, the protocol is highly versatile. It can be applied to various kinds of mammalian samples, such as cultured cells and fresh and frozen tissue. This is important as more and more proteome studies attempt to analyze region-specific protein patterns where material has to be pooled from several sources and thus must be collected over an extended period of time. Third, the protocol is easy to use and allows adjustment to minute amounts of starting material. All protocol steps can be performed in a table microcentrifuge which allows the entire protocol to be scaled down to tissue slices or a few million cells.

Our prefractionation protocol not only reduces the sample complexity but also aims at providing information on the subcellular location of formerly unknown or unannotated proteins by their presence in one of the fractions. It was therefore important to perform an in-depth characterization of the subcellular composition of each fraction. A common approach is the use of immunoblot assays with antibodies for known, compartment-specific marker proteins. Here, we used antibodies against nuclei, ER, the Golgi apparatus, mitochondria and the plasma membrane. The results show a substantial enrichment of each marker protein in a single fraction and its removal from the other fractions close to, or even below, the detection threshold. Despite the robustness of immunoblot assays, they have a major drawback in that

**Table 1.** Proteins of the cytoplasmic fraction from rat cerebellum

Spot no. <sup>a)</sup>	Protein name <sup>a)</sup>	Acc. no. <sup>a)</sup>	Subcellular location	Theoretical $M_r \times 10^{-3} / pI^b)$	Observed $M_r \times 10^{-3} / pI^b)$	No. peptides <sup>b)</sup>	Sequence coverage <sup>b)</sup>	E-value <sup>c)</sup>	Sample peptide <sup>d)</sup>
1	Glyceraldehyde 3-phosphate dehydrogenase	P04797	cytoplasmic	36/8.6	37/7.3	2	8%	1.07	VVDLFAYFAS TR VVDLMAYMAS KE
2	Glyceraldehyde 3-phosphate dehydrogenase	P04797	cytoplasmic	36/8.6	37/7.8	6	22%	$1 \times 10^{-5}$	LLSWYNEDYGY MNL LLSWYDNEYGY SNR
3	Fructose-bisphosphate aldolase C	P09117	cytoplasmic	39/7.1	39/7.6	6	22%	3.8	DNAGAATE YL IKR DNAGAATE EF IKR
4	Aspartate aminotransferase, cytoplasmic	P13221	cytoplasmic	46/6.7	40/7.6	6	19%	0.12	QVEYLV VGS R QVEYLV NE R
5	Triosephosphate isomerase	P48500	cytoplasmic	26/6.9	28/7.5	4	22%	$6 \times 10^{-3}$	IYGGSVTGATGCK IYGGSVTGAT CK

- a) Spot no. corresponds to the position marked on the gel (Fig. 4A). Protein name and Acc. no. were derived from the Swiss-Prot protein knowledge base [22]. Information on the subcellular location was obtained from the Swiss-Prot protein knowledge base
- b) Theoretical  $M_r/pI$  values were calculated from the amino acid sequence, and observed  $M_r/pI$  were derived from the spot position in the gel. No. peptides indicates the number of analyzed peptides that matched the identified protein by sequence and/or mass; their coverage of the entire amino acid sequence is listed under sequence coverage
- c) E-value: A search with the sequence information obtained from the indicated peptides against the NCBI nonredundant database, limited to *Rattus norvegicus*, yielded the listed Expect (E)-values
- d) The sample peptide column lists a representative example of the sequence information obtained for a peptide (upper row) along with the corresponding sequence from the database entry (lower row)

they pinpoint just one out of hundreds or thousands of proteins present in the sample. We therefore used an additional approach and characterized the fractions by 2-DE and MS. By identifying multiple proteins and extracting information about their subcellular location from previous studies, a broad characterization of a fraction is achieved without being limited by the availability and costs of antibodies. The results obtained by this approach comply with those obtained by immunoblot analysis. For the cytoplasmic fraction (supernatant 2), the five analyzed protein spots were all of cytoplasmic origin (Table 1). A total of 18 spots were identified from the nuclear fraction. Eight spots (44%) represented nuclear proteins, and nine spots (50%) represented proteins belonging to, or associated with, the cytoskeleton. Their presence in this fraction is likely due to their association with the nuclear envelope. Only one (6%) of the identified proteins, namely the ATP synthase beta chain, indicated a minor contamination by mitochondrial proteins.

Regarding the membrane fraction, 20 spots were analyzed. The great majority (17, *i.e.*, 85%) represented proteins from organelles, mainly the ER lumen or the mitochondrial matrix. Two proteins (10%) were identified as cytoskeleton components, which likely remained attached to the membranes during fractionation. The only protein that did not seem to fit into the membrane fraction was GAPDH, which is usually considered to be cytoplasmic. Two recent studies, however, showed the association of GAPDH with the postsynaptic density fraction in rat forebrain and with the  $\gamma$ -aminobutyric acid receptor [26, 30]. These findings thus account for its presence in the membrane fraction.

A substantial number of proteins expected in the nuclear fraction and the membrane fraction share characteristics that render them problematic for a display on standard 2-DE. Examples are highly hydrophobic integral membrane proteins and the strongly basic nuclear histones with  $pI$  values  $>11$ . Excluding such proteins from the analysis of fraction specificity due to experimental limitations results in a bias. To overcome such constraints, we employed 16-BAC-SDS gel electrophoresis. Using this approach, we identified the integral membrane protein voltage-dependent anion-selective channel protein 1 and two basic nuclear histones (H1.2 and H1.0). These findings stress the importance of the 16-BAC-SDS gel system when studying certain cellular subproteomes by a gel-supported approach [25, 31].

It is interesting to note that we observed an average deviation of  $\pm 33.6$  kDa ( $\pm 36.5\%$ ) between the mass displayed on the 16-BAC-SDS gel (as indicated by the migration of marker proteins) and the mass derived from the respective database entry. In standard gels, this deviation was only  $\pm 4.3$  kDa ( $\pm 8.2\%$ ). The large deviation in the 16-BAC-SDS gel electrophoresis system was not due to an inherent error, because the separation of seven marker proteins (myosin,  $\beta$ -galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme) by the 16-BAC-SDS gel system yielded the expected sizes. Hydrophobic proteins of the biosynthetic-secretory pathway are often heavily glycosylated which is one possible explanation for the observed increase in  $M_r$  [32]. Since a higher  $M_r$  was also observed for histones, other reasons for the shift must be taken into account as well. The results from our immunoblot analysis agreed with the data obtained by 2-DE and MS. However, the

**Table 2.** Proteins of the nuclear fraction from rat cerebellum

Spot no. <sup>a)</sup>	Protein name <sup>a)</sup>	Acc. no. <sup>a)</sup>	Subcellular location <sup>a)</sup>	Theoretical $M_r \times 10^{-3} / pI^b)$	Observed $M_r \times 10^{-3} / pI^b)$	No. peptides <sup>b)</sup>	Sequence coverage <sup>b)</sup>	E-value <sup>c)</sup>	Sample peptide <sup>d)</sup>
1	High mobility group protein 1	P07155	nuclear [22]	25/5.7	31/6.7	4	12%	0.10	MSSYAFFV SSS R MSSYAFFV QTC R
2	High mobility group protein 1	P07155	nuclear [22]	28/10.8	31/6.5	4	12%	9.67	MS LH AFFVQT GC R MS SY AFFVQT C R
3	Gamma-actin	P02571	cytoskeletal [22]	42/5.4	42/5.3	4	14%	0.02	KN TALA AL TMK EI TALA PS TMK
4	ATP synthase beta chain, mitochondrial	P10719	mitochondrial [22]	56/5.2	48/4.9	8	16%	$7 \times 10^{-5}$	FTQAGSEV SSP LGR FTQAGSEV AL LGR
5	Glial fibrillary acidic protein delta	Q9Z2S0	cytoskeletal [22]	48/5.9	47/5.5	10	29%	$3 \times 10^{-17}$	ESASYQEALAR ESASYQEALAR
6	Alpha-internexin	P23565	cytoskeletal [22]	56/5.3	57/5.3	7	16%	$6 \times 10^{-6}$	LPASDGLDLSQAAAR LPASDGLDLSQAAAR
7	Tubulin beta chain	P04691	cytoskeletal [21]	50/4.8	52/4.7	6	15%	$8 \times 10^{-12}$	NSSYFVEWLP GG NVK NSSYFVEWIP N NVK
8	Tubulin beta chain	P04691	cytoskeletal [21]	50/4.8	52/4.8	4	10%	$4 \times 10^{-6}$	FPGQLNADLR FPGQLNADLR
9	Lamin A	P48679	nuclear [22]	74/6.9	66/6.9	4	7%	–	QSAG SSTP AE TPR SGAQ SSTP LS PTR
10	Lamin A	P48679	nuclear [22]	74/6.9	66/6.8	7	12%	$1 \times 10^{-7}$	SGAQASSTPLSPTR SGAQASSTPLSPTR
11	Lamin A	P48679	nuclear [22]	74/6.9	66/7.1	6	10%	0.05	IDLSAQL EAQ QK IDLSAQL SQL QK
12	Spectrin alpha chain, brain	P16086	cytoskeletal [22]	285/5.3	207	6	3%	0.01	LGESQTLQQFSR LGESQTLQQFSR
13	Spectrin beta chain, brain 1 <sup>n</sup>	XP_240072	cytoskeletal [22]	278/5.6	207	5	3%	$2 \times 10^{-7}$	EIEELQSQAAALSQEGK EIEELQSQAAALSQEGK
14	PTB-associated splicing factor	Q8VIJ6 <sup>m</sup>	nuclear [33]	75/9.7	98	2	3%	$3 \times 10^{-3}$	YGEPGEVFINK YGEPGEVFINK
15	Dynamin-1	P21575	cytoskeletal [22]	96/6.7	77	6	10%	$5 \times 10^{-8}$	VPVGDQPPDLEF GA IR VPVGDQPPDIEF Q IR
16	2',3'-cyclic nucleotide 3'-phosphodiesterase	P13233	Membrane-bound, microtubule associated [34]	45/9.7	44	3	9%	$6 \times 10^{-5}$	GGSQGEEVGELPR GGSQGEEVGELPR
17	Histone H1.2	P15865	nuclear [22]	22/11.4	30	5	13%	0.02	ASGPPVSELITK ASGPPVSELITK
18	Histone H1.0	P43278	nuclear [22]	21/11.2	33	3	15%	0.09	YSD PY VAAIQAEK YSD MI VAAIQAEK

a) Spot no. corresponds to the position marked on the gels (Fig. 4B and Fig. 5A). Protein name and Acc. no. were derived from the Swiss-Prot protein knowledge base [22]; <sup>m</sup>database entry for mouse protein only, <sup>n</sup>no Swiss-Prot entry, Acc. no. and sequence taken from NCBI nr database. Information on subcellular location was obtained from indicated references and databases: Swiss-Prot protein knowledge base [22], Gene Ontology consortium [21], [33], [34]

b) Theoretical  $M_r/pI$  values were calculated from the amino acid sequence, and observed  $M_r/pI$  were derived from the spot position in the gel. No. peptides indicates the number of analyzed peptides that matched the identified protein by sequence and/or mass; their coverage of the entire amino acid sequence is listed under sequence coverage

c) E-value: A search with the sequence information obtained from the indicated peptides against the NCBI nonredundant database, limited to *Rattus norvegicus*, yielded the listed Expect (E)-values

d) The sample peptide column lists a representative example of the sequence information obtained for a peptide (upper row) along with the corresponding sequence from the database entry (lower row)

Entries 1–11 represent spots picked from a conventional 2-D SDS gel (Fig. 4B), whereas entries 12–18 represent spots picked from a 16-BAC-SDS gel (Fig. 5A).

proteomic approach allowed a much better characterization of the subcellular composition of the fractions. Consistent with a previous analysis [19], it revealed the presence of

cytoskeletal proteins in all three fractions. Further fractionation may be envisaged to remove cytoskeletal proteins or to isolate them in a separate fraction.

**Table 3.** Proteins of the membrane fraction from rat cerebellum

Spot no. <sup>a)</sup>	Protein name <sup>a)</sup>	Acc. no. <sup>a)</sup>	Subcellular location <sup>a)</sup>	Theoretical $M_r \times 10^{-3} / pI^b)$	Observed $M_r \times 10^{-3} / pI^b)$	No. peptides <sup>b)</sup>	Sequence coverage <sup>b)</sup>	E-value <sup>c)</sup>	Sample peptide <sup>d)</sup>
1	Peroxiredoxin 2	P35704	cytoplasmic, lysosomal [22]	22/5.5	25/5.2	3	11%	$5 \times 10^{-6}$	QILVNDLPVGR QITVNDLPVGR
2	Phosphatidylethanol-amine-binding protein	P31044	cytoplasmic, membrane-bound [22]	21/5.7	25/5.4	3	25%	$2 \times 10^{-18}$	VDYGGVTVDELGK VDYGGVTVDELGK
3	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 2	P54313	Plasma membrane [22]	37/5.9	36/5.7	2	7%	$2 \times 10^{-3}$	LIIWDSYTTNK LIIWDSYTTNK
4	Protein disulfide isomerase A3	P11598	Endoplasmic reticulum [22]	56/6.1	56/6.1	6	11%	$3 \times 10^{-4}$	YGVSGYPTLK YGVSGYPTLK
5	Glutamate dehydrogenase, mitochondrial	P10860	mitochondrial [22]	61/8.3	52/7.4	3	6%	$9 \times 10^{-7}$	DDGSWEVIEGYR DDGSWEVIEGYR
6	Glutamate dehydrogenase, mitochondrial	P10860	mitochondrial [22]	61/8.3	53/7.6	3	6%	$3 \times 10^{-7}$	YNLGLDLR YNLGLDLR
7	Mitochondrial aconitase	Q9ER34	mitochondrial [22]	85/8.1	95/7.8	4	7%	$3 \times 10^{-3}$	FNPET VY LTGK FNPET DF LTGK
8	Gamma-actin	P02571	cytoskeletal [22]	42/5.4	56/5.3	6	21%	$6 \times 10^{-8}$	SYELPDGQVITIGNER SYELPDGQVITIGNER
9	ATP synthase beta chain, mitochondrial	P10719	mitochondrial [22]	56/5.3	48/4.9	6	14%	$1 \times 10^{-4}$	LVLEVA GA HLGESTVR LVLEVA Q HLGESTVR
10	78 kDa glucose-regulated protein	P06761	Endoplasmic reticulum [22]	72/5.1	77/5.0	8	18%	$2 \times 10^{-12}$	ITPSYVAFTPEGER ITPSYVAFTPEGER
11	Spectrin alpha chain, brain	P16086	cytoskeletal [22]	285/5.3	143	7	3%	$9 \times 10^{-4}$	ITALDEFATK ITALDEFATK
12	Endoplasmin	P08113 <sup>m)</sup>	Endoplasmic reticulum [22]	93/4.8	49	7	10%	$1 \times 10^{-10}$	LISLTDENALAGNEELTVK LISLTDENALAGNEELTVK
13	Heat shock 70k Da protein 1/2	Q07439	Cytoplasmic, organelles [22]	70/5.6	38	4	9%	$2 \times 10^{-5}$	PSYVAFTDER TT PSYVAFTDER
14	60 kDa heat shock protein, mitochondrial	P19226	mitochondrial [22]	61/6.1	34	4	8%	$1 \times 10^{-5}$	VGGTSDVEVNEK VGGTSDVEVNEK
15	ATP synthase alpha chain, mitochondrial	P15999 <sup>x)</sup>	mitochondrial [22]	60/9.5	33	7	17%	$8 \times 10^{-8}$	TGAIVDVPVGDPELLGR TGAIVDVPVGDPELLGR
16	ATP synthase beta chain, mitochondrial	P10719	mitochondrial [22]	56/5.3	33	5	13%	$6 \times 10^{-5}$	FTQAGSEVSALLGR FTQAGSEVSALLGR
17	Creatine kinase, B chain	P07335	cytoplasmic [22], mitochondrial [26]	41/5.5	30	7	21%	$5 \times 10^{-7}$	VLTPELYEARL VLTPELYEARL
18	Citrate synthase, mitochondrial	O75390	mitochondrial [22]	52/8.4	28	2	6%	$2 \times 10^{-5}$	LV G YETSVLDPDEGLR LV YETSVLDPDEGIR
19	Glyceraldehyde 3-phosphate dehydrogenase	P04797	cytoplasmic [22], postsynaptic density [26]	36/8.6	27	7	18%	$6 \times 10^{-8}$	LISWYDNEYGYSNR LISWYDNEYGYSNR
20	Voltage-dependent anion-selective channel protein 1	Q9Z2L0	mitochondrial, plasma membrane [22]	31/8.8	23	4	15%	$1 \times 10^{-3}$	VT GA SNFAVGYK VT Q SNFAVGYK

a) Spot no. corresponds to the position marked on the gels (Fig. 4C and Fig. 5B). Protein name and Acc. no. were derived from the Swiss-Prot protein knowledge base [22]; <sup>m)</sup>database entry for mouse protein only; <sup>x)</sup>fragment, full length entries for other species exist. Information on subcellular location was obtained from the indicated references

b) Theoretical  $M_r/pI$  values were calculated from the amino acid sequence, and observed  $M_r/pI$  were derived from the spot position in the gel. No. peptides indicates the number of analyzed peptides that matched the identified protein by sequence and/or mass; their coverage of the entire amino acid sequence is listed under sequence coverage

c) E-value: A search with the sequence information obtained from the indicated peptides against the NCBI nonredundant database, limited to *Rattus norvegicus*, yielded the listed Expect (E)-values

d) The sample peptide column lists a representative example of the sequence information obtained for a peptide (upper row) along with the corresponding sequence from the database entry (lower row)

Entries 1–10 represent spots picked from a conventional 2-D SDS gel (Fig. 4C), whereas entries 11–20 represent spots picked from a 16-BAC-SDS gel (Fig. 5B).

## 5 Concluding remarks

In summary, our subcellular prefractionation protocol represents an easy, robust, and versatile method to reduce sample complexity while simultaneously retaining subcellular information. This makes it superior to protocols that are based simply on biochemical and/or biophysical properties of proteins. Subcellular location is of prime importance for the understanding of protein function and for defining different physiological and pathophysiological conditions of an organ or tissue. The use of two different types of 2-DE, combined with MS, proved to be an important and essential tool to characterize prefractionation protocols in more detail.

*We thank Jens Schindler for help with the 16-BAC-SDS gel electrophoresis system and Dr. Schlenstedt for the generous gift of the antibody against histones. The monoclonal antibody a5 of Dr. Fambrough was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.*

*Funding for this research project was provided by the DFG Graduate Research School "Molecular, physiological and pharmacological analysis of cellular membrane transport" and by the Nano+Bio-Center Kaiserslautern.*

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