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Proteome analysis of conditioned medium from cultured adult hippocampal progenitors

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It is known that proliferation and survival of neural stem/progenitor cells *in vitro* not only depend on exogenous factors, but also on autocrine factors secreted into the conditioned medium. It is also well known that the identification of bioactive proteins secreted into the conditioned medium poses a substantial challenge. Recently, neural stem/progenitor cells were shown to secrete a survival factor, cystatin C, into the conditioned medium. Here, we demonstrate an approach to identify other low molecular weight proteins in conditioned medium from cultured adult rat hippocampal progenitor cells. A combination of preparative two-dimensional gel electrophoresis (2-DE) and mass spectrometry was utilized in the analysis. We were able to identify a number of proteins, which include Rho-guanine nucleotide dissociation inhibitor 1, phosphatidylethanolamine binding protein (PEBP), also termed Raf-1 kinase interacting protein, polyubiquitin, immunophilin FK506 binding protein 12 (FKBP12) and cystatin C. The presence of PEBP and FKBP12 in conditioned medium was confirmed immunologically. All nestin-positive progenitor cells showed immunoreactivity for antibodies against PEBP and FKBP12. To our knowledge we are the first to use this preparative proteomic approach to search for stem cell factors in conditioned medium. The method could be used to identify novel bioactive proteins secreted by stem/progenitor cells *in vitro*. Identification of bioactive proteins *in vitro* is of potential importance for the understanding of the regulatory mechanisms of the cells *in vivo*. Copyright © 2003 John Wiley & Sons, Ltd.

Most neurons in the central nervous system (CNS) are terminally differentiated and do not divide. However, in the early 1960s, evidence of neurogenesis in the adult rat brain was observed, indicating the existence of neural stem/progenitor cells (NSCs) in the postnatal brain.[1,2] These early findings in the rodent brain were later confirmed.[3–5] A few years ago, neurogenesis in the adult hippocampus of humans[6] and other primates[7] was found, suggesting that neurons are generated throughout life. The mechanisms controlling the neurogenesis are not well understood. The isolation of NSCs from the adult CNS has allowed more detailed characterization of the regulatory mechanisms of survival, differentiation and proliferation *in vitro*, which is of potential importance for the understanding of the regulation of the cells *in vivo*.

Adult rat hippocampal derived progenitor cells (AHPs) can be isolated and propagated with fibroblast growth factor 2 (FGF-2) *in vitro*.[4] Another key factor in the isolation and propagation of AHPs is to understand the cell density dependent survival mechanisms. It is known that proliferation and survival of NSCs depend on exogenous factors as well as on factors secreted into the conditioned medium. Previous studies have demonstrated that unidentified factors affecting the cell culture are present in conditioned medium.[4,8,9] Cystatin C was recently identified as a survival factor in conditioned medium, providing one explanation for the cell density dependence of AHP survival.[9]

The current study employs an approach based on the analysis of conditioned medium. In this study we focused on identification of proteins in the molecular weight range 10–25 kDa, since cystatin C and many other potential secreted bioactive factors are likely to be in this molecular weight region. Challenges in the analysis are associated with the relatively low protein content. The proteins of interest are less abundant than the medium components and they are relatively small as well. Preparative 2-DE was utilized to isolate the proteins prior to mass spectrometric identification.[10–12]

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MATERIALS AND METHODS

Cell culturing

Clonally derived AHPs from rat[4] were received at passage 4 as a gift from Dr Fred Gage (Laboratory of Genetics, The Salk Institute, La Jolla, CA, USA). The cells were cultured in Dulbecco's modified Eagle's medium/Hams' F12 (DMEM/Nut

Mix F12 1:1) plus 2.5 mM L-glutamine, N2 supplement (N2 medium; GIBCO Invitrogen Corp., Paisley, UK), and 20 ng/mL human basic FGF-2 (Peprotech Inc., Rocky Hill, NJ, USA). Cells were cultured in 75-cm^3 flasks coated with polyornithine at a density of 3000 cells per cm^2. Culture medium was replaced 24 h after seeding, and the cells were thereafter allowed to grow for 10 days without further medium replacement, in order to maximize the concentration of secreted proteins. The total volume (50 mL) of conditioned medium was recovered from five flasks of cultured cells. Medium added to flasks containing no cells was incubated in the same way as the conditioned medium and used for comparison. For the experiments confirming the secretion, AHPs were cultured for 2 or 4 days in a similar way with initial medium volume of 12 mL in each of the five flasks. Conditioned medium was collected carefully to avoid contaminating cells, centrifuged to remove any cellular material and stored at −20°C until analysis.

Liquid-phase isoelectric focusing

Liquid-phase isoelectric focusing (IEF) was performed using a Rotofor cell (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's protocol. The medium (45 mL) was dialyzed for 2 h (MW cut-off 6000–8000 Da) against distilled water (4 L, 4°C). Following dialysis, medium samples were brought up to a volume of 55 mL with distilled water. Thereafter octylglycoside (0.1%, Roche, Mannheim, Germany) and Servalyt (2%, pH 3–10, Serva Electrophoresis, Heidelberg, Germany) were added. Focusing was performed at 4°C at 15 W (constant) for approximate 4 h. Twenty fractions (2.8 mL) were harvested and pH was determined using pH indicator strips (Merck, Darmstadt, Germany). Aliquots (100 μL) of the IEF fractions were concentrated and analyzed by the NuPAGE system (Novex, San Diego, CA, USA), as described below. Selected IEF fractions were pooled and dried by vacuum centrifugation.

SDS-PAGE and electroelution

SDS-PAGE and electroelution were performed according to the supplier's protocol. The samples were dissolved in a total volume of 200 μL of NuPAGE sample buffer (0.14 M tris, 0.10 M tris-HCl, 0.4 mM EDTA, pH 8.5, containing 10% glycerol, 2% lithium dodecyl sulfate, 0.08% serva blue, 0.025% phenol red, 3% dithiothreitol), boiled for 3 min and then analyzed by the NuPAGE system using one-well 10% Bis-Tris gels and the NuPAGE MES SDS (1.0 M MES, 1.0 M trisbase, 20.5 mM EDTA, 69.3 mM SDS) running buffer. The electrophoresis was run for 40 min at 200 V (constant). The electroelution was performed at 100 mA (constant) for 30 min using the Mini Whole Gel eluter (Bio-Rad Laboratories) with MOPS-histidine as elution buffer (30 mM MOPS, 25 mM histidine, pH 6.5). Fourteen fractions (0.5 mL) were harvested from the unit. Aliquots (50 μL) were concentrated, dissolved in 15 μL of NuPAGE sample buffer, and analyzed by the NuPAGE system followed by staining with the Novex colloidal blue stain kit. The remaining part of the gel eluter samples was dried and purified from SDS and salt by protein precipitation. The samples were reconstituted in water (200 μL), ice-cold acetone (600 μL) was added, stored at −20°C for 2 h, centrifuged (10 min, 35 000 *g*), and the supernatants carefully removed. The protein pellet was left to dry.

Enzymatic digestion

The fractions were dissolved in 25 μL of digestion buffer (0.05 mM $CaCl_2$, 0.05 M NH_4HCO_3). Sequencing-grade modified trypsin (5 μL, 50 mg/L; Promega, Madison, WI, USA) dissolved in digestion buffer was added and incubated for 4 h at 37°C. The samples were dried and reconstituted in 25 μL of 0.1% trifluoroacetic acid (TFA) in water.

MALDI-TOFMS

Aliquots (15 μL) of the tryptic digests were purified with ZipTip™ C_{18} (Millipore, Bedford, MA, USA) according to supplier's instructions. The samples were eluted with 3 μL saturated α-cyano-4-hydroxycinnamic acid (Aldrich Chemie, Steinheim, Germany) in acetonitrile/0.1% TFA in water (1:1 v/v) directly onto a matrix seed layer.[13] The matrix-assisted laser desorption/ionization (MALDI) analyses were performed using an upgraded Bruker Reflex II instrument (Bruker-Franzen Analytik, Bremen, Germany) equipped with a two-stage electrostatic reflectron, a delayed extraction ion source, a high-resolution detector and a 2 GHz digitizer. The spectra were acquired in reflectron mode. Calibration was performed externally by using a mixture of known peptides or by using two auto-digestion products of trypsin if present in the spectra. Resulting monoisotopic peaks were compared against the NCBInr database.[14] Mass deviations of 75 and 200 ppm were tolerated in the database search of internal and external calibrated mass spectra, respectively. Mammals were specified and missed cuts were between 1–3. Molecular weight was set to 0–300 kDa, but pI was not restricted.

ESI-QTOF-MS/MS

Protein identification/verification was also obtained through acquisition of fragment ion data using an electrospray quadrupole time-of-flight instrument (Q-TOF2; Micromass, Manchester, UK). ZipTip™ C_{18}-enriched samples in acetonitrile/0.1% formic acid (1:1 v/v) were sprayed from gold-coated glass capillaries (Micromass) using a nanoflow electrospray source. Argon was used as collision gas. The instrument calibration was performed in positive ion mode using fragment ions from Glu-fibrinopeptide B and a curve fit polynomial of order 2. Peptide fragment ion spectra were processed using MaxEnt3 (Micromass) and searched against the NCBInr database using MASCOT.[15] Mammals were selected as species. Molecular weight and pI were not restricted in the search.

Immunoblotting of identified proteins in conditioned medium

Immunoblotting of conditioned medium from 2 and 4 days of culturing was performed to confirm the secretion of immunophilin FK506 binding protein 12 (FKBP12) and phosphatidylethanolamine binding protein (PEBP). Conditioned medium (120 or 200 μL) was protein precipitated. Briefly, ice-cold acetone, three times the volume of that of the conditioned

medium, was added to each sample. The samples were stored at −20°C for 2 h, centrifuged (10 min, 35 000 *g*) and the supernatants carefully removed. The protein pellets were dissolved in 12 µL of NuPAGE sample buffer and analyzed by the NuPAGE system. The proteins were transferred to a PVDF membrane (Millipore) using a Trans-blot SD (BioRad) at 0.8 mA/cm^2 for 45 min. The membranes were blocked with 5% milk powder in phosphate-buffered saline (PBS) (50 mM $Na_2HPO_4{\cdot}2H_2O$, 17 mM $Na_2HPO_4{\cdot}H_2O$, 68 mM NaCl, pH 7.4) containing 0.05% Tween-20 and incubated overnight with anti-rabbit antibodies against FKBP12 (Affinity BioReagents, Golden, CO, USA) or PEBP (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:1000 in PBS-Tween. Thereafter, the samples were incubated with anti-rabbit alkaline phosphatase-conjugate (BioRad) diluted 1:1000 and allowed to stand for 1 h and a color reaction was developed.[16]

Immunostaining of identified proteins in AHPs

Cells were cultured for immunohistochemistry experiments to identify the cellular distributions of FKBP12 and PEBP. The cells were washed in PBS at pH 7.3 and fixed using 4% paraformaldehyde in PBS at 4°C for 10 min. After two washes in PBS, followed by blocking in PBS containing 0.05% saponin (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and 3% bovine serum albumin (BSA, Sigma-Aldrich Sweden AB) for 30 min, the cells were incubated with primary antisera. AHPs were incubated with PEBP (1:1000, Upstate Biotechnology) or FKBP12 (1:200, Affinity BioReagents) antibodies together with a purified mouse anti-rat nestin antiserum (1:500; Pharmingen, Becton-Dickinson, Franklin Lakes, NJ, USA) in PBS containing 0.05% saponin and 1% BSA (PBS1%) overnight. After three washes in PBS, cells were incubated for 1 h with secondary antiserum in PBS1% at room temperature. The secondary antisera, fluorescein isothiocyanate-conjugated donkey anti-rabbit antiserum (1:150; Jackson ImmunoResearch Lab, West Grove, PA, USA) and Alexa Fluor594-conjugated goat anti-mouse antiserum (1:500; Molecular Probes, Eugene, OR, USA), were incubated with the nuclear dye bisbenzimide from a stock at 5 µg/mL (1:90, Hoechst 33258, Sigma Chemical Co.). The procedure ended with three washes in PBS followed by mounting using DAKO fluorescent mounting medium (Dakopatts, Glostrup, Denmark).

RESULTS

Identification of proteins in conditioned medium

Preparative 2-DE was utilized to isolate the medium proteins prior to MS analysis. Comparison of the protein content after liquid-phase IEF revealed an increased amount of proteins in the conditioned medium compared with the culture medium. The proteins with apparent molecular weight below 30 kDa were the most markedly increased in the conditioned medium compared with culture medium (Fig. 1). To obtain gel-eluted fractions with higher concentration of the proteins of interest, two consecutive IEF fractions were combined. The

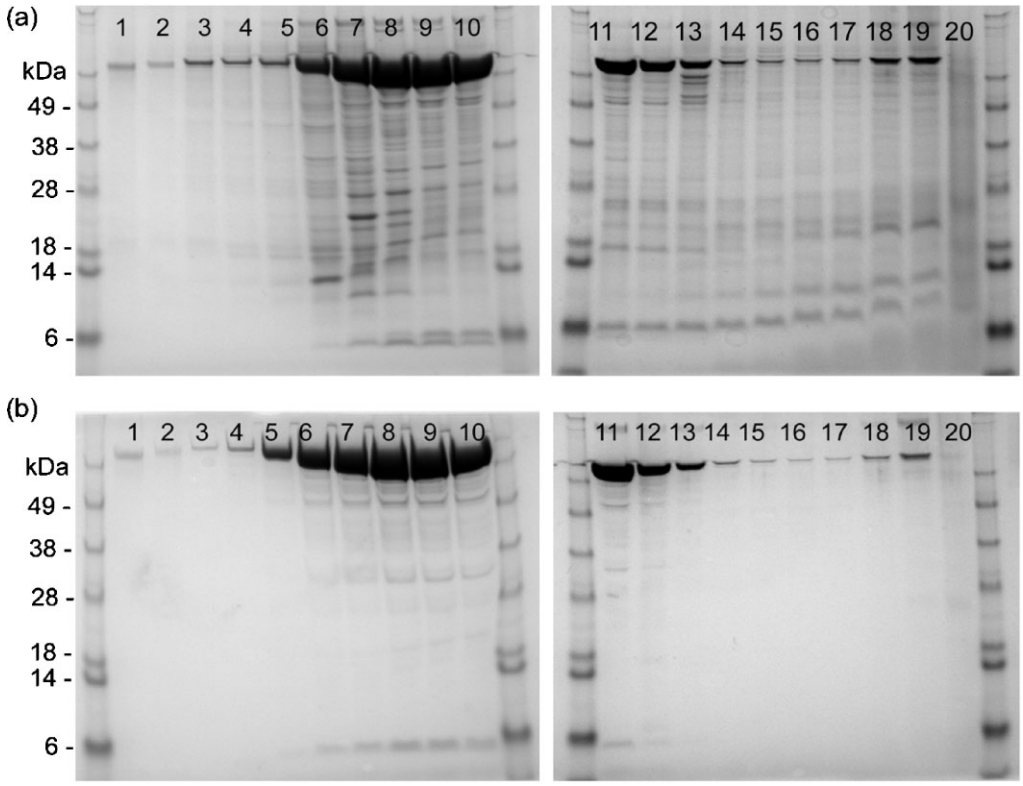

Figure 1. Aliquots of IEF fractions separated by SDS-PAGE. The proteins of the medium were separated according to pI in the liquid phase using the Rotofor cell, yielding 20 IEF fractions. Aliquots of the IEF fractions were separated using SDS-PAGE and visualized with colloidal blue staining: (a) conditioned medium and (b) culture medium. Two consecutive IEF fractions of the conditioned medium were pooled together prior to the 2D separation. The selected IEF fractions were 7 and 8 (IEF set 1), 11 and 12 (IEF set 2), and 17 and 18 (IEF set 3).

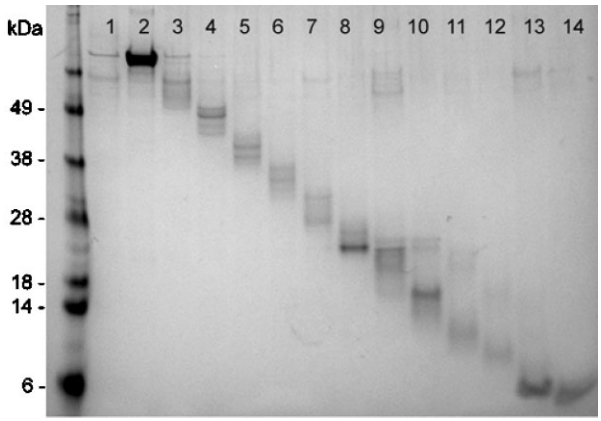

Figure 2. Aliquots of gel-eluted fractions separated by SDS-PAGE. In the second dimension, IEF set 2 (IEF fractions 11 and 12) was further purified by molecular weight using SDS-PAGE and electroelution directly from the gel into 14 fractions in the liquid phase. PEBP and polyubiquitin were identified in gel-eluted fractions 9 and 13, respectively.

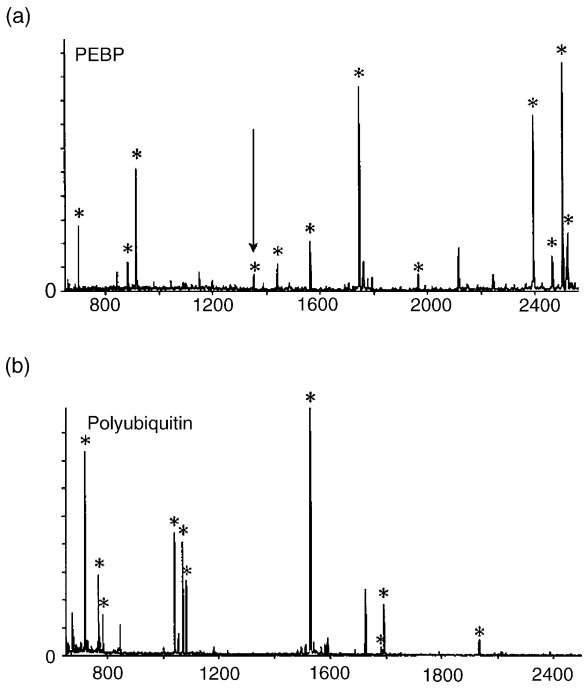

Figure 3. MALDI-TOFMS spectra of tryptic digest of gel-eluted fractions. (a) PEBP found in gel-eluted fraction 9 of IEF set 1 and (b) polyubiquitin found in gel-eluted fraction 13 of IEF set 2. The digestion products originating from PEBP and polyubiquitin are indicated by *. The digestion product indicated by an arrow in the MALDI spectrum was also selected for MS/MS analysis.

IEF fractions were selected by comparing conditioned medium with culture medium. The IEF fractions pooled together contained the same protein bands, while the three IEF sets contained different protein bands in the selected molecular weight range. The pooled fractions were 7 and 8 (IEF set 1), 11 and 12 (IEF set 2), and 17 and 18 (IEF set 3). SDS-PAGE separation combined with electroelution of the IEF fractions allowed isolation of highly enriched protein fractions (Fig. 2).

A number of lower molecular weight proteins were identified by MALDI peptide mapping. The MALDI mass spectra indicate high amounts as well as high purity of these proteins. A relatively large number of digestion products from the specific proteins were detected and almost all of the observed peptides were digestion products of the identified proteins (Fig. 3). Fragmentation analyses of digestion products by ESI-Q-TOF-MS/MS also revealed the identities of some proteins. The sequence tag together with the mass of the whole peptide provided a high specificity in the database search (Fig. 4). Some of the proteins were identified in several fractions, which might suggest post-translational modifications. Cystatin C has previously been identified as a glycosylated protein secreted by AHPs into conditioned medium. A selection of the proteins identified in the conditioned medium in IEF sets 1–3 is shown in Table 1. The identified proteins included Rho-guanine nucleotide dissociation inhibitor 1 (Rho-GDI-1), phosphatidylethanolamine binding protein (PEBP), polyubiquitin, immunophilin FK506 binding protein 12 (FKBP12) and cystatin C.

Immunoblotting of identified proteins in conditioned medium

Immunoblotting of conditioned medium for FKBP12 and PEBP showed presence of these proteins from day 2 of culturing, where they appeared as single bands at molecular weights of 12 and 23 kDa, respectively (Fig. 5). The intensity of the bands increased after 4 days of culturing. In order to evaluate the possible release of intracellular protein due to toxicity or cell death in the AHP cultures, the release of lactate dehydrogenase (LDH) from dying cells was measured. The levels of LDH (an intracellular enzyme) were low in conditioned medium from 2 and 4 days of culturing, and no morphological signs of cell death or toxicity were detected.

Immunostaining of identified proteins in AHPs

The cell cultures were immunostained to evaluate the cellular distributions of FKBP12 and PEBP in the AHPs. Both the proteins showed immunoreactivity in all nestin-positive AHPs. Nestin is a marker for immature NSCs. Immunofluorescent staining with the antibody against FKBP12 indicated weak immunoreactivity and seemed to be located in small vesicles in the cytosol (Plate 1(a)). The immunoreactivity against PEBP was strong and was most pronounced in the cytosol and plasma membrane (Plate 1(b)). No immunoreactivity was found when primary antisera were omitted in the immunofluorescent protocol (Plate 1(c)).

DISCUSSION

Most research on NSCs focuses on identification of factors that facilitate the undifferentiated growth of these cells, and factors that trigger differentiation along specific pathways. The commonly applied strategy to study the regulatory mechanisms is to exogenously add factors to the cell culture. However, systematic testing of factors that are known to affect cell systems other than the NSC culture would be a formidable undertaking. A complementary approach is based on the analysis of conditioned medium in a search for secreted bioactive proteins. One important advantage of

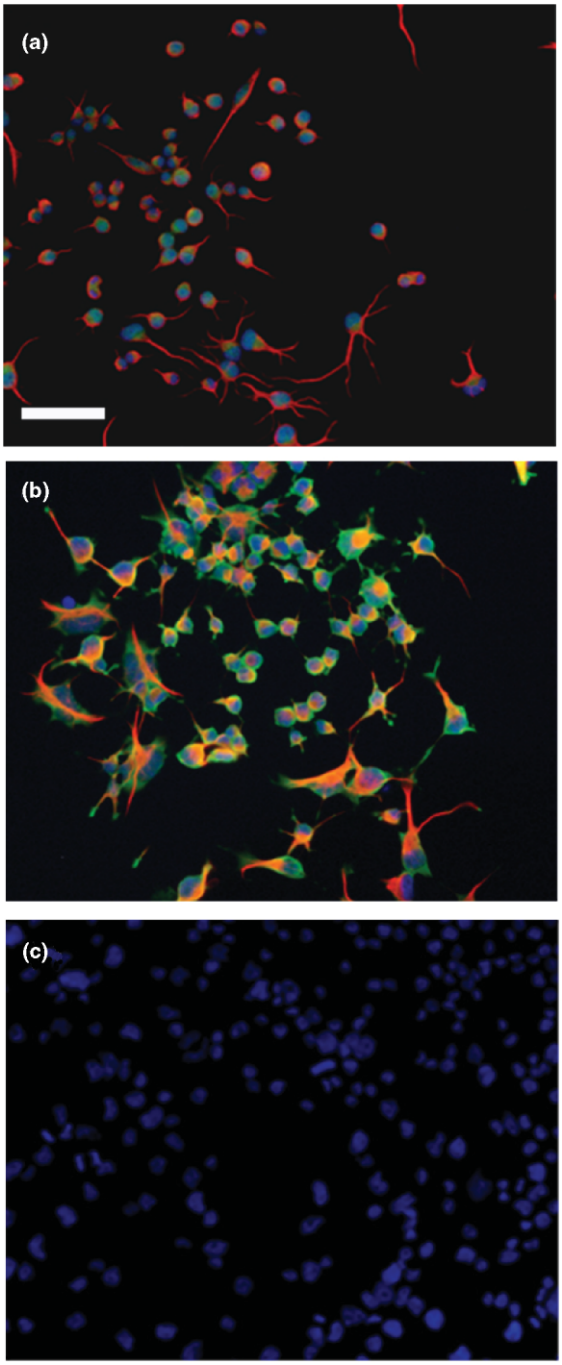

Plate 1. Localization of FKBP12 and PEBP in AHPs. Demonstration of immunostaining (green) for (a) FKBP12 and (b) PEBP. The AHPs are also immunostained with antiserum against nestin (red) and stained by Hoechst 33258 staining (blue). No immunoreactivity was found when primary antisera were omitted (c). Scale bar: 50 μm.

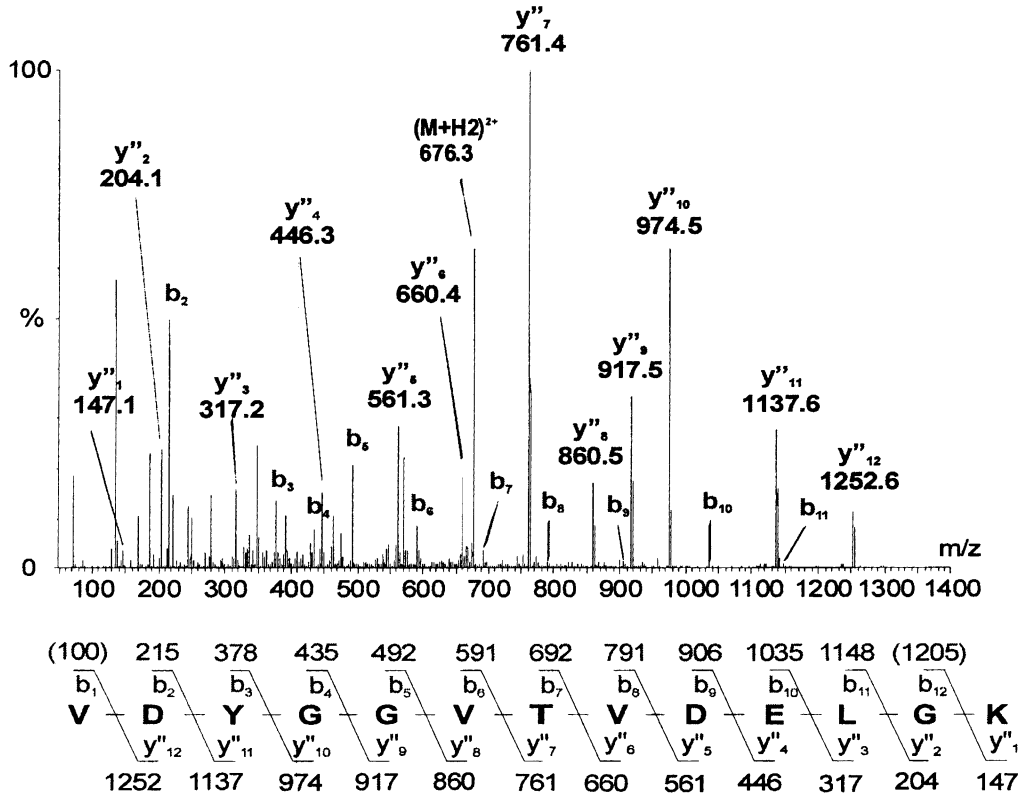

Figure 4. Spectrum from MS/MS analysis of the tryptic digestion product of *m/z* 1351 corresponding to PEBP in gel-eluted fraction 9 of IEF set 1. The spectrum shows the fragmentation pattern of the doubly charged precursor ion. The y-series of ions (C-terminal fragments), as well as those from the b-series (N-terminal fragment), are shown.

this approach is that potentially interesting proteins can be identified and thereby drastically limit the number of compounds that are of interest for bioactivity studies. It also allows identification of novel proteins that might not have been considered as interesting in a hypothesis-driven approach. Some proteins secreted from AHPs can be predicted to have autocrine/paracrine protective or survival effects. Other likely roles include communication with the normal environment of stem/progenitors *in vivo*.

We have demonstrated that it is possible to use a preparative proteomic approach based on mass spectrometry to identify proteins that might serve as examples of proteins in this context. A selection of the identified proteins is shown in Table 1. When potentially interesting proteins have been identified by using this combination of preparative 2-DE and mass spectrometry, complementary methods, e.g. immunohistochemical methods, can be utilized to confirm the secretion of the identified proteins from the AHPs. The approach presented in this study significantly improves the efficiency of identification of low abundance and low molecular weight proteins compared with analytical 2-DE.[10,12] Analysis of conditioned medium yielded highly enriched protein fractions commonly required for detection of a sufficient number of digestion products for a successful identification of these proteins. Furthermore, some of the proteins that can be found in comparative proteomics experiments might not have been discovered as interesting in a bioactivity context with a purely hypothesis-driven approach.

Immunological experiments were performed for two of the identified proteins. Both FKBP12 and PEBP were detected in conditioned medium from cultured AHPs. The fact that the proteins were present in conditioned medium after only 2 days of culturing strengthens the assumption that they are secreted from AHPs. The significant increase in intensity of the protein bands between day 2 and day 4 is caused by an accumulation of the proteins during culturing. The release of LDH was low, and no signs of cell death or toxicity were detected in conditioned medium from either 2 or 4 days of culturing. These results are further evidence that the presence of FKBP12 and PEBP is due to secretion. Earlier studies have detected secretion of FKBP12 and PEBP from cultured human mast cells and Rat-1 fibroblasts, respectively.[17,18] Extracellular FKBP12 was demonstrated to function as a cytokine in cell-to-cell communication by effecting the intracellular Ca^{2+} signaling. High levels of FKBP12 are released in the male reproductive tract and specifically associate with maturing sperm. Recombinant FKBP12 enhances the curvilinear velocity of immature sperm, suggesting a role for FKBP12 in motility initiation. The highest concentrations of soluble FKBP12 in the male reproductive tract occur in the lumen of the vas deferens, a site of sperm storage and the conduit for ejaculated sperm. These results suggest that FKBP12 exerts effects also outside the cell membrane and thus are transported out of cells.[19]

The AHP culture was immunostained to evaluate the distribution of FKBP12 and PEBP. All nestin-positive AHPs exhibited immunoreactivity for the antibodies against

Table 1. Proteins in the conditioned medium identified in the tryptic digest of gel-eluted fractions obtained from IEF sets 1–3. IEF sets 1–3 contain the combinations of IEF fractions 7 and 8, 11 and 12, and 17 and 18, respectively. The proteins originated from rat except for RhoGDI-1, which originated from mouse

IFF set	Gel-eluted fraction	Protein identity	Accession no. (NCBInr)	MALDI-TOF-MS[a]	ESI-Q-TOF-MS/MS[b]	Known function	Ref.
1	8	Rho-guanine nucleotide dissociation inhibitor 1	12597249		x	Regulation of the Rho/Rac family members	37,38
	9	Phosphatidylethanol-amine binding protein	8393910	15	x	Membrane biogenesis Regulation of MARK and NF-κB pathways HCNP peptide precursor Serine protease inhibitor	18,21,31–33,40
2	9	Phosphatidylethanol-amine binding protein	—	8		—	
	13	Polyubiquitin	1050930	12	x	Selective protein degradation	39
3	7	Cystatin C	226712	9		Cysteine protease inhibitor Survival factor of AHPs	9
	8	Cystatin C	—	13		—	
	9	Cystatin C	—	16		—	
	10	Cystatin C	—	9		—	
	11	Immunophilin FK506 binding protein 12	17985953	8		Peptidyl propyl isomerase activity Interacts with TGF-β type 1, IP3R and RyR receptors	17,20,24
	13	Polyubiquitin	—		x	—	

[a]Number of digestion products found in the MALDI mass spectra of the identified protein with a Z score of 1.65 or higher given by ProFound.
[b]Protein identification/verification from peptide fragment ion spectra indicating identity or extensive homology ($p < 0.05$).

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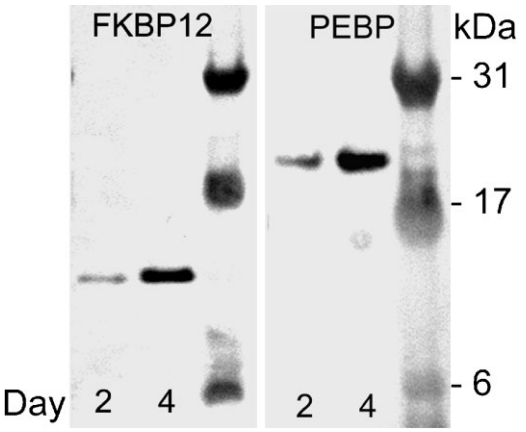

Figure 5. Immunoblotting of FKBP12 and PEBP in conditioned medium from 2 and 4 days of culturing. (a) FKBP12 appeared as a single band at 12 kDa and (b) PEBP as a single band at 23 kDa. The intensity of the bands was significantly increased in conditioned medium from 4 days of culturing.

FKBP12 and PEPB. Both FKBP12 and PEBP had a cytoplasmic localization. These results are supported by previous studies, where PEBP has been localized to the cytoplasm and the plasma membrane, as well as on the cell surface of Rat-1 fibroblast cells.[18] In rat brain, localization of FKBP12 was predominantly neural and the immunoreactivity was strong in the nucleus, cytoplasm and in neural processes of the neurons.[20] Both PEBP and FKBP12 have hippocampal localization in the rodent brain. Very high levels of FKBP12 are localized in neurons in the CA1 layer of the hippocampus.[21] The mRNA expression of PEBP was highest in the CA3 layer and dentate gyrus of the hippocampus.[22] Cystatin C, earlier identified in conditioned medium from AHPs, was demonstrated to stimulate neurogenesis cooperatively with FGF-2 in adult rat brain. Other identified proteins might also have a biological role *in vivo*. The presence of FKBP12 and PEBP in the AHP culture, and in localization in the hippocampus, might suggest such an association. PEBP has been described as the precursor of the hippocampal cholinergic neurostimulating peptide (HCNP) that is involved in the differentiation of neurons in developing hippocampus.[22] The peptide and NGF act cooperatively to enhance the phenotype development of septal cholinergic neurons *in vitro*, although they have different modes of action.[23] One could speculate that PEBP may signal in a retrograde manner to septal neurons from the hippocampus and thus also provide a biochemical link from progenitor cells in the hippocampus to septal cholinergic neurons. The expression of these proteins in the AHPs *in vitro* might be related to their origin and/or function in the hippocampus *in vivo*.

Recently, the gene expression profiles of homatopioetic, neural and embryonic stem cell populations were defined.[24,25] The genes up-regulated in the studied stem cell populations were consistent with the proteins identified in the present study. FKBP12 belongs to the peptidyl-prolyl isomerase family involved in protein folding. Genes of this protein family have been identified as the most up-regulated genes in the stem cell populations.[25] In hematopoietic stem cells, the gene of FKBP12 was up-regulated.[24] However, FKBP12 also binds and regulates the transforming growth factor-β (TGF-β) type 1, inositol 1,4,5-trisphosphate (IP3) and ryanodine (Ry) receptors (R), and the interaction with these receptors has been proposed to be more important than mediating protein folding.[26] The TGF-β family polypeptide also binds to the TGF-β type I receptor and regulates cell growth and differentiation. There is a highly defined expression pattern of IP3R throughout embryogenesis, which suggests important roles of IP3R in developmental events.[27–29] In rat brain, very high levels of FKBP12 and IP3R are localized to the CA1 layer of the hippocampus.[21,30] Several members of the TGF-β pathway and the IP3R were up-regulated in the stem cell populations as well, which might imply roles of FKBP12 in these pathways.[24,25] The protein has also been found in conditioned medium from mouse embryonic fibroblast feeder layers, which support the growth of human embryonic stem cells.[31]

A variety of functions has been suggested for PEBP that include involvement in membrane biogenesis, lipid transfer and inhibitory activity against trypsin-like serine proteases.[18,32,33] Interestingly, PEBP expression has also been implicated in the pathogenesis of Alzheimer's disease.[34] PEBP is also termed Raf-1 kinase interacting protein (RKIP). *In vitro*, PEBP was able to control the activity of the Raf/MEK/ERK mitogen-activated protein kinase (MAPK) pathway by regulating the interaction between Raf-1 and MEK.[35] Several other members of the MAPK pathway were found up-regulated in stem cell populations.[24,25] Primitive hematopoietic progenitor cells require the Raf-1 protein for growth factor stimulated proliferation and differentiation.[36] These findings might suggest that a possible biological function of PEBP in the AHP culture is through this pathway.

Other proteins identified in conditioned medium were RhoGDI-1, polyubiquitin and cystatin C. RhoGDI-1 participates in the regulation of the Rho/Rac family members. The Rho/Rac-family mainly regulates reorganization of the actin cytoskeleton, which is essential for a variety of morphological events associated with developmental processes, but is also involved in transcription, proliferation, apoptosis and membrane trafficking.[37,38] Ubiquitin targets proteins for selective degradation by the attachment of polyubiquitin chains, that is required for a variety of functions such as growth control, transcription and stress response.[39] A number of gene members implicated in their pathways were up-regulated in the studied stem cell cultures.[25] Both the proteins have also been found in conditioned medium from mouse embryonic fibroblasts.[31] A glycoform of cystatin C acts as a survival factor and cooperates with FGF-2 to stimulate proliferation of AHPs.[9]

In summary, preparative 2-DE followed by mass spectrometry can identify low molecular weight proteins in conditioned medium and thus might provide valuable clues in the search for potential bioactive factors. Future studies will test the biological activity of the identified proteins. Further studies are also needed to completely screen all fractions of the culture medium for more potentially interesting proteins and to investigate the role of these proteins in NSC biology *in vivo*. To our knowledge we are the first to successfully analyze conditioned medium in a search

for novel stem cell factors using this preparative proteomic approach.

Acknowledgements

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