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Heat shock protein 70 (Hsp70) subtype expression in neuroendocrine tissue and identification of a neuroendocrine tumour-specific Hsp70 truncation

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Abstract

In order to identify neuroendocrine tumour-specific protein expression, we generated monoclonal antibodies (mAbs) with a tumour-related reaction pattern using a human insulinoma as immunogen. One of the generated mAbs (mAb 1D4) exhibited striking immunoreactivity against various neuroendocrine tumours without staining pancreatic islets of Langerhans. Furthermore, mAb 1D4 immunostained a characteristic subtype of hypothalamic neurones. Using two-dimensional (2-D) gel electrophoresis, mAb 1D4 immunoblotting and mass spectrometry, heat shock protein 70 (Hsp70) isoforms were identified as the mAb 1D4-specific antigen. In hypothalamic tissue, the presence of two different Hsp70 isoforms (Hsp70-8 and Hsp70-1) was revealed by 2-D gel immunoblots and consecutive mass spectrometric peptide analysis. In contrast, insulinoma and other neuroendocrine tumours displayed solely Hsp70-8 expression. Moreover, the tumour-specific presence of an additional mAb 1D4 immunoreactive protein of 40 kDa was observed in eight out of eight tested neuroendocrine tumours. For this variant, exclusively, peptides derived from the C terminus excluding the 299 amino-terminal residues were detected. In cultured tumour-derived fibroblasts, expression of the truncated Hsp70-8 subtype was not present. In conclusion, we have demonstrated a neuroendocrine tumour-specific expression pattern of Hsp70 isoforms and identified an as yet unknown N-terminally truncated Hsp70-8 variant.

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Introduction

Endocrine tumours are characterized by a specific protein and hormone expression pattern leading to tumour characteristic phenotypes. Tumour-specific protein expression is essential for correct histo-pathological classification and diagnosis. Following our long-term goal to identify tumour-specific protein modification and over-expression we generated mouse monoclonal antibodies (mAbs) using a human insulinoma protein extract as immunogen. In this way we selected mAb 1D4 and identified heat shock protein 70 (Hsp70) as its antigen using tumour proteomics.

Heat shock proteins are a diverse family of proteins, which are classified according to their molecular weight. Within these groups, several protein variants have been subclassified recently. Heat shock proteins have been associated with the potential to support cells in overcoming stress such as ischaemia, acidosis and fever. The biological activity of heat shock proteins is exerted at least partly via protein–protein interactions, which are often tissue specific. Depending on their interaction partner, different functional properties have been ascribed to the Hsp70 protein family. Recently identified Hsp70 partner proteins include p53 (Zylicz *et al*. 2001),

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Apaf-1 (Beere *et al*. 2000), and nuclear factor-κB (Malhotra & Wong 2002). Furthermore, Hsp70 was identified as a part of the spliceosome (Zhou *et al*. 2002). At the post-translational level, Hsp70 exerts chaperone activity on newly produced proteins (Hartl & Hayer-Hartl 2002) and is responsible for refolding of protein structures. The diversity of the mostly ATP-dependent biological functions of Hsp70 relies on the characteristic structure of the Hsp70 protein. Whereas the 380 N-terminal amino acids are responsible for ATP binding, the C-terminal part represents the protein–peptide interaction domain (Kiang & Tsokos 1998, Frydman 2001). The importance of this protein family is exemplified by its numerous encodings (11–12 times) in the human genome at different chromosomes (Tavaria *et al*. 1996). However, the exact mechanism of the regulation of Hsp70 subtype expression still has to be elucidated and detailed studies are only available for Hsp70-1, Hsp70-8 and Hsp70-hom (Fourie *et al*. 2001).

Although the underlying molecular basis of neuroendocrine tumour development still has to be characterized (Pavelic *et al*. 1995, Pelengaris & Khan 2001), recent evidence indicates that neuroendocrine neoplasias exhibit aberrant regulation of cell cycle-promoting factors, signalling proteins, oncogenes as well as over-expression of anti-apoptotic proteins, e.g. Hsp70 (Wang 1999, Beer *et al*. 2002, Lichtenfels *et al*. 2002). For example, over-expression of Hsp70 is a common phenomenon in breast cancer cell lines (Nylandsted *et al*. 2000*a*,*b*, Wang *et al*. 2002). Furthermore, a novel 54 kDa splice variant of the Hsp70 gene has been described in human tissue and the U937 promonocytic cell line (Tsukahara *et al*. 2000).

In this study, we have described the mode of antigen identification of the newly produced mAb 1D4. We have elucidated a characteristic neuroendocrine tumour-specific over-expression of the Hsp70-8 isoforms and identified a novel truncated Hsp70-8 subvariant. This was elaborated using the mAb 1D4 in immunohistochemical analysis, two-dimensional (2-D) immunoblots and consecutive mass spectrometric protein analysis. Due to the site of mAb 1D4 antigen recognition within the central part of the protein (amino acids 299–463), we were able to identify a novel N-terminally as well as the already described C-terminally truncated isoform of Hsp70-8 in tumour tissue. We further present data showing that mAb 1D4 has the potential to detect Hsp70-1 and Hsp70-8 isoforms at the 2-D immunoblot. In comparative immunoblot experiments using mAb 1D4 and the commercially available Hsp70-specific mAb (SPA-810) we have shown differences in the detection of Hsp70 variants depending on the mAb.

Materials and methods

Reagents

We obtained the following from their suppliers: rabbit-anti-mouse immunoglobulins, monoclonal mouse peroxidase anti-peroxidase (PAP) and diaminobenzidine tetrahydrochloride chromogen (DAKO, Glostrup, Denmark); aquamount improved (BDH, Poole, Dorset, UK); goat-anti-mouse peroxidase-conjugated and alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO); pGEM-T easy vector system I (Promega, Madison, WI, USA); sample grinding kit and Immobilin DryStrip, pGEX-1λT-protein expression vector and bulk gluthione S-transferase (GST) purification module (Amersham Pharmacia Biotech, Uppsala, Sweden); plasmid purification kits (QIAGEN, Hilden, Germany); ELU-QUIK (Schleicher & Schuell, Dassel, Germany); sodium carbonate, sodium thiosulfate, sodium pyruvate (S-8636), polyethylene glycol 1500 (P-7181), silver nitrate (S-0139), S-Gal/LB agar (C-4478), DL-dithiothreitol (D-0632), HAT media supplement (H-0262), yeast extract (Y-0375), collagenase (C-5138), mAb DM1A specific for α tubulin and penicillin–streptomycin solution (P0781) (Sigma, Vienna, Austria); tryptone (L42; Oxoid, Basingstoke, UK); BM chemoluminescence blotting substrate (POD) and expand high fidelity PCR system (Roche Diagnostics, Mannheim, Germany); RPMI 1640, trypsin–EDTA (1×) in HBSS without Ca and Mg and with EDTA (GibcoBRL, Paisley, Strathclyde, UK); premium fetal bovine serum (Bio Whittaker, Walkersville, MD, USA); anti-Hsp70 mAb (SPA-810; StressGen Biotechnologies Corp., Victoria, BC, Canada); anti-actin affinity-purified rabbit antibody (A2066) (Sigma).

Immunocytochemistry

Cryostat sections of different human tissues were air dried and fixed in acetone (5 min). Fixed tissue sections were either wrapped in aluminium foil and stored at −20°C or stained within 6 h. For immunostaining, slides were brought to room temperature, unwrapped, wetted with phosphate-buffered saline (PBS) and sequentially incubated with the mAb 1D4 (tissue cell culture supernatant, incubation time 2 h), followed by a polyclonal rabbit anti-mouse Ab (diluted 1 : 100 in PBS containing 10 ng/μl bovine serum albumin (BSA), incubation time 45 min), and the mouse PAP complex (diluted 1 : 100 in PBS containing BSA as indicated above, incubation time 45 min). The antigen was visualized by application of diaminobenzidine (5 min). Each incubation step was performed in a moist chamber at room temperature and was followed by a washing step in PBS (3×5 min). Stained tissue sections were mounted with aquamount improved

and cover slipped. Commercially obtained immunoreagents were from DAKO.

Immunoblotting

Human tissue aliquots were homogenized in 200 µl ice-cold homogenization buffer (Tris-buffered saline containing 1% Triton X-100, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 10 µg/ml leupeptin, 15 mM phenylmethylsulfonyl fluoride (PMSF) and 0.8 mM Pefabloc using a Polytron homogenizer (Kinematica, Kriens, Switzerland). Homogenates were then centrifuged at 10 000 ***g*** for 10 min. Total protein content was measured in pre-cleared tissue lysates using a micro BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts (15 µg/lane) were loaded onto a 12% PAGE gel. Electrophoretically separated proteins were transferred to nitrocellulose membranes by semi-dry blotting. Blotted membranes were blocked in skimmed milk for 1 h. Protein detection was performed by sequential incubation of the membranes with mAb 1D4 (3 h at room temperature or overnight at 4 °C) followed by peroxidase-labelled goat anti-mouse Ab diluted 1 : 5000 in PBS (45 min, room temperature). Finally, blots were developed using the BM chemoluminescent reagent (Roche Molecular Biochemicals, Vienna, Austria). Each incubation step was followed by two washes with PBS containing 0.1% Tween 20 for 10 min.

2-D gel electrophoresis, staining and documentation

Tissue samples obtained from tumours removed by microsurgery or human post mortem tissue were immediately frozen in liquid nitrogen. Samples were cut into fragments on dry ice and exposed to lysis buffer (8 M urea, 40 mM Tris base and 4% Triton X-100) and mechanical fragmentation in the 1.5 ml sample preparation tube (PlusOne sample grinding kit; Amersham Biosciences, San Francisco, CA, USA). The solubilized material was then centrifuged for 20 min at 12 000 ***g*** in an Eppendorf centrifuge. After protein measurement, the clear supernatant was either immediately loaded onto the rehydrated immobilized pH gradient (IPG) strip in the strip holder or frozen in 20 µl aliquots at −80 °C. The isoelectric focusing at the IPGphor (Amersham Pharmacia Biotech) was carried out in a three-step mode (500 V, 1 h; 1000 V, 2 h; 8000 V, 8 h) and 50 µA/IPG strip usually leading to a 64 000 Volt hours focusing. The strip was then placed on top of a 12% SDS-PAGE gel after 15 min equilibration in the freshly prepared SDS equilibration buffer (Tris–Cl 50 mM, pH 8.8, urea 6 M, glycerol 30%, SDS 2% and bromophenol blue). The agarose-embedded IPG strip on top of the SDS-PAGE gel was overlaid with running buffer (Tris–glycine–SDS). The second dimension vertical denaturing 12% polyacrylamide SDS gel was run for 6 h at 140 V and then either transferred onto nitrocellulose by semi-dry blotting or fixed in fixative using the silver-staining protocol according to Blum *et al.* (1987). After identification of the antigen recognized by the mAb 1D4 at the immunoblot, the corresponding spots on the silver-stained gel were carefully cut out for preparation of a tryptic digest and subjected to mass spectrometric analysis.

Mass spectrometric protein analysis

Nano HPLC

All nano HPLC separations were performed using a nano HPLC system UltiMate (LC Packings, Amsterdam, The Netherlands). The system consists of an UltiMate micro HPLC pump and an u.v. detection unit, the Switchos micro column-switching device with loading pump and two 10-port valves for pre-concentration, and the FAMOS micro autosampler. The flow rate of the nano HPLC system was 200 nl/min and the u.v. detector was operated at $\lambda = 214$ nm using the nano UV-Z View flow cell; the volume of the u.v. cell is 3 nl.

The Switchos loading pump was operated at 20 µl/min. Connections from the pre-column to the Switchos valve were made using 30 µm internal diameter (ID) tubing. Switchos flow was delivered using 120 µm ID tubing. For the Switchos, pre-columns with 300 µm ID and 5 mm length packed with PepMap C18 were used in the present work.

The FAMOS micro autosampler was operated in the large volume injection mode using a 250 µl sample loop and a 250 µl syringe. After a loading time for the sample of 10 min, the Switchos valve changed the position and switching of the pre-column in-line with the nano separation column. The sample was eluted in back flush mode.

Mobile phases were: A = 95% water (HPLC grade, Supra-Gradient; Merck Darmstadt, Germany), 5% acetonitrile (HPLC grade, Supra-Gradient; Merck Darmstadt, Germany) and 0.1% formic acid (Fluka, Buchs, Switzerland) and B = 30% water, 70% acetonitrile and 0.1% formic acid. The mobile phase for the Switchos loading pump was water with 0.1% trifluoroacetic acid (Pierce, Rockford, USA). The HPLC gradient for separation was 0–50% B in 30 min and 50–100% B in 2 min.

The dimensions of the separation column were 0.075 mm ID × 150 mm length, 3 µm particle size. The sample was loaded for 10 min using the flow of the Switchos loading pump. After 10 min, the valve position

was changed and the sample was eluted onto the nano separation column.

Mass spectrometry

Eluted peptides were transferred on-line to a heated capillary (Pico Tip, FS360-20-10; New Objective, Cambridge, MA, USA) of an ion trap mass spectrometer (LCQ Deca XP, Thermo Electron, Bremen, Germany). The following electro spray ionisation (ESI) parameters were used: spray voltage 1.8 kV, capillary temperature 185 °C, capillary voltage 45 V, tube lens offset voltage 25 V and the electron multiplier at −1050 V. The collision energy was set automatically depending on the mass of the parent ion. Gain control was set to 5×10^7. The data were collected in the centroid mode using a mass spectrometer (MS) experiment (full-MS) followed by four MS/MS experiments of the four most intensive ions (intensity at least 5×10^5). Dynamic exclusion was used for data acquisition with an exclusion duration of 1 min and an exclusion mass width of ±3 Da.

All fluidic connections from UltiMate nano HPLC to the mass spectrometer were made using 20 µm ID fused silica capillaries, connected with low dead volume micro tight connectors from LC Packings.

Fibroblast culture from insulinoma tissue

After preparation of a single-cell suspension by passing insulinoma fragments through a cell strainer, fibroblasts derived from the insulinoma were grown for 2 months using established tissue culture conditions as described for hybridoma cell culture in complete culture medium (Wagner *et al*.. 1998). The absence of insulinoma tumour cells was tested by immunostaining of cytopreparations and insulin measurement in the tissue culture supernatant. Confluent fibroblast cultures were treated with collagenase and trypsin in order to liberate cells from tissue culture flasks. Proteins were extracted after two washing steps in ice-cold PBS as indicated above.

Generation of recombinant Hsp70-8 as a GST-fusion protein

Hsp70-8 cDNA (BC009322) was purchased from the Deutsches Ressourcenzentrum für Genforschung GmbH (Berlin, Germany). The coding region was amplified by PCR using the following primers containing a BamH1 at the 5′-terminus and an EcoR1 site at the 3′-terminus and expand high fidelity PCR system: forward: 5′TCGGATCCATGGCCAAAGCCG 3′; reverse: 5′ TGAATTCCTAATCCACCTCC 3′. The PCR product was ligated into pGEM-T-vector for further amplification. After restriction enzyme digest and gel purification using Schleicher & Schuell ELU-QUICK the Hsp70 coding sequence was ligated into the BamH1/EcoR1 site of the pGEX-1λT protein expression vector. Recombinant *E. coli* containing the Hsp70 coding region in pGEX-1λT was grown for 8 h at 31 °C under constant rotation and induced by isopropyl-1-thio-β-D-galactopyranoside (IPDG) (1.5 mM) for 4 h. Pelleted bacteria were suspended in PBS (4 °C) containing 0.1 mM PMSF and sonicated. After pelleting insoluble fragments, glutathione-Sepharose 4B was added to the supernatant and mixed at 4 °C. Glutathione-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) loaded with GST-HSP70 fusion protein were pelleted at 1000 ***g*** and washed using ice-cold PBS (2×). Finally, beads were incubated with thrombin (Amersham Pharmacia) under constant rotation at room temperature for 1.5 h. Purified protein was then loaded onto a 12% SDS-PAGE gel and either stained by Coomassie blue or transferred onto nitrocellulose for immunoblotting.

Informed consent was obtained from each patient in order to use some parts of the surgically removed tissue for research in accordance with the ethical committee of the Medical University of Vienna.

Results

Development of the murine mAb 1D4

In order to generate neuroendocrine tumour-specific mAbs, 5-week-old Balb-C mice were immunized subcutaneously using homogenized human insulinoma cells. Several insulinoma-reactive mAbs were obtained by fusion of immune spleen cells with the murine myeloma cell line 63A8.653 using polyethylene glycol 1500 by standard techniques (Wagner *et al*. 1998). Wells were screened for insulinoma cell-specific mAb by immunocytochemistry using insulinoma cell cytospin preparations (Fig. 1A). One of these antibodies (mAb 1D4) exhibited a striking, tumour-specific reaction pattern with strong cytoplasmic and nuclear staining of cultured insulinoma cells but absence of β-cell immunoreactivity. Because of its apparent tumour specificity, this antibody was chosen for antigen identification.

Comparison of mAb 1D4 immunostaining between tumour and healthy human tissue

To further analyse mAb 1D4 antigen expression, we screened several human post mortem normal and tumour tissues for mAb 1D4 immunoreactivity. In *in vitro* cultured insulinoma cells initially used as immunogen, a cytoplasmic and nuclear staining was confirmed (Fig. 1A). Pancreatic islet-specific staining was absent. However, remarkable immunostaining was observed in various neuroendocrine tumours: gastrinoma (Fig. 1B), insulinoma, carcinoid and

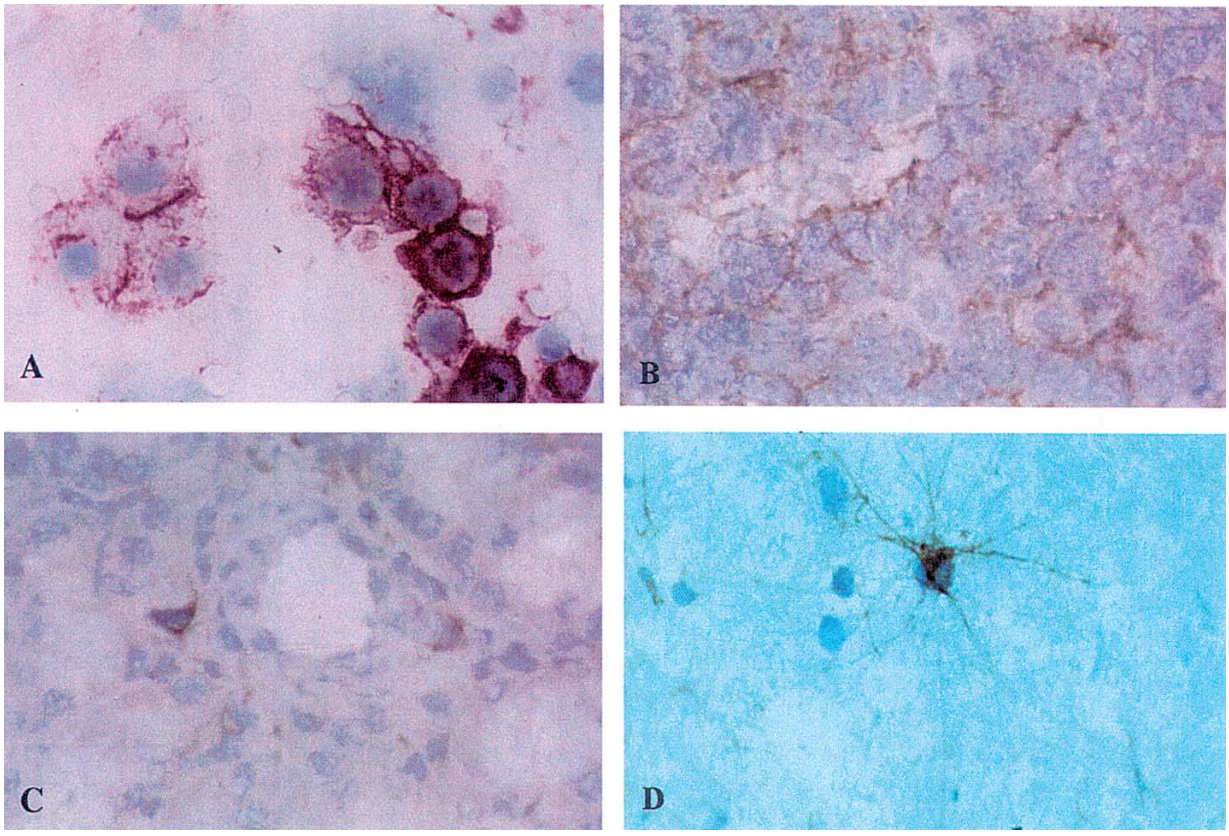

Figure 1 mAb 1D4 immunoreactivity in neuroendocrine tumours, human pancreas and hypothalamic tissue. (A) Cytospin preparations of isolated and human insulinoma cells cultured for 5 days were stained with mAb 1D4 and the APAAP technique. Variable nuclear and cytoplasmic staining intensity at different cells originating from the same tumour is demonstrated (magnification 1200×). (B) Immunostaining of human pancreatic gastrinoma cryosections by mAb 1D4 and the PAP technique was localized at cytoplasmic sites throughout the tumour (magnification 1100×). (C) Post mortem human pancreatic tissue (frozen sections, 4 μm) immunostained by mAb 1D4 and the PAP method using diaminobenzidine revealed positive cells in juxtaposition with pancreatic ducts (magnification 1100 ×). (D) Immunohistochemical localization of mAb 1D4 antigen in hypothalamic tissue. Cryosections of post mortem human hypothalamic tissue revealed mAb 1D4 immunostaining of characteristic polydendritic cells located within the paraventricular nucleus (magnification 1100×).

VIPoma (data not shown). In contrast, post mortem human pancreas revealed immunostaining of only a few periductal and ductal cells (Fig. 1C). In all tumours, mAb 1D4 staining was restricted to the cytoplasm and nuclear staining was absent (Fig. 1B). The staining intensity exhibited a remarkable intra- as well as inter-neoplastic variability, with regions of strong mAb 1D4 staining being directly adjacent to areas of low mAb 1D4 antigen expression.

Additionally, some faint staining was detectable in the anterior lobe of the pituitary gland (data not shown) and in a considerable number of distinct neurones located within the human hypothalamic paraventricular nucleus (Fig. 1D). Cortical neurones tested negative for mAb 1D4 immunostaining. The high extent of mAb 1D4 antigen expression in tumour cells in parallel with the complete absence of this protein in pancreatic islets of Langerhans motivated us to study this protein and the newly produced mAb 1D4 in more detail.

One-dimensional (1-D) immunoblot

In order to further characterize the mAb 1D4 antigen and to evaluate its expression pattern, equal protein quantities of lysates derived from various human endocrine tissues (pituitary, hypothalamic tissue) and tumours (anaplastic thyroid carcinoma (ATC)), ovarian adenoma, insulinoma, pheochromocytoma, pituitary adenoma) were subjected to Western blot analysis. A prominent mAb 1D4-specific band was present at ~70kDa in all of the tissues tested. However, the extent of immunodetected mAb 1D4 antigen showed tissue-specific variations. Considerable expression was detectable in the hypothalamus (Fig. 2A, seventh lane) and in the pituitary gland (Fig. 2A, sixth

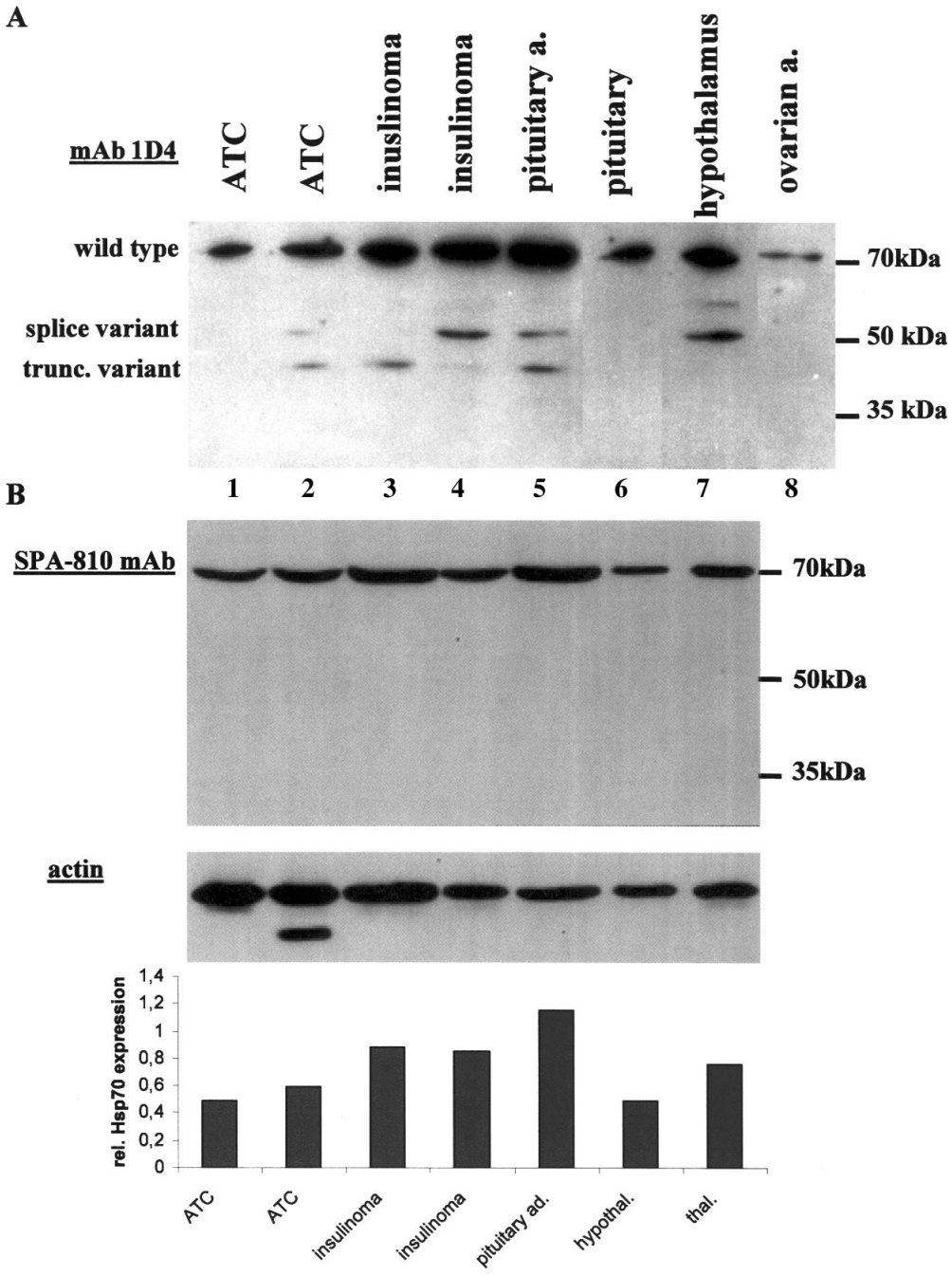

Figure 2 Detection of Hsp70 and Hsp70 subvariants in various endocrine neoplasias using different mAbs. (A) Equal quantities of tissue cell extract were loaded onto 12% SDS-PAGE gel and run under reducing conditions. Separated proteins were transferred to nitrocellulose and mAb 1D4 staining was visualized by mono-specific HRP-conjugated goat anti-mouse Ig and chemoluminescence reagent followed by exposure to X-ray films. Signal intensities were measured by densidometric scanning (lower panel) and set in relation to actin signals. In addition to the 70 kDa mAb 1D4 immunoreactive band the previously described 50 kDa splice variant was detected in tumours as well as in hypothalamus-derived tissue. Furthermore in tumour tissue a 40 kDa band was found with varying intensity depending on the tumour (second to fifth lanes). Pituitary a. = pituitary adenoma; ovarian a. = ovarian adenoma.
(B) Selected tumour extracts presenting with Hsp70 subvariant bands in the mAb 1D4 blot were retested by loading on to a 12% SDS-PAGE and consecutive blotting and development with a commercially available anti-Hsp70 mAb (SPA-810). Only one single band at 70 kDa is detectable. Comparable protein loading is demonstrated by re-probing the blot with anti-actin rabbit polyclonal antibody. Pituitary ad. = pituitary adenoma; hypothal. = hypothalamus; thal. = thalamus.

lane). The highest expression was observed in neuroendocrine tissue-derived tumours (e.g. insulinomas (Fig. 2A, third and fourth lanes) and pituitary adenoma (Fig. 2A, fifth lane). Expression of the mAb 1D4 antigen was lower in endocrine-derived tumours (ATC; Fig. 2A, first and second lanes; ovarian adenoma; Fig. 2A, eighth lane). Similar data were obtained in one medullary thyroid carcinoma (MTC), five further insulinomas and two carcinoid tumours (data not shown).

Surprisingly, two mAb 1D4 immunoreactive bands at ~50 and ~40 kDa were detected in distinct neuroendocrine tumours (insulinoma; Fig. 2A, third and fourth lanes; pituitary adenoma; Fig. 2A, fifth lane; carcinoid and MTC (data not shown)). Whereas the 40 kDa subvariant was completely absent from normal tissue, the ~50 kDa variant was also found in hypothalamic tissue. The immunosignal of these two smaller variants was about 2–4% of that present at 70 kDa. Thus, the immunoblot data were in line with our immunohistochemistry results, revealing remarkable expression of the mAb 1D4 antigen in neuroendocrine tumours.

In order to compare the mAb 1D4 results with a commercially available anti-Hsp70 mAb, we selected tumours showing Hsp70-8 variants at the mAb 1D4 immunoblot (Fig. 2B, upper panel). Interestingly, the same tumours exhibited only one single band at 70 kDa. We stress that all tested tumours were subjected to the same lysis procedure. Loading of comparable amounts of protein was confirmed by re-probing the same blot with a mono-specific anti-actin rabbit Ab (Fig. 2B, lower panel).

2-D electrophoresis and mass spectrometric analysis of mAb 1D4 immunoreactive dots

To identify the mAb 1D4 antigen, we performed 2-D gel electrophoresis using tissue lysates derived from MTC, human insulinoma, carcinoid, phaeochromocytoma, *in vitro* grown neuroendocrine tumour-derived fibroblasts and hypothalamic tissue. The analysed samples were subjected to isoelectric focusing, subsequent vertical SDS gel protein separation and mAb 1D4 immunoblotting. Using a pH gradient of 3–10 for isoelectric focusing only an accumulation of dots was visualized at a molecular weight of about 70 kDa and a pI of 5.4. A corresponding single dot was not identifiable at the silver-stained gel within the area of protein spot accumulation at the site of interest (data not shown). Consequently, we used Immobilon DryStrips with a pH gradient range of 4–7 in order to improve protein separation at the area of interest. The data from these investigations are presented below.

Hypothalamus and MTC

We identified four mAb 1D4 immunoreactive protein dots with modest variation in the molecular size of 70 kDa and differing pIs ranging from 5.37 to 5.48 when human hypothalamic tissue was the subject of investigation (Fig. 3A and B). In parallel, tissue of a human MTC was analyzed (Fig. 4). At the immunoblot, only one prominent and a juxtaposed weaker protein spot were identified at a molecular size of 70 kDa. This represented a remarkable difference from the results obtained from the hypothalamus. Subsequent mass spectrometric analysis (depicted in the left panel of Fig. 3A and B; identified matching peptides are shown underlined) of these separated dots identified Hsp70 subtypes as the antigen recognized by the mAb 1D4 (hypothalamus: dot 1 representing Hsp70-1, dots 2 and 3 representing Hsp70-8; MTC: all three dots representing Hsp70-8). Interestingly, we observed in both tissues the smaller mAb 1D4 immunoreactive protein spot of a molecular weight of 50 kDa and a pI of 5.6 (Figs 4B and 5B). A corresponding mAb 1D4 immunosignal was visualized in the 1-D immunoblot (Fig. 2A, second, fourth, fifth and seventh lanes). This dot represents the already described splice variant of Hsp70-8 (Tsukahara *et al*. 2000) which has a calculated pI of 5.64. Moreover, in lysates derived from the MTC, a 40kDa protein spot with a pI of 4.8 was detectable at the mAb 1D4 immunoblot (Fig. 5B). Corresponding immunosignals were already detected at the 1-D gel (Fig. 2A, second to fifth lanes). Mass spectrometric analysis of the corresponding silver-stained spot revealed peptides which mapped exclusively to the 346 C-terminal amino acids of Hsp70-8, indicating that this mAb 1D4-reactive protein moiety represents an N-terminally truncated Hsp70-8 variant. Bioinformatic analysis of this Hsp70-8 variant confirmed its appearence in 2-D gel electrophoresis.

Protein Hsp70-1 and Hsp70-8 alignment

In order to evaluate the opportunity to differentiate between Hsp70-1 and Hsp70-8 using 2-D gel protein separation and subsequent mass spectrometric analysis, the protein sequences of Hsp70-1 and Hsp70-8 were aligned. Both proteins exhibit a high degree (64%) of identity (Fig. 3C). However, the difference between the primary structure of Hsp70-1 and Hsp70-8 provides the possibility of confining all neuroendocrine tumour-derived identified endopeptides to Hsp70-8, whereas no tumour-derived endopeptide matched to a specific Hsp70-1 amino acid sequence.

Insulinoma E

Protein 2-D gel separation of the microsurgically removed tumour (Fig. 5A) resulted in visualization of three different mAb 1D4-immunoreactive protein dots (Fig. 5B). In accordance with the data obtained from the MTC analysis, we found a 40 kDa protein moiety exhibiting a pI of ~4.8. Mass spectrometry of this protein spot revealed

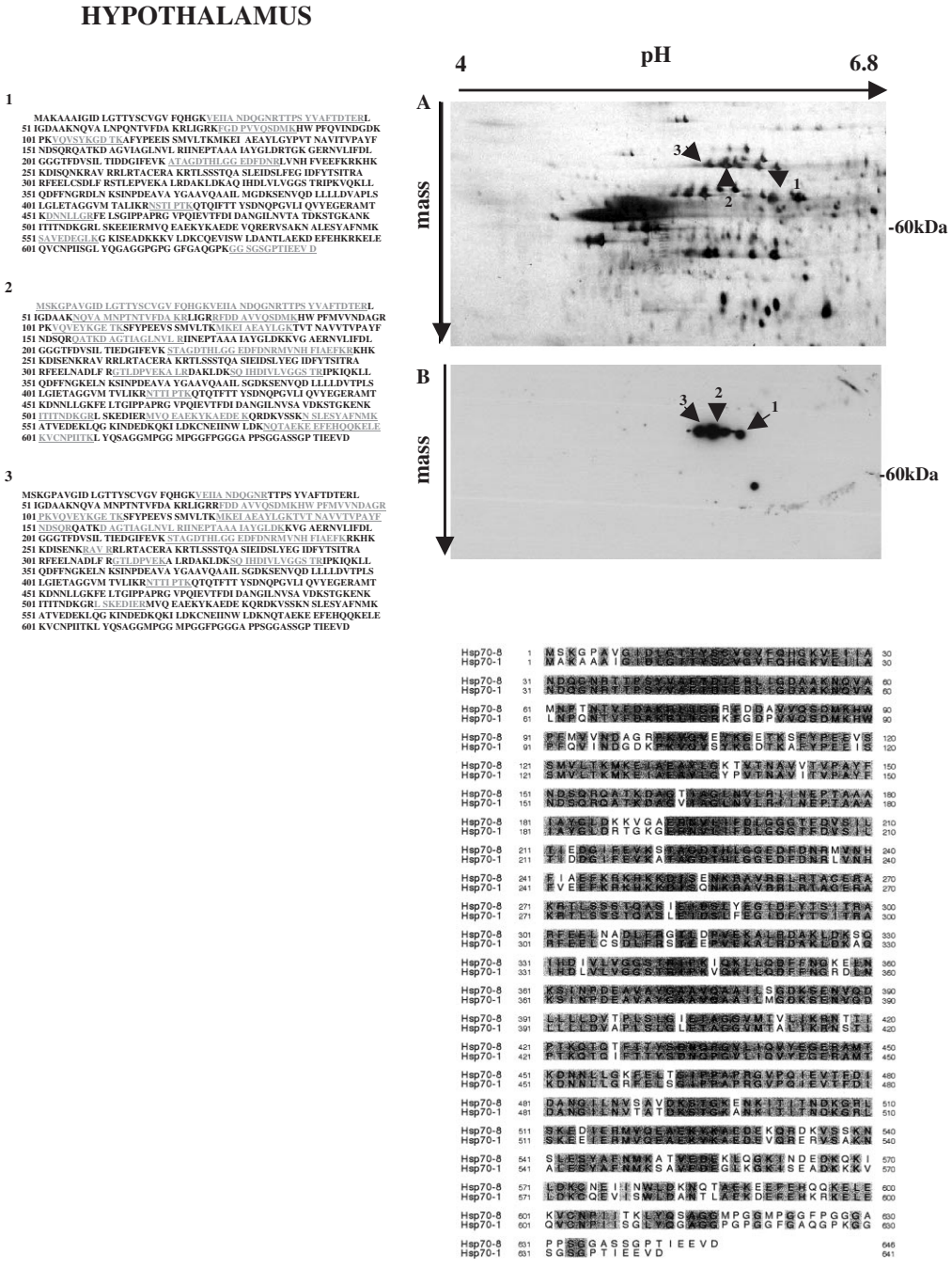

Figure 3 Identification of Hsp70-1 and Hsp70-8 as mAb 1D4 antigens using 2-D gel electrophoresis and subsequent mass spectrometric analysis of hypothalamic tissue extract. (A) Silver stain: hypothalamic tissue extract separated by 2-D electrophoresis, fixed and stained was used to cut out the putative mAb 1D4 immunoreactive protein spots for mass spectrometric analysis. Amino acid sequences of recovered peptides underlined matched to Hsp70-1 (dot 1) and 8 (dots 2 and 3) isoforms (left part of the figure). (B) mAb 1D4 immunoblot: hypothalamic tissue extract was separated by 2-D electrophoresis and transferred to nitrocellulose membranes. The mAb 1D4 immunoreactive spots were used as a template to identify the relevant spots in the silver-stained gel. Three dots (nos 1–3) at a molecular mass of 70 kDa only differed in their pI (4.4–4.5). An additional dot was located at ~50 kDa. Dot no.1 was identified as Hsp70-1 by mass spectrometric analysis. Peptides derived from dot nos 2 and 3 were identified as Hsp70-8 gene product. (C) Hsp70-1 and Hsp70-8 protein alignment: a high degree of protein identity (shaded regions) between Hsp70-1 and Hsp70-8 can be seen (64%). However, differences in the amino acid sequence are present all over the protein, with the highest extent of sequence disparity at the C terminus.

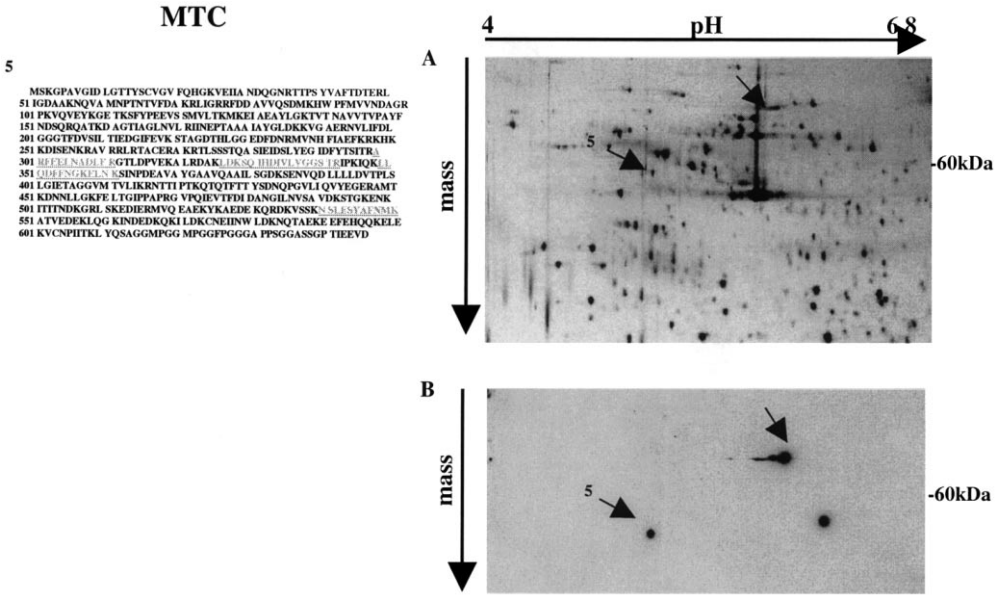

Figure 4 Identification of a new, neuroendocrine tumour-specific Hsp70-8 variant. Silver staining of medullary thyroid carcinoma (MTC) cell extract (A) separated by isoelectric focusing (pH gradient 4–6.8) followed by 12% SDS-PAGE gel and consecutive mass spectrometric analysis of corresponding mAb 1D4 immunoreactive dots (B) reveals, besides the full-length Hsp70-8 dot at 70 kDa, an additional dot (no. 5) at 40 kDa, corresponding to a truncated Hsp70-8 variant (346 carboxy-terminal amino acids). The protein/peptide match is indicated in the left part of the figure. Recovered peptides are indicated underlined.

peptides mapping to Hsp70-8 subtype constituted by the 346 C-terminal amino acids already identified in the tested MTC (Fig. 4). This truncated Hsp70-8 version was also visible at the 1-D immunoblot, thus confirming our 2-D gel findings. It is important to emphasize that the same truncation could be identified in the insulinoma tumour as in MTC. Thus it has to be assumed that the same mechanism is involved in these two different tumour tissues.

Insulinoma E-derived fibroblasts

In order to further investigate if this mAb 1D4 reaction pattern at the 2-D gel immunoblot represents a tumour-specific variation, we isolated fibroblasts from the tumour tissue and cultured them for 2 months. The fibroblast tissue culture supernatant was tested for absence of human insulin to confirm a tumour cell-free fibroblast population. Consequently, lysate of cultured fibroblasts was subjected to 2-D gel electrophoresis and mAb 1D4 immunoblotting. One single protein spot was recovered at a molecular size of 70 kDa. This protein spot was located at the same position within the 2-D gel as the Hsp70-8 spot visualized when the insulinoma E was the subject of testing. However, the quantity of expression was lower when compared with that observed in the insulinoma (Fig. 5C). Remarkably, the other two Hsp70-specific protein spots at lower molecular mass (50 and 40 kDa) were absent from insulinoma-derived fibroblasts (Fig. 5C and Table 1). This further confirmed the endocrine tumour cell-specific modification of the Hsp70-8 protein.

Insulinomas

The immunoblot analysis of a further three insulinoma tumours revealed similar results, showing the dominant Hsp70-8 isoform at 70 kDa with the additional immunoreactive dots at ~50 and 40 kDa (Table1).

Phaeochromocytoma

Whereas the distribution of the mAb 1D4 immunoreactive dots was identical to that observed in the majority of the other tested neuroendocrine tumours, the staining intensity varied. The native Hsp70-representing dot exhibited highest staining intensity. At 40 and 50 kDa, only marginal protein quantities were detectable (Fig. 6A).

Carcinoid

Consistent with our findings in MTC (Fig. 4B), insulinoma E (Fig. 5B) and phaeochromocytoma (Fig. 6A), carcinoid-derived lysates similarly exhibited the three-dot pattern when tested in 2-D gel immunoblots using mAb 1D4 (Fig. 6B).

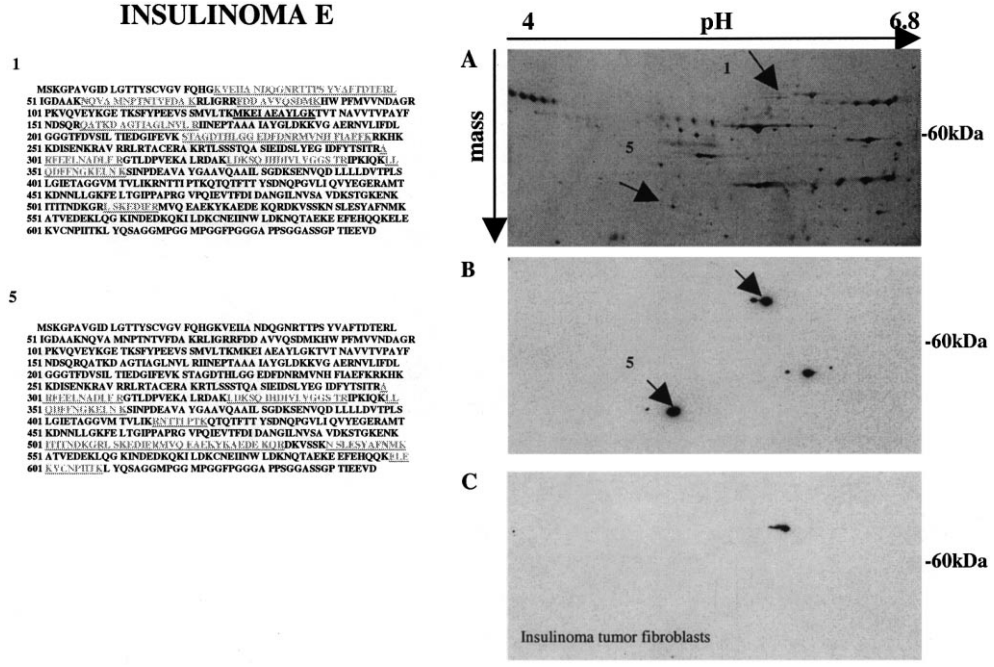

Figure 5 Silver staining and mAb 1D4 immunoblot of human insulinoma E and insulinoma E-derived *in vitro* grown fibroblasts. (A) Silver staining of insulinoma cell extract separated by 2-D gel (pH gradient 4–6.8). Indicated protein spots corresponded with the mAb 1D4 immunoreactive dots in the parallel blot and were chosen for mass spectrometry. (B) mAb 1D4 immunoblot of insulinoma E cell extract separated by isoelectric focusing (pH gradient 4–6.8) followed by SDS vertical PAGE gels. The separated proteins were transferred onto nitrocellulose and developed by mAb 1D4 and HRP-conjugated goat anti-mouse Ab. The 70 kDa mAb 1D4 reactive material was identified as Hsp70-8. The protein dot with a molecular mass of ~40 kDa was identified as Hsp70-8 protein. However, peptides only mapped to the 346 C-terminal amino acids. The protein dot at about 54 kDa represents an already described splice variant of Hsp70-8. (C) mAb 1D4 2-D gel immunoblot of insulinoma-derived and *in vitro*-grown fibroblasts. There was only one single 70 kDa immunoreactive dot representing full-length Hsp70-8, but no further dots at 40 or 54 kDa were present. Remarkably, expression of full-length (70 kDa) Hsp70-8 was lower in tumour-derived fibroblasts than in the tumour tissue derived from the same patient.

In vitro expression of the recombinant protein

In order to confirm antibody specificity, and to investigate whether the tissue lysate preparation is responsible for native protein fragmentation, we generated recombinant Hsp70-8 and performed Western blot analysis under the same protein-denaturing conditions as for tumour tissue treatment. Hsp70-GST fusion protein as well as thrombin cut, recombinant Hsp70 were subjected to 1-D gel immunoblotting. As expected, the recombinantly expressed thrombin cut Hsp70 and GST-Hsp70 fusion protein was detected by the mAb 1D4 as single bands at

Table 1 Differential expression of Hsp70 isoforms in different neuroendocrine tumour tissue, insulinoma-derived fibroblasts and hypothalamic tissue

	Hsp70-8			
Tissues	**40 kDa variant**	**Native protein (70 kDa)**	**50 kDa splice variant**	**Hsp70-1**
Hypothalamus	–	+	+	+
MTC	+	+	+	–
Phaeochromcytoma	+	+	+	–
Carcinoid	+	+	+	–
Insulinoma E	+	+	+	–
Fibroblasts	–	+	–	–
Insulinoma F	+	+	–	–
Insulinoma G	+	+	+	–
Insulinoma H	+	+	–	–

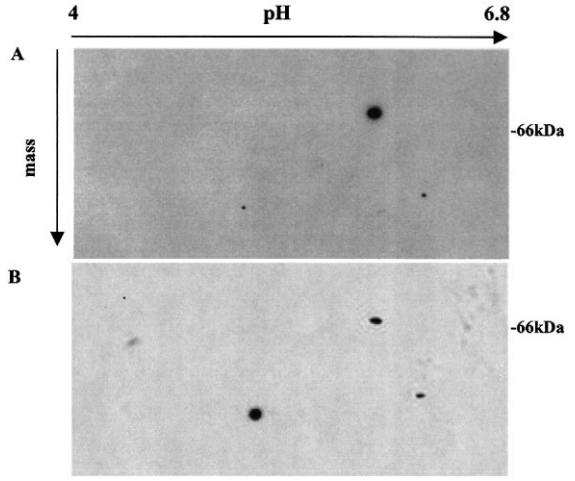

Figure 6 mAb 1D4 immunoblot of human phaeochromocytoma and carcinoid. (A) Proteins of a human phaeochromocytoma were extracted and separated by 2-D gel electrophoresis followed by mAb 1D4 immunoblotting. The prominent immunoreactive dot was located at 70 kDa and minor mAb 1D4-reactive protein dots were visualized at 50 and 40 kDa. (B) A human pancreas-derived carcinoid tumour was extracted and separated proteins were immunoblotted using the mAb 1D4 mAb. The three-dot pattern already observed in the MTC, insulinomas and a phaeochromocytoma was visualized, with dominance of the 40 kDa Hsp70-8 variant.

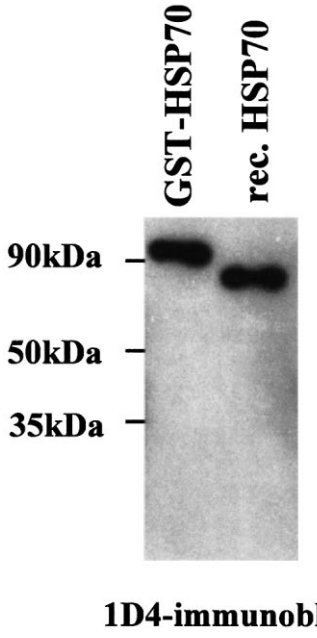

Figure 7 Preparation of human recombinant Hsp70-8 as a GST-fusion protein. Purified recombinant Hsp70-8 protein was loaded onto a 10% SDS-PAGE and transferred onto nitrocellulose for mAb 1D4 immunoblotting. Lane 1: mAb 1D4 immunoblot of GST-Hsp70 fusion protein; lane 2: mAb 1D4 immunoblot of thrombin cleaved recombinant (rec.) Hsp70-8.

a molecular size of 70 and 90 kDa respectively (Fig. 7). No protein fragmentation was detectable in the course of this analysis.

Discussion

In this paper we present evidence of Hsp70-8 subtype over-expression in various human neuroendocrine tumours. Furthermore, using 2-D gel electrophoresis, we identify a novel truncated Hsp70-8 variant detected specifically in endocrine neoplasias.

Over-expression of distinct proteins is a characteristic phenomenon in tumour biology. Identification of genes specifically over-expressed in neoplasias represents the basis of histopathologic diagnosis and is essential for further elucidation of mechanism leading to exaggerated cell proliferation. With the aim of identifying genes specifically expressed in neuroendocrine tumours, we selected a monoclonal antibody (mAb 1D4) generated against a human insulinoma which detected a protein strongly expressed in the insulinoma but absent from pancreatic β cells. As this finding suggested specific over-expression of the mAb 1D4 antigen in neoplastic neuroendocrine tissue, the identification as well as the expression pattern of the immunodetected protein was of interest. Using 2-D gel electrophoresis and subsequent mass spectrometry, we were able to identify Hsp70 subtypes as the antigens specifically detected by mAb 1D4. Hsp70-1 and Hsp70-8 represent isoforms, which differ only by some amino acids. Thus, it is difficult to differentiate between these two protein variants by means of immunological techniques. However, 2-D gel electrophoresis in combination with the newly generated mAb 1D4 allowed clear differentiation between Hsp70-1 and Hsp70-8. The tumour-specific presence of Hsp70-8 might be of importance as the C-terminal tail, which differs in its sequence from Hsp70-1, provides specific protein interaction sites and therefore plays a central role in Hsp70-8 function (Freeman *et al*. 1995).

In addition to our finding of Hsp70-8 expression in neuroendocrine tumours, the mAb 1D4 recognizes truncated variants of Hsp70 in distinct endocrine tissues. MAb 1D4 not only detects the recently described 54 kDa Hsp70 splice variant which was found in the promonocytic cell line U937 and in other tissues (Tsukahara *et al*. 2000), but similarly mAb 1D4 visualizes a 40 kDa Hsp70 variant. The previously described variant (~50 kDa) was expressed inconsistently in our tested tumours (Table 1). We stress that both variants were exclusively detected by

the mAb 1D4, but not by the commercially available anti-Hsp70 antibody SPA-810.

Variations in post-transcriptional protein processing (splicing), leading to an altered, often tumour-specific protein expression pattern, is a well-described phenomenon and can contribute to tumorigenesis or immune recognition of malignant cells. Despite the fact that an Hsp70 splice variant has been described earlier, the observation of an N-terminal Hsp70 truncation in the tested neuroendocrine tumours was unexpected. Protein structure analysis reveals that the truncation is located in the region between the ATP-binding domain and the peptide-binding domain (Frydman 2001). Therefore, it has to be assumed that the truncation has a considerable impact on protein function. We stress that the level at which the protein modification occurs still has to be identified. Whereas the already described 54 kDa protein variant is the result of post-transcriptional splicing, the newly identified 40 kDa Hsp70 subtype might also be the result of tumour-specific post-translational protein modification as specific protease cleavage.

Heat shock proteins constitute a large group of inducible compounds which fulfil a wide range of intracellular functions (Gabai *et al*. 1997). Initially identified as protectors against cellular stress (Wang *et al*. 2002), there is growing evidence that the members of this protein family are involved in diverse biological functions, ranging from involvement in inflammatory processes (Asea *et al*. 2002, Vabulas *et al*. 2002) to anti-apoptotic effects (Li *et al*. 2000, Nylandsted *et al*. 2000*b*). A reduced rate of apoptosis is a well-known component of neuroendocrine neoplastic cells. Thus, the over-expression of Hsp70 variants in these tissues might be one factor involved in apoptosis inhibition.

Our extensive immunohistochemical Hsp70 tumour tissue investigation has demonstrated that the status of Hsp70 over-expression is not equally distributed throughout the tumour cell population. The Hsp70 expression pattern observed within single tumours might represent a varying extent of tumour cell differentiation or polyclonal mitotic centres, resulting in differing clonal extent of Hsp70 transcription and post-transcriptional processing. However, we are not able to fully rule out that the intra-neoplastic expression patterns of Hsp70 are the result of a varying degree of hypoxia in the different areas of the tumour.

Surprisingly, we observed high hypothalamic expression of Hsp70 subtypes. A possible explanation might be that protein folding and protection against aggregate formation is of particular importance in the central nervous system. Secretion and immunostimulatory effects via the Hsp70-specific receptor to improve clearance of amyloid-β could further support this process, as already suggested by earlier experimental work (Kakimura *et al*. 2002).

In conclusion, we have described the development of a new Hsp70 monoclonal antibody. This antibody provides a tool for differentiation between distinct Hsp70 subtypes. We were able to identify characteristic Hsp70 subtype expression patterns in neuroendocrine tumours and tissues, with characterization of a new truncated Hsp70 variant found solely in neuroendocrine neoplasias. Our findings provide the basis for further elucidation of the functional impact of Hsp70 subtype over-expression in neuroendocrine neoplasias.

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