

# Sub-proteome Differential Display: Single Gel Comparison by 2D Electrophoresis and Mass Spectrometry

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Two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) have been used in comparative proteomics but inherent problems of the 2D electrophoresis technique lead to difficulties when comparing two samples. We describe a method (sub-proteome differential display) for comparing the proteins from two sources simultaneously. Proteins from one source are mixed with radiolabelled proteins from a second source in a ratio of 100:1. These combined proteomes are fractionated simultaneously using column chromatographic methods, followed by analysis of the pre-fractionated proteomes (designated sub-proteomes) using 2D gel electrophoresis. Silver staining and <sup>35</sup>S autoradiography of a single gel allows precise discrimination between members of each sub-proteome, using commonly available computer software. This is followed by MS identification of individual proteins. We have demonstrated the utility of the technology by identifying the product of a transfected gene and several proteins expressed differentially between two renal carcinoma proteomes. The procedure has the capacity to enrich proteins prior to 2D electrophoresis and provides a simple, inexpensive approach to compare proteomes. The single gel approach eliminates differences that might arise if separate proteome fractionations or 2D gels are employed.

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*Keywords:* proteome; 2D electrophoresis; mass spectrometry; cancer; kidney

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## Introduction

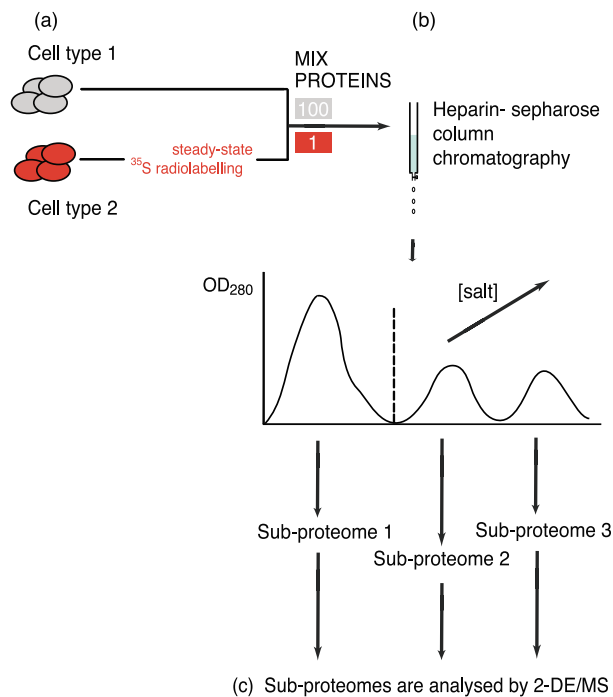
The completion of the first draft of the human genome<sup>1,2</sup> will allow genes to be identified directly and their protein products predicted. These studies have already shown that there are fewer genes than expected but it is apparent that the number of proteins is higher than predicted. This discrepancy is probably due to a combination of post-translational changes and different, but related, proteins resulting from alternatively spliced mRNA forms. As the number of proteins in a cell exceeds 100,000, the identification of all

these molecules is beyond the capability of most currently available technologies.

The genome projects provide a basis for many proteomic studies in which the expressed protein profiles are the focus. Proteomics objectives range from describing all proteins found in a specific cell type, to describing the proteins found during specific stages of development or those found in disease tissues. In the latter, the most important molecules are those expressed differentially (that is, up or down-regulated), those with mutations or those expressed ectopically in the abnormal setting compared with their normal counterparts. Thus, while whole proteomes are important targets to describe and quantify, finding the differences between disease and normal tissue will focus attention on proteins with a potential pathogenic role in disease development. This is an important objective in cancer biology. In this disease, the karyotypes of commonly occurring solid tumours (i.e. tumours of epithelial origin such as breast cancer) are generally highly complex, showing

Abbreviations used: CHO, Chinese hamster ovary; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; RCC, renal cell carcinoma; TCP, T-complex protein.

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**Figure 1.** Sub-proteome differential display strategy. The sub-proteome differential display strategy comprises of three steps. (a) Whole-cell proteins from cell-type 1 are mixed with radiolabelled proteins from cell-type 2 (in a ratio of 100:1). (b) The protein mixture is fractionated using heparin-Sepharose columns, collecting sub-proteome 1 (flow-through) and eluting sub-proteome 2 and 3 with an increasing concentration of NaCl (0.2 and 2 M, respectively). Any appropriate chromatography system could be used. (c) The sub-proteomes are fractionated by 2D gel electrophoresis; the gel is silver-stained, dried and subjected to autoradiography. The two images are scanned and Adobe Photoshop images created and overlaid (the scanned computer images of silver-stained gels are represented grey throughout and those of the autoradiograph images red). The two images can be moved manually with respect to each other on the computer screen, allowing the investigator to assess potential differentially expressed proteins. In general, it is necessary to perform reciprocal labelling experiments, with each pair of proteomes being compared to obviate identification of differences due to labelling inefficiencies, for example proteins low in methionine and/or cysteine.

chromosomal translocations, deletions and amplified regions<sup>†</sup>.<sup>3,4</sup>

The latter two types of chromosomal change are associated with large and variable lengths of chromosomes. In addition, chromosomal translocations in epithelial tumours are often not consistent, recurring events, unlike translocations found in leukaemias and sarcomas. Therefore, DNA cloning to obtain probes is an unattractive proposition in most cases. Proteomics offers a distinct approach to these problems, directly at the protein level.

<sup>†</sup> See also <http://cgap.nci.nih.gov/Chromosomes/Mitelman>

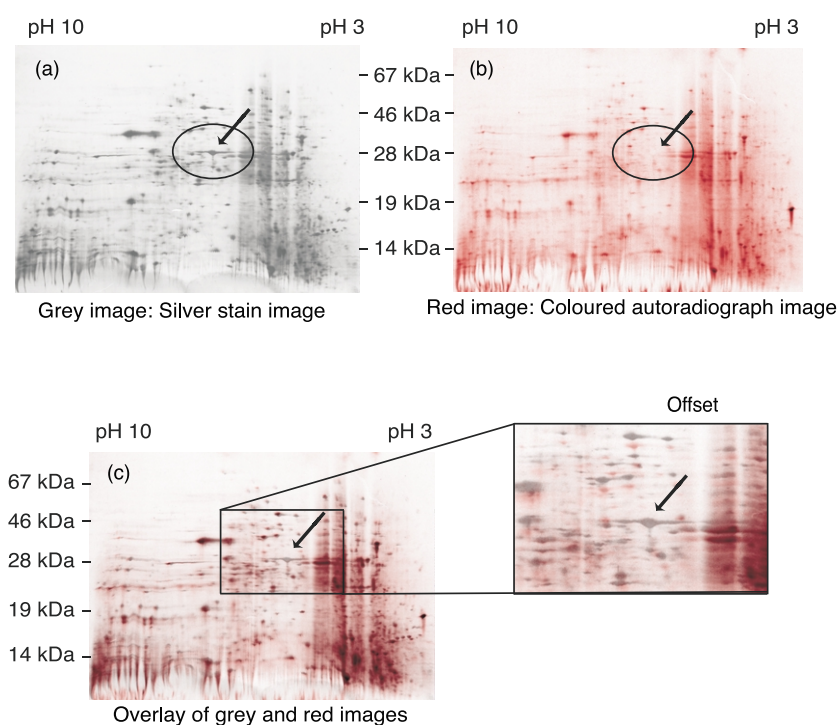
Proteomics utilises various analytical tools such as two-dimensional (2D) electrophoresis,<sup>5</sup> mass spectrometry (MS)<sup>6,7</sup> and various informatics tools.<sup>8</sup> A plethora of proteins can be separated by 2D electrophoresis followed by MS of protein spots to determine the masses of peptides derived from a protein. The masses of the peptides can be matched to theoretical peptide masses, and the protein is identified.<sup>9,10</sup> However, there are severe limitations in the 2D gel methodologies available for comparison of two proteomes. Firstly, the loading capacity of the 2D gels is poor, leading to ineffective protein identification.<sup>11</sup> For this reason, fractionation of complicated protein samples such as whole-cell proteins is necessary prior to analysis by 2D electrophoresis. Also, through fractionation, enrichment of proteins is achieved. This results in the visualisation of proteins that would not normally be visualised from analysis of whole-protein extracts. Secondly, comparison between two proteomes is difficult because of irreproducibility between separations.<sup>12–14</sup> In addition, comparing two gels with complicated patterns usually requires the use of expensive and sophisticated image analysis software.

Here, we describe a simple technique, sub-proteome differential display, which is useful for comparison of two proteomes, focusing only on the proteins, which are expressed differentially. The strategy employs mixing of the two protein populations prior to analysis and single-gel comparison of the proteins to avoid artefactual differences resulting from sample handling. We describe some differences found in proteomes from two different renal cell carcinoma cell lines.

## Results

### The sub-proteome differential display strategy

Our strategy for comparison of two proteomes has three steps (Figure 1) in which protein mixtures are fractionated by column chromatography prior to 2D gel separation and MS analysis of protein spots. First, the proteins from one cell-type are radiolabelled and mixed with 100-fold more unlabelled proteins from a second cell-type (Figure 1(a)). This is a key element of the methodology, since the proteins from the two cell-types subsequently undergo the same treatment, eliminating differences that can arise in parallel handling. The second step is to prepare sub-proteomes from the mixture by chromatographic procedures (we have used heparin-Sepharose column chromatography, Figure 1(b)) to raise the proportional concentration of each protein, thus allowing larger amounts (calculated in cell equivalents) to be applied in the 2D analysis. The third step (Figure 1(c)) is comparison of the sub-proteomes within a single 2D gel, using commonly available computer software (Adobe PhotoShop), followed by MS identification of proteins.



**Figure 2.** Proteome differential display of transfected CHO cells. Whole-cell proteins from  $10^6$  CHO cells transfected with the GFP expression vector pEGFPc-1 were mixed with  $^{35}\text{S}$ -labelled proteins from  $10^4$  untransfected CHO cells. The mixture was fractionated by 2D gel electrophoresis (isoelectric focussing step pH 3–10), the gel was silver-stained (image shown in (a)) and autoradiographed (image shown in (b)). The circled area includes a stained spot that is not visible in the corresponding area of the autoradiograph, indicating the presence of the GFP protein in the transfected cells. (c) The stained and autoradiography image have been overlaid using Adobe PhotoShop and the two images offset manually, highlighted in the inset. This shows that the arrowed spot is present in stained (grey) but not autoradiographic (red) images. This protein is GFP, as determined by mass spectrometry. The size markers used were prestained, high molecular mass protein markers (Gibco BRL).

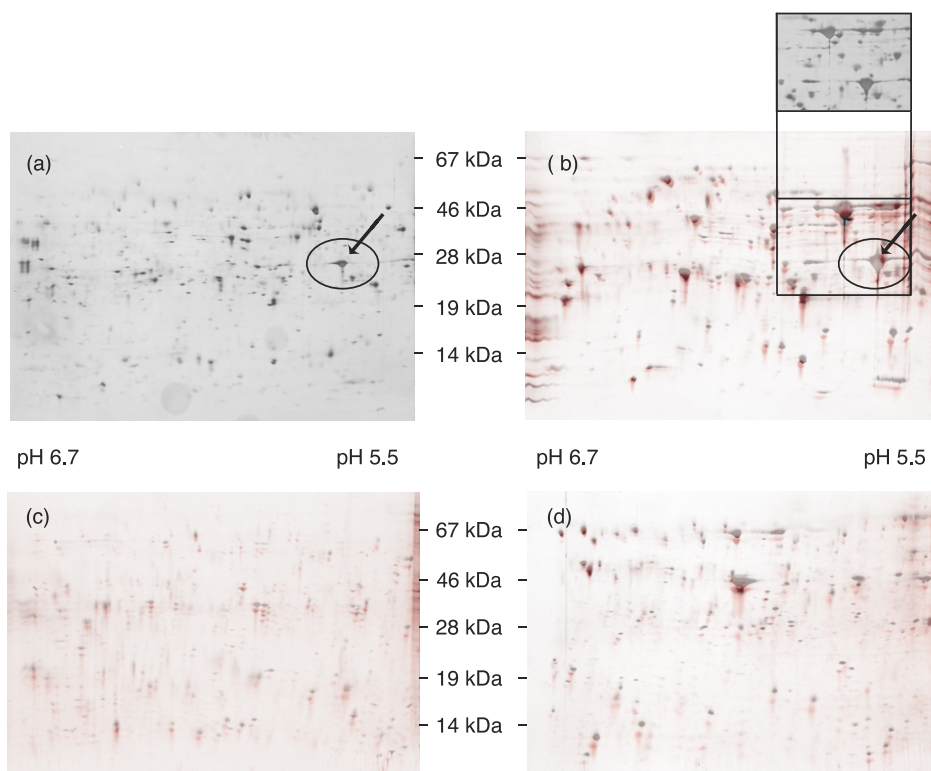
### Identification of a transfected gene product

Our protein differential display strategy has been validated in model experiments designed to identify the product of a transfected gene, the green fluorescent protein (GFP), from amongst whole-cell proteins. Chinese hamster ovary (CHO) cells were transfected with a GFP expression vector and whole cell protein extracts made. These were mixed with extracts from untransfected CHO cells, which had been labelled radioactively with [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine (in a ratio of 100:1 unlabelled to radiolabelled). This mixture of proteins was subjected to 2D gel electrophoresis (Figure 2). The silver-stained and autoradiographic images (Figure 2(a) and (b), respectively; note the silver-stained images throughout are shown in grey and autoradiographic images in red) were scanned and converted to Adobe PhotoShop files, which were superimposed (Figure 2(c)). The use of Adobe PhotoShop allows manual relative movement of the two images, thus allowing identification of differences and the inset in Figure 2(c) shows the silver-stained and autoradiograph images slightly offset for ease of visualisation. The arrows indicate a protein spot present in the transfected proteome image (grey) but not in the untransfected one (red). This protein occurs in the pI region around 6 and molecular mass of around 28 kDa, as expected for GFP.

We have employed heparin-Sepharose column chromatography to prepare sub-proteomes, prior to the 2D electrophoresis step, using mixtures of

whole-cell protein extracts from CHO cells transfected with the GFP expression vector and  $^{35}\text{S}$ -radiolabelled untransfected CHO cells (ratio 100:1). The patterns of proteins in the three heparin-Sepharose chromatography fractions are distinct (Figure 3(b), unbound sub-proteome; (c), sub-proteome eluted with low salt; (d), sub-proteome eluted with high salt) reflecting fractionation into three different sub-proteomes. The images in Figure 3(b)–(d) have been offset slightly for ease of comparison and this reveals a protein present in the extract from the transfected cell sub-proteome (grey) that does not bind to heparin (indicated by the arrow, Figure 3(b)). This spot has a pI of 5.5–6.0 and molecular mass of around 28 kDa, as expected for GFP. Furthermore, the relative amount of this protein has been increased in the sub-proteome compared to the same spot in the whole proteome (Figure 3(a) shows the whole proteome fractionation of these transfected cells). This protein spot was subsequently confirmed by MS as GFP from a separate heparin fractionation experiment (Figure 3(b), inset). Thus, the protein differential display strategy allows simultaneous image analysis of enriched sub-proteomes.

A measure of the sensitivity of the sub-proteome differential display was obtained in a dilution experiment. Transfected cell proteins were diluted with proteins from untransfected cells (ratio of 1:50 transfected to untransfected) and then mixed with radiolabelled, untransfected cell proteins (100:1 unlabelled to radiolabelled). Sub-proteomes

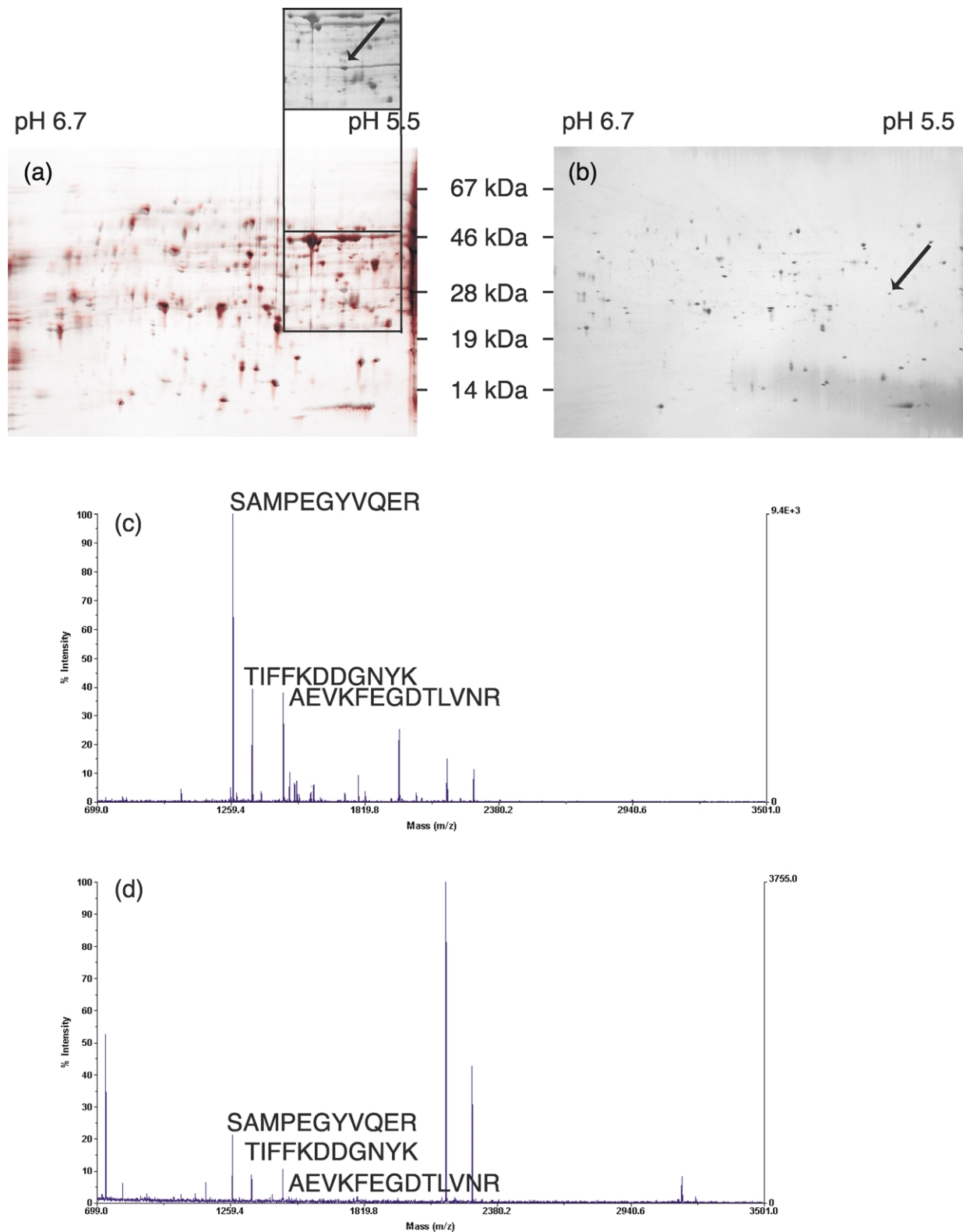


**Figure 3.** Sub-proteome differential display of transfected CHO cells. CHO cells were transfected with the GFP expression vector pEGFPC-1 and proteins from  $10^6$  cells were separated by 2D electrophoresis (isoelectric focussing first dimension from pH 5.5–6.7) and silver-stained. The putative GFP protein is indicated by the arrow in (a). For differential display analysis, proteins from  $10^8$  transfected cells were mixed with  $10^6$   $^{35}\text{S}$ -radiolabelled untransfected cell proteins and fractionated on heparin-Sepharose. Unbound proteins were collected and separated by 2D electrophoresis, and the stained (grey) and autoradiography (red) images were overlaid (the putative GFP spot is arrowed in (b)). The analogous spot from a heparin-Sepharose fractionation of only transfected CHO proteins was eluted from a 2D gel, stained (inset in (b)) and subsequently identified as GFP by MS. (c) and (d) Superimposed staining (grey) and autoradiograph (red) images of the heparin-Sepharose fractionation eluates ((c) 0.2 M NaCl eluted fraction; (d) 2 M NaCl eluted fraction). The images have been offset slightly to illustrate the similarity in the patterns between heparin-Sepharose-bound proteins of transfected and untransfected proteins. Cell equivalents are used throughout for the estimation of amounts of protein.

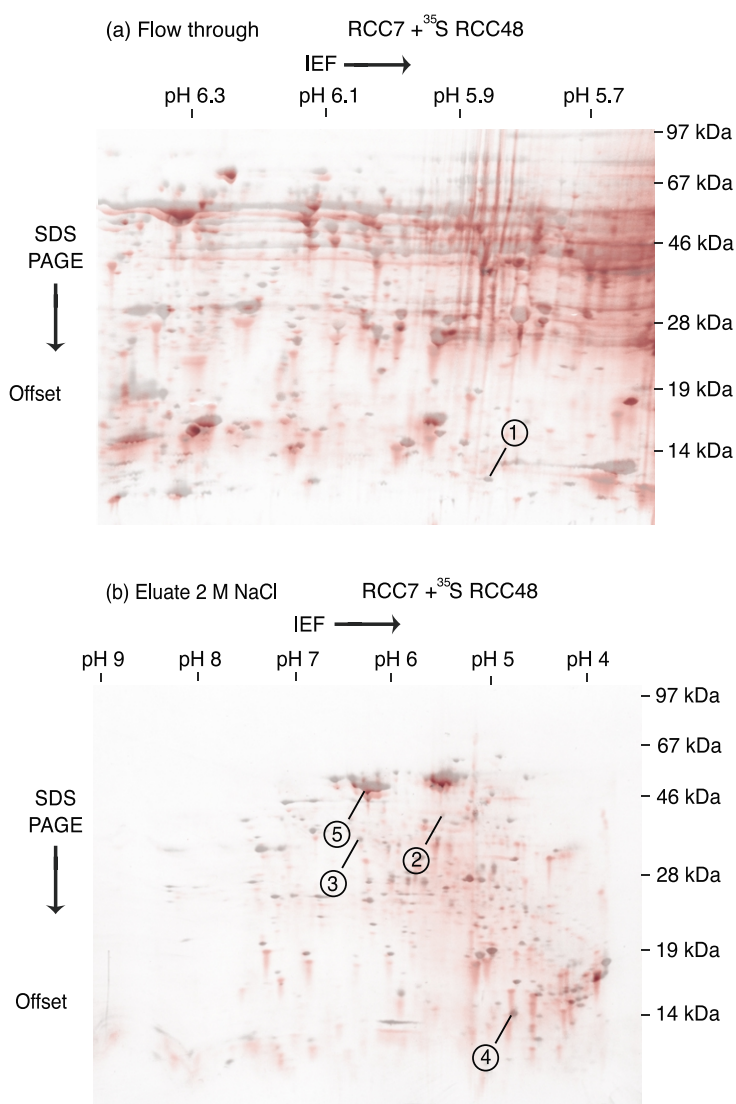
were made by heparin chromatography. After 2D separation, the stained and autoradiographic images were superimposed. The non-bound sub-proteome (Figure 4(a)) contained a stained spot in the region expected for GFP (see the inset). The equivalent spot was identified by MS after repeating the separation of a 1:50 mixture of transfected/untransfected proteins (without the radiolabelled proteins) (Figure 4(a), inset). The spot arrowed in the inset in Figure 4(a) was analysed by MS and Figure 4(c) shows the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) peptide map with identified GFP peptides for this protein spot. The sensitivity of the analytical procedure was further tested by 2D analysis of whole-cell protein extracts of the 1:50 dilution (i.e. not using sub-proteome preparation prior to 2D separation). A low-intensity putative GFP protein spot was observed (Figure 4(b)). This protein (arrowed) was eluted and the MALDI-TOF peptide map trace is shown in Figure 4(d), verifying tryptic peptides from GFP.

### Difference between sub-proteomes of renal cell carcinomas

The application of the technology to a comparison of endogenous proteins was carried out by comparing proteomes from two renal cell carcinoma (RCC) cell lines. Whole-cell proteins from one line (RCC7) were mixed with those from a second line (RCC48), which had been radiolabelled (in a ratio of 100:1 RCC7 to radiolabelled RCC48) and the mixture fractionated using heparin-Sepharose chromatography. The unbound and 2 M NaCl eluate sub-proteomes were analysed on 2D gels and the silver-stained and autoradiograph images compared by computer (Figure 5(a) and (b), respectively, show offset images). This revealed several protein spots (numbered 1–5) that appeared to be expressed more highly in RCC7 compared with RCC48. However, the radiolabelling does not give a quantitative estimate of protein amount in comparison to staining. Therefore, the reciprocal analysis was done to compare the ratio



**Figure 4.** Sensitivity of the sub-proteome differential display. Proteins from CHO cells transfected with the GFP expression vector were diluted 1:50 with proteins from untransfected cells and, in turn, mixed with protein from  $10^6$   $^{35}\text{S}$ -radiolabelled untransfected cells. These proteins were fractionated on heparin-Sepharose and unbound proteins (which contain GFP, see Figure 3) were analysed by 2D gel electrophoresis (isoelectric focussing step, pH 5.5–6.7). Identification of the putative GFP spot was achieved by superimposing the images of stained and autoradiographed gels and manual alignment using Adobe PhotoShop (a) silver-stained, grey; autoradiograph, red). In the inset, the relevant region of a similar gel prepared for MS with only the 1:50 diluted, unlabelled proteins is shown and this was used to identify GFP-specific peptides. (c) The MALDI-TOF spectrum and GFP peptide peaks. (b) The transfected/untransfected protein mixture from the equivalent of  $10^6$  cells was separated by 2D gel electrophoresis, without prior heparin-Sepharose fractionation, and silver-stained. The arrow indicates the putative GFP protein spot, which was excised and analysed by MS. (d) The MALDI-TOF spectrum and GFP peptide peaks.



**Figure 5.** Comparison of RCC7 with  $^{35}\text{S}$ -radiolabelled RCC48 sub-proteomes. The  $10^8$  RCC7 proteins were prepared and mixed with  $10^6$   $^{35}\text{S}$ -radiolabelled RCC48 proteins. The mixture was fractionated by heparin-Sepharose chromatography, and (a) the flow-through and (b) 2 M NaCl eluted fractions were analysed by 2D gel electrophoresis. The indicated protein spots appear to be expressed differentially between RCC7 and RCC48. The spots marked were identified by MALDI-TOF MS and the results are shown in [Table 1](#).

of stained and labelled spots. Furthermore, this precludes differences that might have arisen due to inefficiencies in the labelling and silver-staining procedures.

The reciprocal labelling and fractionation was carried out, followed by 2D separation of heparin column flow-through and 2 M eluate sub-proteomes ([Figure 6\(a\) and \(b\)](#)). This confirmed spots 1–5 and further identified differentially expressed spots 6–10, which have an apparent higher expression in RCC48 compared with RCC7.

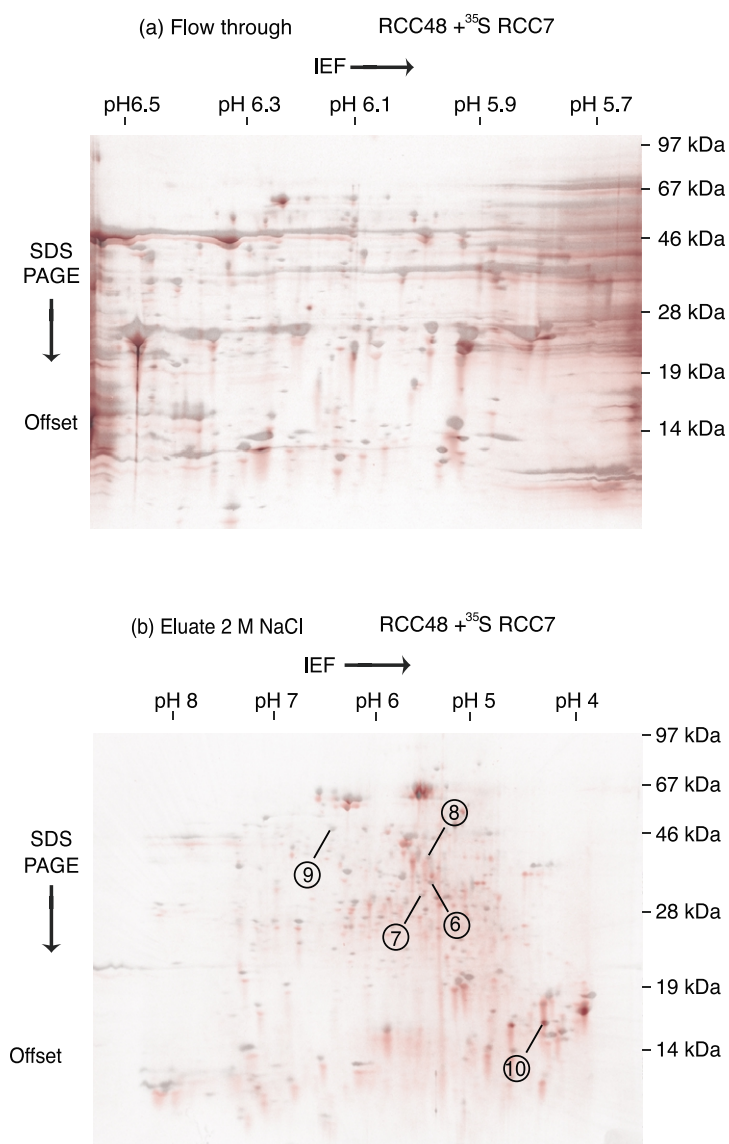
The spots corresponding to differentially expressed proteins were prepared in separate fractionations of proteins from the RCC7 and RCC48 cell lines, and analysed by MS. This identified several proteins, representing species present in nuclear, cytoplasmic or mitochondrial compartments ([Table 1](#)). Examples of nuclear proteins were replication protein A3 and B23 nucleophosmin, which bind to single-stranded nucleic acids, and nucleolin, which induces chromatin decondensation. Prohibitin and proliferation-associated 2G4, which both regulate proliferation,

are located in the cytoplasm and nucleus, respectively. Histidine triad nucleotide-binding protein is a cytoplasmic molecule involved in cell-signalling. TCP-1 (T-complex protein 1) is a cytoplasmic protein that has been found to interact with cyclin E. ETIF3, subunit 5 and ETIF3, subunit 2 are ribosome-associated proteins and MRP-S22 is found in mitochondria.

A number of other differences can be seen on the gels in [Figures 5 and 6](#), which have not been discussed. These had been excised from silver-stained gels using the preparative procedure and analysed by MS but no confident identification was obtained.

#### RNA analysis of differentially expressed genes in renal cell carcinoma

The analysis of protein gels identifies species that differ quantitatively between two sources and here we have found a number of differences between two tumour cell-lines. Further confirmation that these differences are due to differential



**Figure 6.** Reciprocal comparison of renal cell carcinoma sub-proteomes. The reciprocal labelling was carried out in which  $10^8$  RCC48 proteins were mixed with  $10^6$  <sup>35</sup>S-radiolabeled RCC7 proteins. This mixture was fractionated by heparin-Sepharose chromatography, and (a) the flow-through and (b) 2 M NaCl eluted fractions were analysed by 2D gel electrophoresis. The protein spots marked are expressed differentially. The spots marked were identified by MALDI-TOF MS and the results are shown in Table 1.

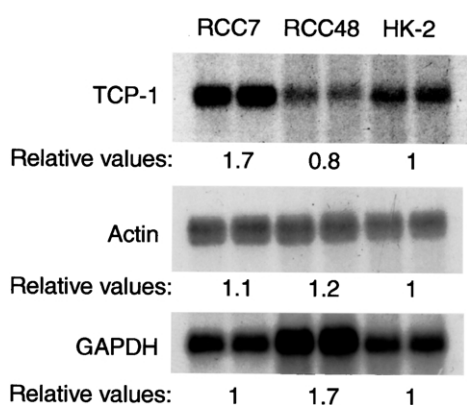
**Table 1.** Renal cell carcinoma proteins identified by mass spectrometry (MALDI-TOF)

Protein name	Putative subcellular location	No. identified peptide masses	pI		Molecular mass (kDa)		Coverage (%) <sup>a</sup>
			O <sup>b</sup>	T <sup>b</sup>	O <sup>b</sup>	T <sup>b</sup>	
1. Histidine triad nucleotide-binding protein	Cytoplasm	4	5.87	6.2	10	15.9	54
2. ETIF3, subunit 5	Cytoplasm	4	5.7	5.2	45	37.6	16
3. MRP-S22	Mitochondrial	20	6.5	7.7	40	41.3	36
4. Replication protein A3 (14 kDa)	Nucleus	6	4.9	5.0	12.5	13.6	61
5. TCP-1	Cytoplasm	22	6.1	6.0	55	57.5	45
6. Nucleolin	Nucleus	14	5.6	8.6	32	39.5	39
7. Prohibitin	Cytoplasm	7	5.7	5.6	30	29.8	31
8. ETIF3, subunit 2	Cytoplasm	11	5.6	5.4	40	36.5	38
9. Proliferation associated 2G4	Nucleus	8	6.6	6.1	48	43.8	11
10. B23 nucleophosmin	Nucleus	11	4.6	4.7	13	30.9	23

The NCBI Entrez numbers for the proteins are the following: 1, 12719168; 2, 4503519; 3, 9910244; 4, 4506587; 5, 5453603; 6, 12728692; 7, 4505773; 8, 4503513; 9, 5453842; 10, 825671.

<sup>a</sup> Percentage coverage of the whole protein sequence from the peptides identified.

<sup>b</sup> O, observed; T, theoretical.



**Figure 7.** RNA expression of TCP-1 in renal cell carcinomas. Duplicate samples of total RNA (10 µg) from the renal cell carcinoma lines RCC7 and RCC48, and the normal kidney epithelial line HK-2 were fractionated on agarose and transferred to nylon membranes. These were hybridised with TCP-1 followed by actin and GAPDH probes. The autoradiographs were exposed to a phosphorimager and the relative values calculated from the average counts of the two lanes, for each cell-line.

expression was sought using RNA analysis. We chose one protein, TCP-1, which is over-represented in RCC7 and for which no antibody was available. Northern filter hybridisation was carried out using a TCP-1 probe in comparison with two control RNAs, actin and glyceraldehyde-3-phosphate dehydrogenase GAPDH (Figure 7). Duplicate samples of each RNA were separated on the filters and a comparison of the RCC RNAs was made with a normal kidney epithelial cell line HK-2.<sup>15</sup> Levels of TCP-1 RNA are about 2.1-fold higher in RCC7 than in RCC48 when normalised to actin and about 3.4-fold when normalised to GAPDH, reflecting the levels of protein observed in the sub-proteome differential display.

## Discussion

Progress in biological research requires a combination of technical improvements applied to key biological processes. The completion of the human genome sequence is a landmark in providing the basic information on which to build future experimental strategies and the key area of expression profiling is now tractable with the growth of cDNA expression microarrays and techniques in proteomics. Whole expression proteome profiles are difficult and require a major resource. Nevertheless, the ability to identify proteins using 2D electrophoresis has resulted in the analysis of many hundreds of proteins<sup>16</sup> and holds major promise for the future. However, a shorter-term goal is the identification of proteins that differ in two situations, such as normal and disease cells. The sub-proteome differential display strategy, involving the mixing and fractionation of two proteomes prior to simultaneous comparison in a

single gel, should be useful in this latter type of study.

The protein differential display approach removes the difficulty of comparing separate 2D images and increases the sensitivity by comparison of sub-populations of proteins. Comparison of proteomes within a single 2D gel have been described that employ either dual radiolabelling of total cell proteins before 2D gel electrophoresis<sup>17</sup> or fluorescent dye modification of two total proteomes (called difference gel electrophoresis).<sup>18</sup> The approach based on the dual radiolabelling of proteins from *Escherichia coli* grown under two different conditions. Cultures of exponentially growing *E. coli*, grown in the two different conditions, were labelled using the distinguishable [<sup>3</sup>H]leucine and [<sup>14</sup>C]leucine. The protein samples were mixed and analysed on a single 2D gel. When using difference gel electrophoresis, two different populations of whole-cell proteins can be compared by labelling each population with a different cyanine dye (Cy3 and Cy5) and comparing the expression profile on a single gel. Proteins can be detected by fluorescence imaging immediately after electrophoresis with a sensitivity equal to that obtainable by silver-staining. However, the apparatus needed for visualisation of both dyes when using difference gel electrophoresis is very expensive. In addition, the dye-to-protein ratio has to be calculated so that there is one dye molecule binding per protein molecule.

Our strategy allows for mixing of two populations prior to fractionation into sub-proteomes, thereby obviating any differences that might occur if parallel fractionations are used. The resulting mixed sub-proteomes are thus inherently identical, except with respect to protein differences that may have existed initially. The use of sub-proteomes allows for intrinsically greater loading amounts to be analysed on the 2D gels. In addition, our current approach takes advantage of commonly available software to analyse the differences between two proteomes, facilitating the identification of differences, which can be analysed subsequently by mass spectrometry.

Although we have employed steady-state radiolabelling of cellular proteins, the reciprocal comparison is very important in the comparison of two unknown samples, as demonstrated here for the RCC cell lines, because any differences due to the inefficiencies in the radiolabelling procedure can be overcome. In addition, the reciprocal comparison circumvents the fact that silver-staining is a semi-quantitative method.

This approach should allow proteomic comparison of any two cell-lines of interest, such as cancer cell-lines with or without a chromosomal translocation. Improvements in the sensitivity and automation of mass spectrometry will also be key features,<sup>19,20</sup> as will robust techniques for differential labelling of proteomes<sup>21</sup> or using other methods, such as 2D liquid chromatography<sup>22,23</sup>



prior to the MS analysis. As a strategy that can be applied in most laboratories, the sub-proteome differential display technique can provide information about differentially expressed genes and thus provide valuable information for identification of proteins that are involved in human disease.

## Materials and Methods

### Cell culture and protein preparation

CHO cells were transfected transiently for 48 hours with pEGFPC-1, a GFP expression vector (Clontech), using lipofectamine (Gibco BRL). Nuclear and cytoplasmic extraction of proteins were prepared as described.<sup>24</sup> In outline, the method involved cell lysis in 0.1% (v/v) NP-40, 20 mM Hepes (pH 7.9 at 4 °C), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl (immediately before use, 0.5 mM DTT, 10 µg/ml of pepstatin A, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin and 1 mM PMSF were added), and recovery of nuclei and extraction of nuclear proteins using 20 mM Hepes (pH 7.9 at 4 °C), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA (immediately before use, 0.5 mM DTT, 10 µg/ml of pepstatin A, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin and 0.5 mM PMSF were added). After estimation of protein yield, the nuclear and cytoplasmic extracts were pooled.

### Renal carcinomas and normal kidney cell culture

The RCC7 and RCC48 cell-lines were grown in RPMI (1640 with Glutamax-I, Gibco BRL), 10% (v/v) fetal calf serum, penicillin and streptomycin.<sup>25</sup> The HK-2 cell-line was grown in Keratinocyte SFM medium (Gibco) supplemented with recombinant human epidermal growth factor (EGF) and bovine pituitary extract in the presence of penicillin and streptomycin.

### Steady-state *in vivo* radiolabelling of cellular proteins

Steady-state labelling of cellular proteins from CHO cells (untransfected) was achieved using a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (<sup>35</sup>S Protein Labelling Mix-Redivue Promix L (100 µCi/ml; 1000 Ci/mmol specific activity). Cultures at approximately 60% confluence (about 2 × 10<sup>6</sup> cells in a 75 cm<sup>2</sup> flask) were incubated for 24 hours at 37 °C.

### Heparin fractionation for preparation of sub-proteomes

Heparin-Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) was used. Heparin is a naturally occurring glycosaminoglycan, which serves as an effective affinity-binding and ion-exchange ligand for a wide range of biomolecules.<sup>26</sup> The column was equilibrated with 20 mM Hepes (pH 7.9 at 4 °C), 7% (v/v) glycerol, 0.12 M NaCl, 1.5 mM MgCl<sub>2</sub>, 7 mM KCl, 0.06 mM EDTA. Proteins obtained from 10<sup>8</sup> cells (roughly 15 mg of protein, estimated by the Bradford assay) were applied onto a 23 ml column. The protein was filtered through a 0.22 µm low protein binding membrane (Steriflip™, Millipore) before application to the column.

The protein that did not bind to the column (flow-through) was roughly 7 mg. Bound proteins were eluted with equilibration buffer containing 0.2 M NaCl (protein eluted was roughly 1 mg) and subsequently with equilibration buffer containing 2 M NaCl (protein eluted was roughly 1 mg).

### Two-dimensional gel electrophoresis

Protein samples were prepared for 2D electrophoresis,<sup>5</sup> by incubation with ribonuclease A (1 µg/ml) at 37 °C for 30 minutes. The proteins were concentrated using a Millipore Ultrafree-15 Concentrator (5 kDa), and precipitated for 20 minutes on ice after addition of 80% (v/v) ice-cold acetone. The precipitate was recovered at 24,000 g for ten minutes at 4 °C. The pellet was air-dried for five minutes and the protein resuspended in 350 µl of 8 M urea, 2% (w/v) Chaps, 0.5% (v/v) immobilised pH gradient (IPG) buffer (pH 3–10 or pH 5.5–6.7, depending on the pH range of the IPG strip to be rehydrated) (Amersham-Pharmacia Biotech), 10 mM DTT. Solubilised proteins were electrophoresed in the first dimension by using a commercial flatbed electrophoresis system (Multiphor II, Amersham-Pharmacia Biotech) with IPG dry strips (Amersham). Different linear pH ranges of 18 cm IPG strips were used (pH 3–10 and pH 5.5–6.7). The IPG strips were rehydrated with the samples overnight at room temperature. The samples on the pH 3–10 linear strips were run at 500 V, 1 mA, 5 W for three hours and at 3500 V, 1 mA, 5 W for 17.5 hours, whilst the samples on the pH 5.5–6.7 linear strips were run at 500 V, 2 mA, 5 W for 1.5 hours and at 3500 V, 2 mA, 5 W for 16 hours. Samples on both pH range strips were run in gradient mode using an EPS 3501 XL power supply (Amersham Pharmacia Biotech). After the isoelectric focusing, the IPG strips were re-equilibrated for 30 minutes in 2% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, 0.05 M Tris-HCl (pH 6.8), 2% (w/v) DTT. The strips were placed onto gradient SDS-PAGE gels (12%–14% (w/v) polyacrylamide) and run at 1000 V, 20 mA, 40 W for 40 minutes, at 1000 V, 40 mA, 40 W for five minutes and finally at 1000 V, 40 mA, 40 W for 2.25 hours. The proteins were visualised by silver-staining for analytical purposes.<sup>27</sup> and as described for MS analysis.<sup>28</sup> When radiolabelled proteins were used, the gel was photographed after staining, dried onto 3 mm paper and exposed to X-ray film (Biomax MS, Kodak) for 4–14 days. The photographic and autoradiographic films were scanned (using a Hi-Scan scanner, Eurocore) and images overlaid using Adobe PhotoShop, allowing manual movement of each image in relation to the other. Features of Adobe PhotoShop such as magnification of specific fields of view assist greatly in the process of visualisation of proteins that differ between the two populations.

### Mass spectrometry

Protein spots were excised from the gel, washed and digested in-gel with trypsin<sup>10</sup> (sequencing grade, Boehringer Mannheim). All MALDI-TOF mass spectra were acquired on a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA) mass spectrometer.

### RNA analysis

The Trizol method (Gibco BRL) was used for total RNA preparation from cell lines, according to the

manufacturer's instructions. Total RNA samples were run in 1.4% (w/v) agarose in 10 mM NaPO<sub>4</sub>. Total RNA (10 µg) was glyoxylated<sup>29</sup> with 20 µl of a mix consisting of 5 µl of 6 M glyoxal, 13.5 µl of water, 1.5 µl of 0.2 M NaPO<sub>4</sub> (pH 7.0), in a final volume of 30 µl, for one hour at 50 °C. Samples were mixed with 8 µl of 50% 10 mM NaPO<sub>4</sub>, 50% glycerol, and separated in the 1.4% gel with 10 mM NaPO<sub>4</sub> buffer at 100 mA until the Fast grey dye reached the bottom of the gel. The running buffer was circulated throughout the procedure by the use of magnetic stirrers at both ends of the gel tank. RNA was transferred from 1.4% agarose gels onto Hybond-N+ membranes (Amersham Pharmacia Biotech) directly following electrophoresis.<sup>29</sup> RNA was cross-linked to the filter using a Stratalinker 2400 and the filter was baked at 80 °C for one hour, between a double layer of 3 mM paper (Whatman). Filters were stored at -20 °C, covered in Saran wrap.

### Preparation of radioactive probes for Northern analysis

For the preparation of the probes, a 350 bp fragment of TCP-1<sup>30</sup> was amplified from RCC7 cDNA using PCR. This fragment was cloned into pBluescript KS+, and sequenced. The vector containing the appropriate insert was digested enzymatically, and the fragment was gel-purified using the QIAEX II kit (QIAGEN), according to the manufacturer's instructions: 5–25 ng of this purified fragment was used to make a radioactive probe using the Rediprime II labelling kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

### TCP-1 primers and cloning procedure

The primers used for the cloning of the TCP-1 fragment were the following:

TCP-1 forward primer: 5' gatggatccatggcttccttccctccacact 3';

TCP-1 reverse primer 5' gatgaattcattaaagattctgcttc-ccggagc 3'.

These primers were used in order to amplify the first 350 bp from the TCP-1 coding sequence by reverse transcription (RT)-PCR using RCC7 cDNA as a template and then cloned into a pBluescript KS+ vector. This was sequenced to verify the clone, and the fragment was used to make the probe.

### Acknowledgments

We thank Dr L. J. Old for generously providing the renal cell carcinoma cell lines. We especially thank Dr Martin R. Stocks for suggestions regarding the use of Adobe PhotoShop, and Dr David R. Quinn for his help with 2D electrophoresis. We thank Dr Sew Yeu Peak-Chew and Farida Begum for the detailed mass spectrometry analysis. We thank Alan Forster for help and advice, Dr Paul H. Dear for helpful comments on the manuscript, and the LMB Visual Aids Department for the photography and preparation of the Figs. A.S. is the recipient of a Medical Research Council Post-Graduate Studentship and the work was supported by the Medical Research Council.

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*Edited by J. Karn*

(Received 8 October 2001; received in revised form 6 February 2002; accepted 7 February 2002)