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14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking

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14-3-3-interacting proteins were isolated from extracts of proliferating HeLa cells using 14-3-3 affinity chromatography, eluting with a phosphopeptide that competes with targets for 14-3-3 binding. The isolated proteins did not bind to 14-3-3 proteins (14-3-3s) after dephosphorylation with protein phosphatase 2A (PP2A), indicating that binding to 14-3-3s requires their phosphorylation. The binding proteins identified by tryptic mass fingerprinting and Western blotting include many enzymes involved in generating precursors such as purines (AMP, GMP and ATP), FAD, NADPH, cysteine and *S*-adenosylmethionine, which are needed for cell growth, regulators of cell proliferation, including enzymes of DNA replication, proteins of anti-oxidative metabolism, regulators of actin dynamics and cellular trafficking, and proteins whose deregulation has been implicated in cancers, diabetes, Parkinsonism and other neurological diseases. Several proteins bound to 14-3-3–Sepharose in extracts of proliferating cells, but not in non-proliferating, serum-starved cells, including a novel microtubule-interacting protein ELP95 (EMAP-like protein of 95 kDa) and a small HVA22/Yop1p-related protein. In contrast, the interactions of 14-3-3s with the *N*-methyl-D-aspartate receptor 2A subunit and NuMA (nuclear mitotic apparatus protein) were not regulated by serum. Overall, our findings suggest that 14-3-3s may be central to integrating the regulation of biosynthetic metabolism, cell proliferation, survival, and other processes in human cells.

Key words: affinity chromatography, cell proliferation, folate metabolism, 14-3-3 protein, proteomics, purine biosynthesis.

INTRODUCTION

14-3-3 proteins (14-3-3s) are highly conserved eukaryotic proteins that regulate many cellular processes by binding to phosphorylated sites in diverse target proteins [1,2]. Two canonical 14-3-3-binding motifs have been defined as R(S/X)XpSXP and RXXXpSXP (where 'X' denotes 'any amino acid residue' and pS denotes phosphorylated serine) [3,4], although other binding motifs are being discovered, including unphosphorylated sites on a few proteins [5,6].

In plants, 14-3-3s have emerged as important regulators of phosphorylated enzymes of biosynthetic metabolism, ion channels and regulators of plant growth [6–9]. In contrast, most of the >60 known 14-3-3-binding (phospho)proteins in mammalian cells are components of intracellular signalling pathways. In fact, mammalian 14-3-3s are best known for promoting cell survival through their interactions with signalling proteins, such as Raf-1, the pro-apoptotic protein BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death), the FOXO transcription factor FKHR (forkhead in rhabdosarcoma) and the cell-cycle phosphatase cdc25 [1,10]. Consistent with cell survival roles for 14-3-3s, expression of an artificial protein that prevents 14-3-3s from binding to their targets in mammalian cells triggers apoptosis [11].

While there are bound to be Kingdom-specific functions for 14-3-3s [12], the stark differences in the types of 14-3-3-binding partners found in plants and animals seemed odd for such highly conserved proteins. We wondered whether the preponderance of signalling proteins identified as 14-3-3 targets might reflect the intensity of research on mammalian signalling, which means, for example, that signalling proteins are often used as baits in two-hybrid screens. In contrast, two-hybrid screens have not proven useful for searching systematically for 14-3-3-interacting proteins, possibly because 14-3-3s tend to bind preferentially to 14-3-3s from the target library. Therefore, to redress the possible sampling bias, we isolated human proteins that bound to 14-3-3–Sepharose and were eluted specifically with a competing 14-3-3-binding phosphopeptide, ARAApSAPA [7,13]. Using this method, we recently identified the cardiac isoform of PFK-2 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), which generates the glycolysis-stimulating metabolite fructose 2,6-bisphosphate, as a human 14-3-3-binding phosphoprotein. The 14-3-3s bind to PFK-2 at a site that is phosphorylated by protein kinase B/Akt in response to insulin and growth factors. Moreover, the effects of a cell-permeable form of the ARAApSAPA peptide indicated that 14-3-3s are essential for insulin/growth factor activation of glycolysis [13]. These findings gave the first indication that regulation of cellular metabolism by 14-3-3s may contribute to the pro-survival functions of human 14-3-3s.

Our experience with PFK-2 enhanced our confidence in the sensitivity and selectivity of the 14-3-3 affinity chromatography procedure. Here, we have scaled up the preparations and show that the ARAApSAPA elution pool is highly enriched in many human proteins that can bind directly to 14-3-3s in a phosphorylation-dependent manner. Amongst the >200 14-3-3-affinity-purified proteins identified, many are enzymes of primary biosynthetic metabolism, signalling components, proteins of vesicle and protein trafficking, and proteins that regulate chromatin function.

Abbreviations used: BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; BiP, immunoglobulin heavy-chain binding protein; DIG, digoxigenin; ELP95, EMAP-like protein of 95 kDa; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC4, histone deacetylase 4; KHC, kinesin heavy chain; KLC, kinesin light chain; MALDI-TOF/TOF MS, matrix-assisted laser-desorption ionization–time-of-flight/time-of-flight mass spectrometry; MRCKβ, myotonic dystrophy kinase-related Cdc42-binding kinase β; NMDA, *N*-methyl-D-aspartate; NuMA, nuclear mitotic apparatus protein; PFK-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PP2A, protein serine/threonine phosphatase 2A; vps, vacuolar protein sorting.

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Isolating so many enzymes involved in the growth, survival and proliferation of cells led us to investigate how 14-3-3/phosphoprotein interactions are regulated with respect to the proliferation status of cells.

EXPERIMENTAL

Materials

Dr Graham Bloomberg at the School of Medical Sciences, University of Bristol (Bristol, U.K.) produced synthetic peptides. Microcystin–LR was provided by Dr Linda Lawton at the School of Life Sciences, Robert Gordon's University in Aberdeen (Scotland, U.K.); Vivaspin concentrators were from Vivascience (Lincoln, U.K.); sulphorodamine B protein stain was from Molecular Probes (Eugene, OR, USA); tissue culture reagents and pre-poured Novex gels were from Invitrogen; protease inhibitor mixture tablets (catalogue number 1 697 498) and sequencing grade trypsin were from Roche Molecular Biochemicals; and calyculin A was from Calbiochem. The antibody against the NMDA (*N*-methyl-D-aspartate) receptor 2A was ab108 from Abcam Ltd (Cambridge, U.K.); anti-NuMA (where NuMA represents nuclear mitotic apparatus protein) antibody was from Calbiochem, and anti-Tiam1 was monoclonal antibody SC-872 (obtained from Santa Cruz). The MANDM1 antibody that recognizes MRCKβ (myotonic dystrophy kinase-related Cdc42-binding kinase β; [14]) was kindly given by Professor Glenn Morris (MRIC Biochemistry Group, North East Wales Institute, Wrexham, Wales, U.K.), and the antibodies that recognize Tip49a and Tip49b were gifts from Professor Taka-aki Tamura (Department of Biology, Faculty of Science, Chiba University, Japan). Other chemicals were from BDH Chemicals or Sigma–Aldrich (Poole, U.K.).

Western blotting and digoxigenin (DIG)-14-3-3 overlays

Proteins were separated by SDS/PAGE on NuPage 10 % Bistris gels (Invitrogen), transferred to nitrocellulose membranes (Schleicher and Schuell), and blots were probed with antibodies that were detected with secondary antibodies conjugated to horseradish peroxidase, and exposed using the ECL® system (Amersham Biosciences). For 14-3-3 overlays, DIG-labelled 14-3-3s were used in place of primary antibody, followed by an anti-DIG-horseradish peroxidase antibody [7,13].

14-3-3 affinity and anion-exchange chromatographies

14-3-3-Sepharose was prepared by binding 2 mg of mixed BMH1 and BMH2 (the 14-3-3 isoforms from *Saccharomyces cerevisiae*) per ml of CH-Sepharose activated with *N*-hydroxysuccinimide ester groups to couple with primary amines (Amersham Biosciences; 17-0490-01). An extract of 20 ml prepared from HeLa cells (5×10^9 frozen cells from 4c Biotech, Ghent, Belgium) was mixed end-over-end with 6 ml of 14-3-3-Sepharose [7,13]. The mixture was poured into a column of 1.5 cm diameter, and washed in 6 litres of 0.5 M NaCl in 25 mM Tris/HCl, pH 7.5 (4 °C)/25 mM NaF, with flow assisted by a water aspirator vacuum. The column was 'mock-eluted' after being incubated for 1 h in 12 ml of 1 mM WFYpSPFLE phosphopeptide, which does not bind to 14-3-3s. Proteins that bind to the phosphopeptide-binding site of 14-3-3s were eluted after incubating for 1 h in 12 ml of 1 mM ARAApSAPA phosphopeptide [13]. Samples (12 ml) from the middle and end of the 0.5 M NaCl wash, and both phosphopeptide elution pools (12 ml each) were concentrated to ≈ 150 μl in Vivaspin 10 000 concentrators, of which 2 μl was run on gels (see Figures 1 and 4). Microcystin–LR was not included beyond the lysis step when preparations were to be used for dephosphorylation experiments.

Where indicated, 14-3-3-affinity-purified proteins were fractionated further by anion-exchange chromatography on a Mono Q 1.6/5 column (Amersham Biosciences) run in 25 mM Hepes/KOH containing 1 mM dithiothreitol (where fractions 1–15 were the flow through and wash), and developed with a 1.5 ml gradient from 0–0.5 M NaCl (fractions 16–31), and 0.4 ml from 0.5–1 M NaCl (fractions 32–35). The flow rate was 0.1 ml/min, and fractions of 0.1 ml were collected. Fractions were frozen, concentrated to ≈ 20 μl by rotary evaporation under vacuum in a Speedivac and dialysed in 20 mM Hepes/KOH containing 20 mM NaF, 5 mM sodium pyrophosphate, 0.1 % (v/v) 2-mercaptoethanol and 0.1 % (w/v) benzamidine in Slide-A-Lyzer mini-dialysis units (Perbio Science UK Ltd, Tattenhall, Cheshire, U.K.).

Tryptic mass fingerprinting

Proteins were alkylated with iodoacetamide, run on Novex 4–12 % Bistris/SDS gels, excised and digested with trypsin. In the first round (see the Results section), tryptic peptide masses were analysed as described previously [13,15]. In the second-round experiment, peptides were analysed by combined MALDI-TOF/TOF MS (matrix-assisted laser-desorption ionization–time-of-flight/time-of-flight mass spectrometry) analysis on an Applied Biosystems 4700 ToF/ToF Proteomics Analyser. In the second round, the human database of the European Bioinformatics Institute International Protein Index (http://www.ebi.ac.uk/IPI/) was searched using the Mascot search algorithm (http://www.matrixscience.com; [16]) and GI accession numbers were assigned by comparisons with the NCBI (National Center for Biotechnology Information) databases. We performed BLAST searches and referred to the original literature where available, as a guide to assigning protein names. Note, however, that many of the proteins identified here have not been studied previously, so assignments of names should be regarded as provisional. Where possible, we indicate which isoform or splice variant is most likely to have been isolated.

Antibody production and affinity purification

Polyclonal antibodies that recognize human vps (vacuolar protein sorting) 33b protein were raised against two peptides: G^{106}RTRKYKVIFSPQKFYAC^{123} (where numbers indicate residues in human vps33b) and C^{494}IPRVDGEYDLKVP^{506} (cysteine for coupling plus residues 494–507 of human vps33b). Antibodies against ELP95 (EMAP-like protein of 95 kDa) were raised against the peptide D^{166}PLSSPGGPGSRRSNY^{181}. Antiserum was produced in sheep at Diagnostics Scotland (Carluke, Ayrshire, U.K.), and antibodies were affinity-purified on peptide–CH-Sepharose columns.

For the 29 kDa HVA22/YOP1p-related protein (FLJ22246, gi:13376836), sheep were injected with a phosphopeptide (C-R^{245}TRKKpTVPSDVDSA^{258}; pT is phosphorylated Thr^{249}), because this site looked like a potential 14-3-3-binding site. Using the affinity-purified antibodies, we failed to find any phosphate at this site on the 14-3-3-affinity purified protein, although the resulting antibody does recognize the protein.

RESULTS

Affinity purification of phosphorylated HeLa proteins that bind to 14-3-3s in competition with a 14-3-3-binding phosphopeptide

A crude extract of human HeLa cells was passed through a 14-3-3–Sepharose column. The column was washed extensively with

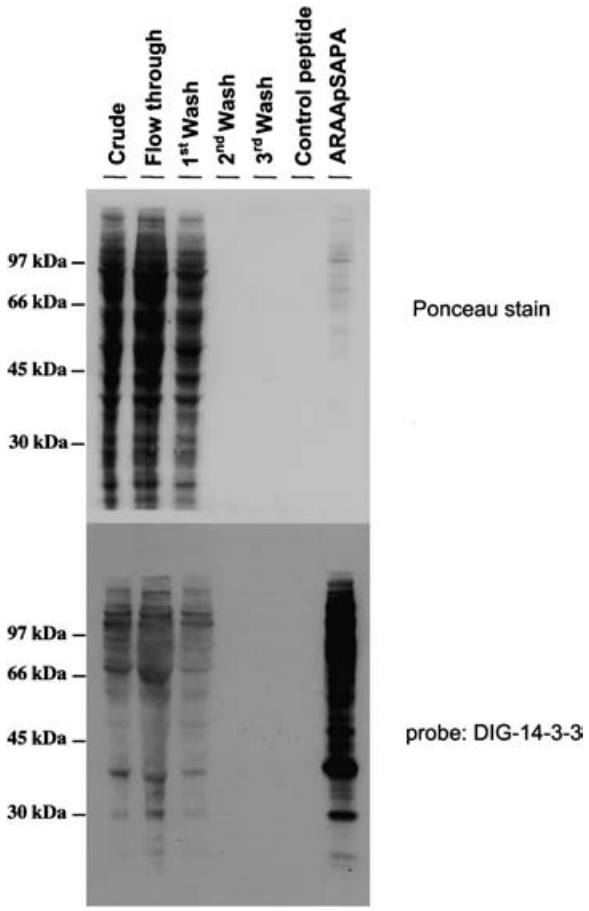

Figure 1 14-3-3 affinity chromatography of human HeLa cell extracts

Clarified HeLa cell extract was chromatographed on 14-3-3-Sepharose, as described in the Experimental section. Column fractions were run on SDS/PAGE using NuPage 10% Bistris gels, and transferred to nitrocellulose membranes. Amounts of protein run on SDS/PAGE were as follows: extract, flow through and beginning of salt wash (1st Wash), 40 μg of each; middle and end of salt wash (2nd Wash and 3rd Wash respectively), protein undetectable; control (phospho)peptide pool, <1 μg; and ARAApSAPA elution pool, 2 μg. Membranes were stained for protein (top panel) and binding to DIG-14-3-3s (bottom panel).

salt-containing buffer (500 mM), followed by a 'mock' elution with control peptides that do not bind to 14-3-3 proteins (see the Experimental section). Finally, proteins were eluted with a 14-3-3-binding phosphopeptide (ARAApSAPA, where pS is phosphorylated serine), with the aim of selectively purifying those proteins that were bound to the phosphopeptide-binding site on the immobilized 14-3-3s. Consistent with recent findings [13], 14-3-3 overlays showed that the ARAApSAPA elution pool was highly enriched in 14-3-3-binding proteins compared with the crude extract (Figure 1), and that 14-3-3-binding proteins were not eluted with any of the control non-binding (phospho)peptides tested in different experiments (Figure 1, and results not shown).

Dephosphorylated proteins do not bind 14-3-3s, and calyculin A treatment enhances binding of cellular protein to 14-3-3s

While 14-3-3s generally bind to phosphorylated sites on target proteins, non-phosphorylated 14-3-3 binding sequences have been identified by phage display and in interacting proteins [5]. We therefore tested the phosphorylation-dependence of 14-3-3 binding of the pool of HeLa cell proteins. The binding of purified proteins to 14-3-3s was abolished by incubation with PP2A (protein serine/threonine phosphatase 2A; Figure 2, and results not shown). This finding demonstrated that, for the majority of the proteins purified by this method, phosphorylation was essential for binding to 14-3-3s.

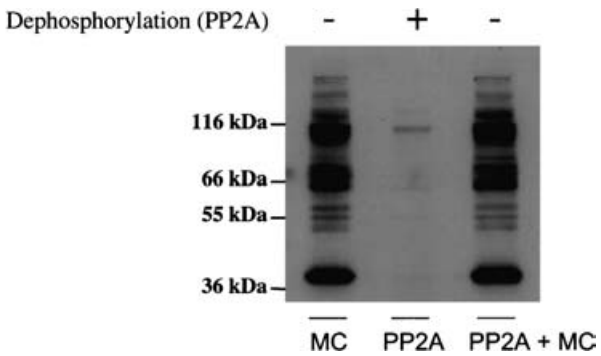

Figure 2 Dephosphorylation of 14-3-3 binding proteins

The 14-3-3-binding proteins that were eluted with ARAApSAPA from a 14-3-3 affinity column (2 μg per sample) were incubated with no additions, or with 50 m-unit/ml PP2A for 30 min at 30 °C, and dephosphorylation was stopped by adding 5 μM microcystin (MC)-LR. Lane 3 is a control experiment, where the MC-LR and PP2A were pre-mixed prior to incubation with the 14-3-3-column elution pool. Proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed for binding to DIG-14-3-3s.

A second indication that the vast majority of cellular binding partners interact with 14-3-3s in a phosphorylation-dependent manner is shown in Figure 3. When cells were treated with the protein serine/threonine phosphatase inhibitor calyculin A, the 14-3-3 binding signals in overlay assays of the cell extracts were dramatically enhanced. When protein phosphatases are inhibited, phosphate is trapped in phosphorylated substrates, and accumulates if the relevant protein kinases are at least slightly active. Thus many proteins become phosphorylated and are capable of binding to 14-3-3s in calyculin-A-treated cells.

First-round identification of 14-3-3-affinity-purified proteins by tryptic mass fingerprinting and Western blotting

Because so many proteins were present in the elution pool, we adopted a multi-tier strategy for their identification. In the first round, the complex mixture was simply separated on one-dimensional SDS/PAGE, and protein bands were excised, digested with trypsin and identified by comparing MALDI mass spectra with the masses of virtual tryptic digests of proteins from the human sequence databases using the MS-FIT and MS-X programs [15]. Western blotting generally confirmed the presence of the corresponding protein with the appropriate molecular mass in the ARAApSAPA elution pool (see the proteins highlighted by asterisks in Table 1 for molecular masses), as shown in Figure 4 for the NMDA receptor subunit 2A (unexpected in HeLa cells, since NMDA receptors operate in the central nervous system), vps33b [17], HDAC4 (histone deacetylase 4; previously identified as a 14-3-3-regulated protein [18,19]), KHC (kinesin heavy chain) 5; KLC (kinesin light chain) 2 (previously identified as a 14-3-3-binding protein [20]); GAPDH (glyceraldehyde-3-phosphate dehydrogenase), BiP (immunoglobulin heavy-chain binding protein), NuMA [21], MRCKβ {[14,22]; a human protein that is related to the vesicle trafficking proteins HVA22 (in plants) and Yop1p (in yeast [23]; GI: 13376836)} and an EMAP-like protein [24] that we named ELP95. Some proteins, particularly the

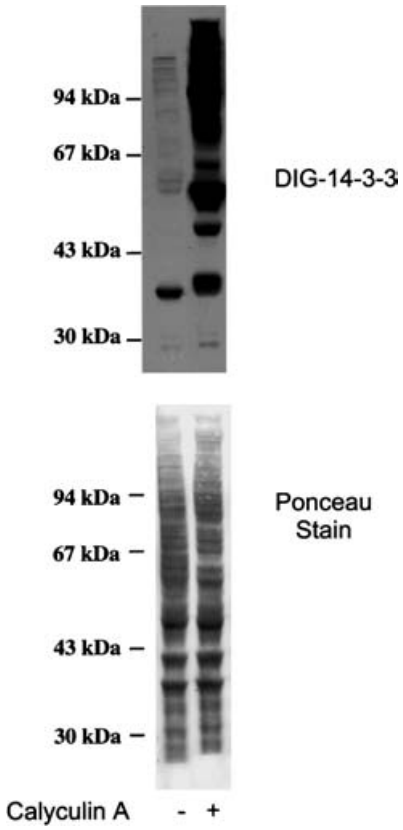

Figure 3 Binding of cellular proteins to 14-3-3s in extracts of cells treated with the protein phosphatase inhibitor, calyculin A

HeLa cells were incubated in the presence or absence of 300 nM calyculin A for 1 h before cells were washed and lysed. Lysate proteins (40 μg per lane) were separated by SDS/PAGE, transferred to nitrocellulose and probed for binding to DIG-14-3-3s (top panel). The blot stained for protein is shown in the bottom panel.

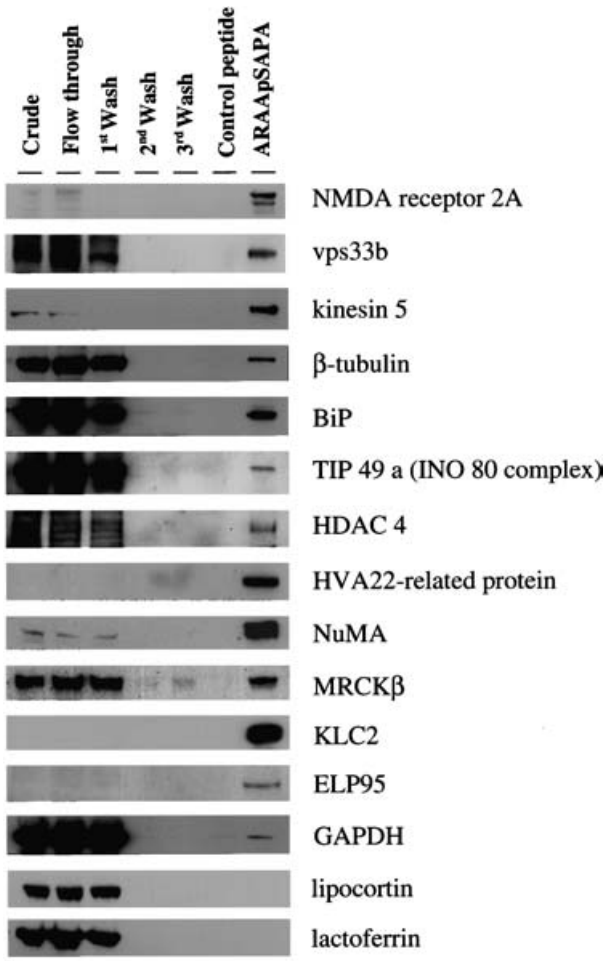

Figure 4 Western blots of 14-3-3 affinity column fractions

Column fractions were prepared as described in the legend to Figure 1, and Western blots were probed with antibodies against the indicated proteins. The molecular masses of the proteins indicated by Western blotting were in agreement with the masses of the proteins cut out for the original tryptic mass fingerprints and the masses predicted from the gene sequences (Table 1). For the 29 kDa HVA22-related protein, an additional signal at $\approx$20 kDa was sometimes seen, presumably a proteolytic fragment. Two proteins that were identified at least twice from MALDI-TOF spectra in different experiments, namely annexin A2 and lactoferrin (bottom two panels), could not be detected in the ARAApSAPA phosphopeptide elution pool by Western-blotting these proteins. These may represent false positives, or perhaps only trace amounts of these proteins are phosphorylated and can bind 14-3-3s in extracts of cells grown continuously in serum.

NMDA receptor subunit 2A, the HVA22/Yop1p-related protein, KHC5, KLC2, NuMA and ELP95, were particularly highly enriched in the ARAApSAPA elution pool compared with the crude extract (Figure 4).

We had identified the Ino80 chromatin-remodelling ATPase by tryptic mass fingerprinting in the ARAApSAPA elution pool. In *S. cerevisiae*, Ino80p is a component of a large multiprotein complex that includes Rvb1 and Rvb2 [25]. Western blotting showed that the human counterparts of these two proteins [26], namely Tip49a (Figure 4) and Tip49b (not shown), were both specifically eluted from the 14-3-3 column by the ARAApSAPA peptide.

None of these proteins were eluted from the column by either extensive washing under high-salt conditions or mock elution with control phosphopeptides that do not bind to 14-3-3 proteins (Figure 4, and results not shown). These results indicate that the majority of the isolated proteins bind to the phosphopeptide-binding site on the 14-3-3s, either directly or as components of protein complexes.

GAPDH is an abundant protein, so it seemed particularly important to establish whether or not its interaction with 14-3-3s is specific. It was found that 92 % of GAPDH in the 14-3-3 affinity-isolated pool of proteins could rebind to 14-3-3–Sepharose in the absence, but not the presence, of the ARAApSAPA peptide. In contrast, less than 5 % of the total GAPDH in a crude cell extract or the original 14-3-3 column flow-through was precipitated by 14-3-3–Sepharose, even after anion exchange chromatography to remove endogenous 14-3-3s (results not shown). These findings indicate that we had isolated a distinct subpopulation of the GAPDH, which was in a form that could bind specifically to 14-3-3s.

Second-round identification of 14-3-3-affinity-purified proteins: enzymes associated with cellular growth and proliferation

Many of the protein bands in the first round of identification contained complex mixtures of proteins (results not shown). Therefore, 14-3-3-affinity-purified proteins were fractionated further by anion-exchange chromatography, followed by SDS/PAGE. Two separate preparations gave very similar patterns of protein bands, and protein bands were analysed, this time using

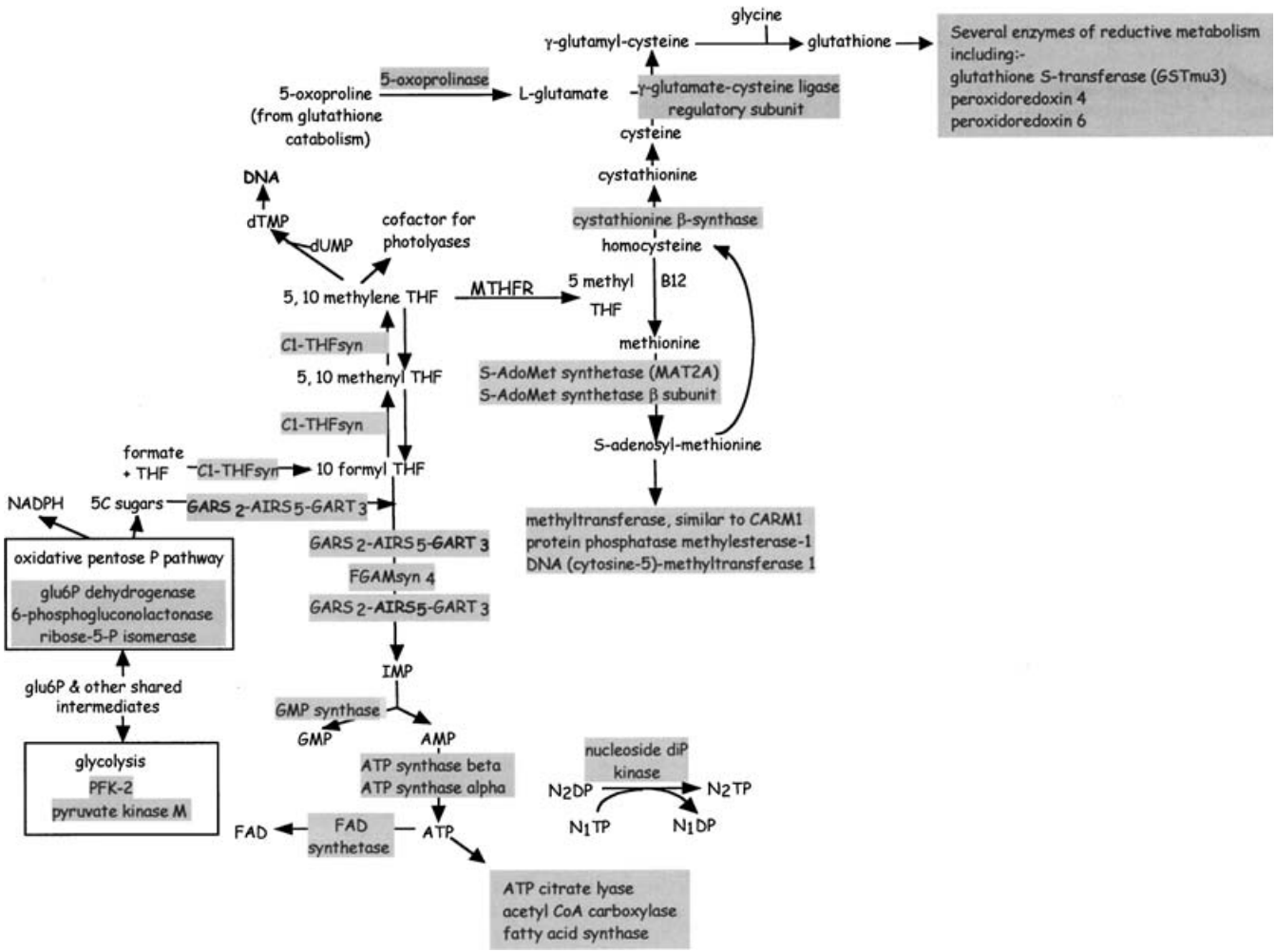

Figure 5 Abbreviated metabolic map of 14-3-3 affinity-purified proteins involved in the synthesis of cellular precursors

Enzymes shown in grey boxes (or proteins with sequences similar to these enzymes) have been identified in the 14-3-3 ARAApSAPA elution pool. Starting in the bottom left-hand corner, in addition to PFK-2 [13], we identified further enzymes of glycolysis, as well as three enzymes in the oxidative pentose phosphate pathway, which generate both NADPH (antioxidant and biosynthetic precursor) and the 5C sugar precursor for synthesis of purines. Also, in the *de novo* synthesis of purines, we have identified the trifunctional enzyme GARS–AIRS–GART (phosphoribosylglycinamide synthetase–phosphoribosylaminoimidazole synthetase–phosphoribosylglycinamide formyltransferase), which catalyses the second, third and fifth steps, and phosphoribosylformylglycinamidine synthase (FGAM synthase), which catalyses the fourth step, in which the purine ring is built up atom by atom upon the ribose 5-phosphate sugar precursor. C and N donors for these steps are a component [10-formyl tetrahydrofolate (THF)] of the folate-one-carbon pool (atom 2), the amide nitrogen of glutamine (atom 3) and glycine (atoms 4 and 5). The first purine to be generated is IMP (inositol monophosphate), which is a precursor for GMP, AMP and their more phosphorylated derivatives: in this area of metabolism, we also isolated a GMP synthase, ATP synthase α, ATP synthase β (see [8] for information on regulation of ATP synthase β by 14-3-3s in plants), nucleoside diphosphate kinase and FAD synthetase, which makes FAD from ATP and flavin mononucleotide. In addition to AIRS–GARS–GART, other 14-3-3 affinity-purified enzymes from the pathways that channel one carbon units into biosynthesis were C1-THF synthase, which catalyses three reactions that combine formate with the C_1 carrier THF and convert the THF into different oxidation states via NADP(H)-dependent reactions; and *S*-adenosylmethionine (S-AdoMet) synthetase (MAT2A gene product and the β-regulatory subunit), which generates S-AdoMet from methionine. We also identified an enzyme that uses S-AdoMet as a methyl donor to methylate PP2A, found in the same anion-exchange fraction (see Table 1), a DNA methyltransferase and a protein similar to co-activator-associated arginine methyltransferase 1 (CARM1), which methylates and alters the specificities of transcriptional regulators [63]. Competing for substrate with S-AdoMet synthetase is the pathway that generates cysteine for synthesis of glutathione and proteins, and in this pathway we have affinity-purified cystathionine β-synthase, as well as an enzyme similar to 5-oxoprolinase (which recycles breakdown products of glutathione catabolism back into glutathione synthesis), a regulatory subunit of glutamate–cysteine ligase and several enzymes that use glutathione in cellular redox regulation.

both MS and MALDI-TOF/TOF MS tryptic mass fingerprinting. The proteins that were identified are summarized in Figure 5 and Table 1.

Prominent in the specific 14-3-3-binding pool were enzymes that synthesize the major precursors required for cellular biosynthesis, energy, growth and antioxidant pathways, and for the hundreds of methylation reactions in cells, namely NADPH, purines (GMP, AMP, ATP), FAD, cysteine, glutathione and *S*-adenosylmethionine (Figure 5). In addition, we isolated select enzymes in pathways that use these precursors to counteract oxidative stresses, and to generate the major components of cells, including fatty acids, DNA, RNA and proteins (Table 1). For example, for DNA synthesis, we isolated at least one of the DNA-replication licensing factors, MCM3 (minichromosome maintenance 3) protein [27]. We also isolated several proteins that share the common feature of binding with high affinity to poly(C) sequences in RNA, and whose roles include regulating the stability of mRNA and the efficiency of its translation; namely poly(C)-binding protein, an ATP-dependent helicase (DDX1) and heterogeneous nuclear ribonucleoprotein K ([28]; Table 1). We also identified components of the RNA processing exosome complex (Table 1).

Table 1 14-3-3-affinity-isolated proteins from human HeLa cells (grouped loosely by function)

Proteins indicated with an asterisk were identified in the first-round experiments (see the Results section), and if assigned a fraction number were also found in anion-exchange fractions in the second-round experiment. In the second round, data were searched against the European Bioinformatics Institute/International Protein Index human database using the Mascot search algorithm (see the Experimental section). Protein scores and the number of peptides that were confirmed by MALDI-TOF/TOF MS data are indicated. Where proteins were found in more than one fraction, the highest score is reported. A protein score of > 60 was considered to be significant ($P < 0.05$; see http://www.matrixscience.com [16]). Sample ID numbers are given to aid readers who may wish to request the raw data. Fraction 2 is representative of the flow-through fractions. This list represents $< 50\%$ of the protein bands on an SDS gel of the anion-exchange column fractions. See Figure 5 for further details concerning the precursors for biosynthesis and methylation, and the text and Figure legends for explanations of abbreviations not otherwise specified in this Table.

Function	GI accession no.	Provisional name	Mass (kDa: apparent/ predicted)	Fraction no.	Protein score (Peptides confirmed by MALDI-TOF/TOF MS)	Sample ID
Precursors for biosynthesis and methylation. . .	12 643 333	*Cardiac PFK-2 (regulates fructose 2,6-bisphosphate)	50/59	2/21–25	420 (8)	KG031/81/128/ 161/163/203
	20 178 296	Pyruvate kinase (M isoform)	60/58	2	327 (5)	KG028
	21 614 520	Glucose-6-phosphate dehydrogenase (pentose phosphate pathway)	55/64	2/21	441 (8)	KG079/80
	6 912 586	6-Phosphogluconolactonase (pentose phosphate pathway)	31/28	2	116 (2)	KG044
	21 389 337	Ribose 5-phosphate isomerase (pentose phosphate pathway)	33/36	2	88 (2)	KG041
	4 505 409	Nucleoside diphosphate kinase B (NM23 protein)	20/17	2	92 (3)	KG050
	4 503 915	GARS–AIRS–GART (steps 2, 3 and 5 in purine synthesis)	116/109	2/21	379 (7)	KG015
	31 657 129	FGAM synthase (step 4 purine synthesis)	146/140	21	379 (8)	KG060
	32 189 394	*ATP synthase β (mitochondrial gene)	51/56	25 to 28	408 (6)	KG335/336/388
	4 757 810	ATP synthase α chain 'mitochondrial precursor'	52/60	31	157 (3)	KG545
	4 557 415	Cystathionine β-synthase (possibly splice isoform 2)	58/61	24/25/28	85 (2)	KG199/245/246
	30 150 507	5-Oxoprolinase	120/98	21	103 (2)	KG061
	4 504 011	Glutamate–cysteine ligase regulatory subunit	34/31	21	292 (5)	KG096
	400 245	*S*-Adenosylmethionine synthetase (MAT2A gene product)	45/44	21	105 (0)	KG086
	11 034 825	*S*-Adenosylmethionine synthetase β regulatory subunit	40/38	21	64 (0)	KG091
	6 912 598	Cytosolic purine 5′-nucleotidase	54/65	32	101 (2)	KG579
	120 649	*Glyceraldehyde-3-phosphate dehydrogenase	40/36	2	74 (1)	KG039
	4 504 035	GMP synthase	70/77	2	371 (6)	KG023
	2 887 579	FAD synthetase	53/61	21	207 (5)	KG082
	36 796 743	C1 THF synthase	110/94	2	94 (2)	KG016
	22 095 395	Bifunctional CoA synthase	60/67	22	67 (2)	KG123
	18 601 084	Similar to CARM1 (co-activator-associated Arg methyltransferase)	60/66	2	89 (2)	KG026
	7 706 645	Protein phosphatase methylesterase-1 (methylates PP2A)	50/43	26	282 (5)	KG298
	12 231 019	DNA (cytosine-5)-methyltransferase 1 (Dnmt1) (HsaI)	150/147	25	72 (2)	KG223
	17 440 180	Similar to mNN-84AG (predicted methylase, SET domain)	49/49	30	169 (2)	KG502
Fatty acid synthesis. . .	2 493 311	Acetyl-CoA carboxylase α	200/267	28	210 (4)	KG357
	4 501 865	ATP citrate lyase	116/121	2/21	782 (9)	KG014/62
	21 618 359	Fatty acid synthase	200/275	21 to 24	860 (10)	KG56/57/104/146/ 176/177/220
Reductive metabolism. . .	7 705 696	Thioredoxin-like p19 (ER-thioredoxin superfamily precursor)	21/22	21	93	KG103
	4 759 274	Thioredoxin-like (TRP32)	35/33	28	527 (6)	KG397/398
	21 361 837	Thioredoxin-like	26/24	28	297 (3)	KG403/404
	33 519 432	Thioredoxin reductase 1; KM-102-derived factor; GRIM12	55/61	2	106 (3)	KG030
	23 065 552	Glutathione S-transferase (GST Mu 3)	28/27	21	113	KG100
	10 835 189	Glutathione reductase (mitochondrial precursor)	50/57	2	573 (6)	KG032
	4 505 591	Peroxiredoxin 1	24/22	2	247 (3)	KG047
	5 453 549	Peroxiredoxin 4	28/31	26/28	75 (1)	KG263/403
	4 758 638	Peroxiredoxin 6	25/25	2	332 (6)	KG046
	31 982 925	Thioredoxin-domain-containing 5; muted	48/48	25 to 28	441 (5)	KG253/254/389
	4 502 599	NADPH-dependent carbonyl reductase	33/40	2	156 (3)	KG042
Iron metabolism. . .	8 659 555	Iron-responsive element bp 1 (aconitase family)	97/98	2	81	KG019
	136 191	*Transferrin precursor (or 4 557 871) (fragment?)	62/84	2	71	KG090
Other metabolism. . .	20 149 680	Fructosamine-3-kinase-like (protein deglycation)	36/35	2	209 (2)	KG040
	3 258 631	GDP-D-mannose-4,6-dehyratase (fucose synthesis)	40/42	2	251 (2)	KG037
	4 557 283	Glycogen-debranching enzyme (gene symbol AGL)	180/174	2	83	KG008
	20 270 371	Hypothetical protein in diene lactone hydrolase family	30/28	2	454 (6)	KG045
	4 505 891	Catalyses 3 steps in hydroxylysine-linked carbohydrate formation	75/85	27	157 (3)	KG325/376
	1 703 068	Acyl-CoA dehydrogenase, very-long-chain specific	60/71	25	127 (3)	KG244
	4 758 426	Guanine deaminase	50/51	21	147 (2)	KG084
	9 910 280	UDP–glucose:glycoprotein glucosyltransferase-like	160/178	27	329 (7)	KG315

Table 1 (contd.)

Function	GI accession no.	Provisional name	Mass (kDa: apparent/ predicted)	Fraction no.	Protein score (Peptides confirmed by MALDI-TOF/TOF MS)	Sample ID
DNA/chromatin, including transcription factors. . .	18 105 045	Histone H2a family member (isoform/allelic variant unknown)	21/14–17	34	108 (2)	KG037
	7 662 142	Ku86-related protein-1 (KARP-1-binding protein)	185/176	27/28	65 (2)	KG312/313/359
	4 503 841	Ku antigen (fragment?)	36/70	27	86 (1)	KG345
	4 557 641	HDAC 2	56/56	25/27	92 (2)	KG523
	5 174 481	*HDAC 4	67/119	25	175 (4)	KG230/240
	7 662 198	TBC1 domain family, member 4	150/148	31	414 (7)	KG331/332/363/ 364/365
	6 330 933	*Ino80 (chromatin-remodelling ATPase)	120/119			
	7 662 198	TBC1 domain family, member 4	150/148	27/28	92 (3)	KG316/363
	7 019 339	CCR4–NOT transcription complex, subunit 7 isoform 1	34/30	26	132 (1)	KG305
	29 789 090	Similar to regulator of chromosome condensation (RCC1)	61/55	?	160 (4)	KG159
	20 540 128	Forkhead box protein K1 (FOXK1; myocyte nuclear factor)	80/65	2	279 (5)	KG020
	30 173 386	Structural maintenance of chromosome 4-like protein	141/180	2	101 (3)	KG009
	6 631 095	DNA-replication licensing factor MCM3	92/91	28	65 (1)	KG373/532
	23 510 283	Elongation protein 3 homologue (transcription)	56/57	?	111 (2)	KG498
	5 032 179	KRAB-associated protein 1(transcriptional regulation)	97/90	?	68 (1)	KG371
RNA binding. . .	4 885 511	Nucleolin (fragment?)	96/76	32	111 (3)	KG573
	31 543 380	DJ-1 (mutated in PARK7)	24/24	2	318 (3)	KG047
	7 657 431	EBNA-2 co-activator p100	97/103	25	70	KG233
	5 453 854	Poly(rC) binding protein 1 (PCBP1, αCP-1)	40/38	2/21	100 (6)	KG011/38/90
	14 165 439	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	56/51	25	152 (1)	KG248
	4 826 686	DDX1 (ATP-dependent helicase; DEAD box-1)	75/83	21	783 (9)	KG070/149
	5 031 753	Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	50/50	21	341 (6)	KG083
	23 308 499	FUSE-binding protein 2 (KH-type splicing)	80/73	2	65 (2)	KG021
	13 124 667	DDX15 (pre-mRNA splicing factor, RNA helicase)	85/92	21/24	171 (4)	KG068/190
	24 307 969	Fragile X mental retardation (FMRP)-interacting protein 1	(125,140)/149	27/28	193 (3)	KG317/366
	4 505 575	Poly(A)-binding protein 1 (or 12 229 876, PABP2)	70/70 (59)	34	217 (4)	KG616
	9 994 185	Putative RNA-binding protein 7	32/30	28	181 (3)	KG399
	2 430 805	KIAA0052 (RNA helicase-like)	116/119	28	298 (6)	KG368
	19 923 403	RRP4 in RNA-processing exosome complex	34/33	28	177 (3)	KG398
	4 826 922	Scleroderma autoantigen 1 (RRP45 in exosome complex)	60/48	28	112 (2)	KG383
	9 506 689	RRP41 in exosome complex	28/28	28	158 (2)	KG402
Protein synthesis. . .	4 503 471	Elongation factor-1 α 1	50/50	2	193 (3)	KG034
	11 136 628	Elongation factor-1 β	32/25	32	148 (1)	KG585
	4 503 481	Elongation factor-1 γ	49/50	31/32	333 (5)	KG548/582
	25 453 472	Elongation factor-1 δ (GEF)	37/31	32	79 (2)	KG584
	119 172	Elongation factor 2 (EF2)	97/96	2/21	121 (7)	KG019/66
	21 264 523	Glycyl-tRNA synthetase	66/84	21	244 (6)	KG073
	4 758 294	Bifunctional (Glu/Pro) tRNA synthetase	140/164	25	287 (5)	KG225
	21 041 641	Cysteinyl-tRNA synthetase spice isoform 2	97/96	2	67 (2)	KG021
	18 575 985	Similar to ribosomal protein L7a	40	34	71 (1)	KG622
Protein folding and processing. . .	6 005 727	T-complex protein 1 τ subunit (cytosolic chaperone)	55/60	21/22/24	190 (4)	KG125/201
	31 542 947	60 kDa heat-shock protein (mitochondrial precursor)	58/61	2/21/27/28	287 (3)	KG27/78/331/384
	5 123 454	Hsp70 (NP_006588/NP_005336)	60/71	21/24	174	KG197
	4 885 431	Hsp70	60/70	22 to 24/25/27/28	778 (9)	KG024/155/ 197/328/381
	24 234 686	Heat-shock cognate 71 kDa protein	64/71	2/21 to 25/28/31	757 (8)	KG24/75/120/ 196/242/327/380
	24 234 688	Stress-70 protein (mitochondrial precursor)	68/74	24	93 (3)	KG074/153/195
	16 507 237	BiP/glucose-regulated protein 78 in ER	67/72	25/27/28/31	561 (7)	KG241/248/249/ 327/378/379/537
	21 361 657	ER-protein-disulphide isomerase A3 precursor (ER60)	53/57	27	184 (4)	KG249/333
	5 031 973	ER-protein-disulphide isomerase A6 precursor	45/48	28/31	399 (6)	KG389/390/504/549
	119 360	Tumour rejection antigen-1 (gp96) (ER)	95/93	31	291 (4)	KG532
Proteolysis, protease inhibitors, and ubiquitin metabolism. . .	124 157	Insulin-degrading enzyme (IDE)	100/118	21	147 (1)	KG063
	5 453 990	Proteasome activator PA28α (interferon gamma up-regulated)	34/29	2/25	374 (7)	KG042/97/ 212/261
	30 410 792	Proteasome activator PA28β	32/28	22/24/25	450 (5)	KG098/135/ 212/262

Table 1 (contd.)

Function	GI accession no.	Provisional name	Mass (kDa: apparent/ predicted)	Fraction no.	Protein score (Peptides confirmed by MALDI-TOF/TOF MS)	Sample ID
	22 538 465	Proteasome subunit β (and/or NP_002785, NP_002788)	24/23	29	168 (2)	KG462/463
	6 912 248	Aspartyl aminopeptidase (DAP)	50/53	2	369 (5)	KG033
	4 503 143	Cathepsin D preproprotein (lysosomal)	31/45	2	175 (4)	KG044
	13 489 087	Monocyte/neutrophil protease inhibitor (MNEI)	40/43	2	353 (5)	KG037
	7 661 744	F-box-only protein 3	45/55	27	97 (2)	KG338
	7 661 744	Basic leucine zipper and W2 domains 2; HSPC028 protein	45/48	27	98 (2)	KG338
	8 923 114	Ubiquitin-specific protease otubain 1	36/32	21	571 (8)	KG095
	16 753 205	Ubiquitin 1 isoform 2 (ataxin/ubiquitin binding protein)	60/60	2/21	108 (3)	KG026
	23 510 338	Ubiquitin-activating enzyme E1	116/119	24	113 (3)	KG185
	21 361 472	Ubiquitin–protein ligase NEDD4-like	100/105	27	113 (3)	KG320
	30 923 268	UBA/UBX protein (ubiquitin-related)	40/34	25	152 (4)	KG257
Cellular signalling/apoptosis. . .	5 803 225	14-3-3ε (and possibly other isoforms)	32/29	31	343 (5)	KG400/401/509/ 510/558
	20 545 807	Similar to insulin receptor substrate 2	160/138	21	279 (7)	KG059/363
	21 618 340	Signal transducer and activator of transcription STAT3 isoform 1	75/88	21	116 (1)	KG069
	1 711 556	Signal transducer and activator of transcription STAT5B	85/90	25	241 (4)	KG236
	5 579 478	Mitogen-activated protein kinase kinase 1 (MEK)	40/44	2	66 (2)	KG036
	1 169 094	Crk-like protein (CrkL)	40/34	21	710 (8)	KG091/92/93
	3 915 771	*NMDA receptor subtype 2A	190/176			
	4 502 735	Cell-division protein kinase 4 (CDK4)	36/34	21	112 (0)	KG095
	4 506 539	TNF receptor-interacting Ser/Thr kinase 2 (RIP)	66/80	21	98	KG074
	7 019 485	Programmed cell death protein 6/apoptosis-linked gene 2 (ALG2)	22/22	2	310 (4)	KG048/102
	6 912 582	PEFLIN	31/30	2	332 (6)	KG043
	32 469 770	Dedicator of cytokinesis protein 6	195/232	28	90	KG330
	32 469 768	Dedicator of cytokinesis protein 7	195/233	28	78 (2)	KG358
	13 639 697	*Yes-associated protein 65 (YAP65)	65/65			
	72 061	Platelet glycoprotein 1b α (possible fragment or variant)	28/69	21	130 (1)	KG100
	4 758 952	PP2A catalytic β subunit (PP2A)	36/36	2	228 (4)	KG094
	4 506 017	PP2A catalytic α subunit (PP2A)	37/36	27	253 (5)	KG344
	5 915 686	PR65-β isoform (regulatory subunit of PP2A)	59/67	27	175 (3)	KG330
	4 506 007	PP 1 catalytic subunit (α or γ)	39/39	25	64 (1)	KG258
	7 661 712	G-protein-coupled receptor kinase-interactor 1 (GIT1)	85/85	27	82 (2)	KG323
	20 357 556	Cortactin isoform b (oncogene EMS1)	61/58	28	159 (3)	KG377/378
	19 923 746	Casein kinase 1α	38/39	28	199 (4)	KG395
	4 758 516	Hepatoma-derived growth factor	37/27	28	198 (2)	KG396
	1 006 668	Identical or similar to TRK-fused gene (and/or 21 361 320)	51/(66/44)	25	178 (3)	KG204/252
	5 453 710	LIM and SH3 protein 1; Lasp-1	38/30	25	220 (3)	KG259
	40 254 446	Vasopressin-activated calcium-mobilizing receptor-1;cullin-5	75/94	25	184 (3)	KG204
	33 286 446	ζ-type opioid receptor	90/74	31	138 (2)	KG533
	7 656 924	Crk-associated substrate (p130Cas) (and/or 8 134 339)	97/94	21/23	155 (4)	KG064/187
	23 503 295	Casein kinase II β chain	26/25	30	97 (2)	KG511
	25 777 713	S-phase kinase-associated protein 1A isoform B	21/19	28	278 (3)	KG407/464
	4 885 109	Calmodulin (NP_005175)	16/17	29	123 (1)	KG466
	37 539 570	Similar to KIAA0456 (rho-GAP-like and SH3 domain)	125/130	27	113 (2)	KG318
	21 070 978	Second mitochondria-derived activator of caspase (Smac-β) (or 9 845 297Smac-α)	22(21/27)	28	298 (5)	KG406
	21 361 670	HIP-55 (SH3 domain protein)	53/49	28	237 (4)	KG387
Actin dynamics. . .	4 501 885	Actin (and/or 4 501 887)	44/42	22/24/26/27/ 31/32/33	547 (6)	KG088/132/167/ 209/341/256/505/ 506/551/583
	7 657 365	Rho-GEF 16 (overexpressed in neuroblastoma)	70/49	2/21/24	670 (9)	KG022/72/ 117/151/193
	4 505 573	Rho-GEF 7 (PAK-interacting)	70/74	27	104 (2)	KG326
	2 494 863	*Tiam 1	178/205			
	14 133 241	*Myotonic dystrophy-related cdc42-binding protein kinase β	195/200			
	11 414 808	Vasodilator-stimulated phosphoprotein (VASP)	40/40	2	161 (4)	KG035
	4 508 047	Zyxin	66/62	21	88 (3)	KG074
	13 124 696	F-actin-capping protein β (possibly splice isoform 2)	35/32	25	283 (5)	KG260
	1 705 650	F-actin-capping protein α-1 subunit (CapZ α-1)	38/33	25	220 (3)	KG211/259
	4 503 747	β-Filamin (filamin A; actin-binding protein 278)	240/280	24/25	189	KG175/218
	12 025 678	Actinin, α 4	95/105	27	485 (6)	KG321/322
	30 148 842	Similar to FKSG30 (contains actin-like domain)	40/66	27	183 (2)	KG341
	22 040 999	Similar to KIAA0456, (rho-GAP and SH3 domains)	130/129	27/28	113 (2)	KG318/366
	32 880 195	Smooth muscle myosin phosphatase-/Rho-interacting protein 3	120/84	21	84 (2)	KG061
	4 758 018	Calponin 2	36/34	21	149 (2)	KG094

Table 1 (contd.)

Function	GI accession no.	Provisional name	Mass (kDa: apparent/ predicted)	Fraction no.	Protein score (Peptides confirmed by MALDI-TOF/TOF MS)	Sample ID
	21 748 542	FLJ00343; two calponin and multiple filamin domains	200/283	25	90 (3)	KG218
	16 163 690	Similar to WAVE-associated Rac-GAP	125/121	28	144 (3)	KG366
Microtubule-associated...	135 395	Tubulin α	50/51	31/32/33	423 (5)	KG388/546/580/598
	135 448	Tubulin β	60/50	31/32/34	368 (6)	KG581/618
	21 361 322	Tubulin β5	50/50	30	173 (4)	KG501
	13 878 553	*KLC2	70/69	26		
	4 758 648	*KHC5B	110/110	27/28	242 (4)	KG231/319/320/ 368
	743 447	*NuMA	220/240			
	23 397 564	*ELP95	95/96	23/24/25	269 (5)	KG065/188/234
Other cellular trafficking...	5 454 042	sec23A (ER-to-Golgi transport)	70/87	21/24	358 (7)	KG071/192
	145 919 248	sec23B	70/87	25	219 (5)	KG239
	37 550 192	Similar to sec24A	110/120	25	147 (3)	KG231
	27 735 256	sec24C	116/119	24/25	121 (3)	KG185
	4 506 411	Ran-GTPase-activating protein 1	60/64	32	84 (2)	KG578
	8 248 765	*vps33b	70/71			
	13 376 836	*Hypothetical HVA22/Yop1p-like	20/29			
	24 797 086	Importin β3/RanBP5/karyopherin 3	100/127	25	103 (2)	KG232
	4 504 897	Importin α (karyopherin alpha 2)	56/58	28	89 (2)	KG385/386/578
	4 758 012	Clathrin heavy chain	170/193	27/28	232 (4)	KG314/361
	5 729 953	Related to nuclear distribution gene C (NudC; *A. nidulans)*	46/38	25/26	89 (1)	KG208
Transporters...	7 705 837	NY-REN-45 antigen; putative potassium channel	85/90	25	83 (2)	KG020/236
	19 913 424	Vacuolar ATPase, H^+ transporting, subunit A, isoform 1	60/69	25	133 (3)	KG244
Miscellaneous...	4 757 756	*Annexin A2 (lipocortin)	40/39	2	730 (9)	KG246
	4 504 919	Keratin 8	50/54	24	180 (4)	KG205
	4 557 701	*Keratin 17	48/48	21	134 (3)	KG085
	4 557 888	Keratin 18	42/49	24	181 (4)	KG207
	9 845 502	Laminin receptor like protein (37 kDa form)	39/32	26	172 (2)	KG343
	6 005 711	Annexin A10; annexin 14	38/38	26	95 (1)	KG303
	5 031 863	Galectin 3 binding protein; L3 antigen; Mac-2-binding protein	60/66	27	89 (2)	KG329
	11 545 731	Gigaxonin (mutated in giant axonal neuropathy)	60/69	27	86 (3)	KG329
	136 085	Tropomyosin α3 chain	34/29	27	217 (3)	KG346
	19 923 613	Hypothetical protein FLJ21128 (YjeF-related protein N-terminus)	58/57	25	119 (2)	KG246
	37 551 404	Hypothetical protein related to peptidases	50/51	21	192 (4)	KG084 29 789 090
		Hypothetical protein KIAA1470	55/61	23	160 (4)	KG159
	37 546 025	Hypothetical protein KIAA0052	116/119	28	298 (6)	KG368
	4 827 071	Zinc-finger protein 9 (zfp 273)	24/31	2	134 (2)	KG047/172/265
	27 486 360	Haloacid dehalogenase-like hydrolase family	36/34	2	261 (6)	KG040
	4 757 714	Tyrosine/acid phosphatase 1 isoform c	20/18	2	324 (5)	KG049
	23 097 250	Hypothetical hydrolase-like protein	34/32	21	82 (1)	KG096
	12 052 752	Hypothetical protein (DUF689-like domain)	38/34	24	184 (3)	KG210
	No entry	IPI00335979	100/86	28	270 (6)	KG370/371
	22 040 999	KIAA0456 (or could be 37 539 570)	120/125	28	89 (2)	KG367
	29 736 132	Similar to phosphofusin acidic cluster sorting protein	105/106	28	124 (3)	KG369
	38 044 290	FLJ90157 (uncharacterized conserved protein with PSP domain)	92/97	28	270 (6)	KG373

14-3-3s are already well known as regulators of signalling components that control cell growth and cell division, and they also have central roles in inhibiting apoptosis. Here, we identified further signalling enzymes, including: the NMDA receptor 2A subunit; regulators of apoptosis, such as RIP {the TNF (tumour necrosis factor) receptor-interacting serine/threonine protein kinase [29,30]} and programmed cell death protein 6/ALG2 (apoptosis-linked gene 2) [31]; PP2A and its regulatory subunits; CDK4 (cyclin-dependent kinase 4), which regulates the $G_1 \rightarrow S$ transition and progression through S-phase [32]; and several signalling adaptors.

Several of the signalling proteins are involved in regulating actin dynamics, including a guanine-exchange factor of Rho (Rho-GEF16), p130cas and MRCKβ (Table 1, and [22,33]). By analogy with the closely related MRCKα [33], it is possible that MRCKβ acts upstream of LIM kinase and cofilin, which were identified previously as 14-3-3-binding proteins [34,35]. In addition, we have identified several proteins that operate closely together to regulate actin structure, including zyxin, VASP (vasodilator-stimulated phosphoprotein), which promotes the formation of long actin filaments by preventing the capping of barbed ends, the F-actin capping protein β and filamin A, one of the proteins which stabilizes the actin network ([36], and references cited therein). Thus 14-3-3s may have multiple roles in connecting signalling pathways to the regulation of actin-based cellular shape changes.

Other 'clusters' of 14-3-3 affinity-purified proteins comprise microtubule-associated proteins, proteins involved in the cellular

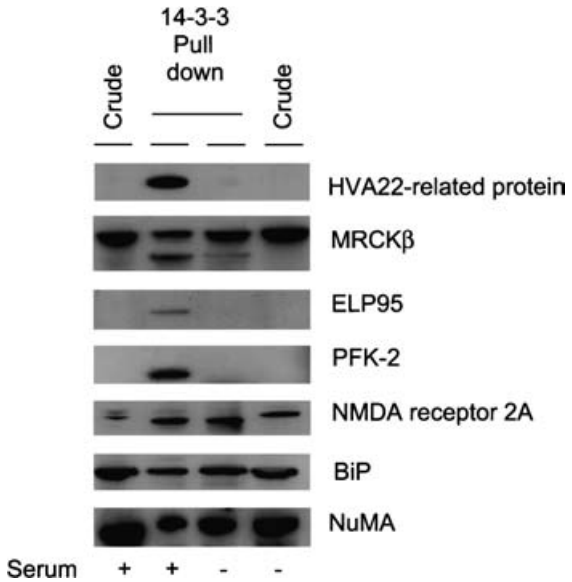

Figure 6 Effects of serum on the ability of proteins to bind to 14-3-3–Sepharose

HeLa cells that had been grown continuously in the presence of serum were serum-starved for 12 h or kept in serum, as indicated. Cell extracts (3 mg of protein) were incubated for 1 h with 50 μl of 14-3-3–Sepharose, and washed pellets were extracted with SDS-sample buffer, run on SDS/PAGE and probed with antibodies against the small HVA22/Yop1p-related protein, the protein kinase MRCKβ, the EMAP-related protein ELP95, PFK-2, whose binding to 14-3-3s is regulated by growth factors [13], the NMDA receptor 2A subunit, BiP and the mitotic apparatus protein, NuMA. Note that inclusion of the cysteine protease inhibitor, MG132 (20 mM), in extraction buffers was essential to prevent degradation of 14-3-3 affinity-purified proteins in extracts of serum-deprived cells (results not shown). Whether the MG132-sensitive degradation was linked to 14-3-3 interactions with the proteases and proteasome regulators shown in Table 1 is unknown.

trafficking of proteins and vesicles, proteins involved in (de)ubiquitination and the proteasome (Table 1).

14-3-3 binding of the small HVA22/Yop1p-related protein and ELP95 are regulated by serum

Because so many of the 14-3-3 affinity-purified proteins are involved in processes that are up-regulated to promote cell growth and proliferation (Figure 5 and Table 1), and because all, or at least most, of the proteins bound to 14-3-3–Sepharose because they had been phosphorylated *in vivo* (Figure 2, and results not shown), we became curious to know whether the phosphorylation of 14-3-3-binding sites on these proteins was connected with the proliferation status of the cells.

The cells used for these experiments had been grown in the presence of serum, which contains growth factors and other components that stimulate cell growth and division. We found that the small HVA22/Yop1p-related protein and ELP95 were present in 14-3-3–Sepharose precipitates from extracts of cells that had been grown continuously in serum. However, these proteins did not bind to 14-3-3–Sepharose in extracts of cells that had been deprived of serum overnight (Figure 6). In contrast, serum did not regulate the ability of the NMDA receptor 2A subunit, NuMA, BiP or MRCKβ to bind to 14-3-3–Sepharose, though an unknown protein of lower molecular mass than MRCKβ that was recognized by the MANDM1 antibody was only seen in the 14-3-3–Sepharose precipitates from serum-grown cells (Figure 6).

vps33b, HDAC4, KLC2, MRCKβ and Tiam1 bind directly to 14-3-3s in a phosphorylation-dependent manner

Another general question about the 14-3-3 affinity-purified proteins is which of the proteins bind directly to 14-3-3s and which of them bind as components of multi-subunit complexes. The majority (> 95 %) of the isolated proteins aligned with signals in 14-3-3 overlays. For example, Figure 7(A) shows the striking co-incidence of protein bands and 14-3-3 overlay signals for the anion-exchange fraction that contained vps33b and HDAC4 (Figure 7A). No signals were seen on the 14-3-3 overlay after the proteins had been dephosphorylated (results not shown). These findings confirmed that the majority of the isolated proteins do bind directly to 14-3-3s in a phosphorylation-dependent manner. However, even after anion-exchange chromatography, many protein bands still contained more than one protein, and aligning 14-3-3 overlays with blots and gels of so many proteins was not trivial. We therefore fractionated the samples further by two-dimensional gel electrophoresis, which enabled us to pinpoint KLC2 as a protein that binds directly to 14-3-3s (Figure 7B), in agreement with the results of Ichimura et al. [20]. However, two-dimensional gel electrophoresis is a relatively low capacity procedure and did not prove to be useful for tracking the lower abundance proteins, so we turned to more specific methods for individual proteins. The anti-MRCKβ and anti-Tiam1 antibodies were found to immunoprecipitate the proteins from cell extracts, and the immunoprecipitated MRCKβ and Tiam1 both bound directly to 14-3-3s in overlay assays (Figures 7C and 7D). Furthermore, the interaction of these proteins with 14-3-3s was phosphorylation-dependent, because binding was abolished by dephosphorylating MRCKβ and Tiam1with PP2A (Figures 7C and D).

DISCUSSION

Although the specificity of binding to 14-3-3s of every individual protein must be checked, these results have shown that most of the proteins isolated here bind specifically to the phosphopeptide-binding site of 14-3-3s, either directly or as components of multiprotein complexes: (a) several are known 14-3-3-interacting phosphoproteins, including HDAC4 [18,19], YAP65 (Yes-associated protein 65) [37], platelet glycoprotein 1bα (e.g. see [38]), keratin 18 [39] and PFK-2 [13], whereas MAP kinase kinase 1 was found in a multi-protein complex that also contained 14-3-3s [40]; (b) the binding of proteins to 14-3-3s in overlay assays was abolished by dephosphorylation (Figures 2 and 7, and results not shown); (c) 14-3-3 overlays (Figure 1) and Western blotting (Figure 4) indicated specific elution from the 14-3-3 affinity column of most of the proteins tested; and (d) isolated vsp33b, HDAC4, KLC2, MRCKβ and Tiam1 were able to bind directly to 14-3-3s in a phosphorylation-dependent manner (Figure 7).

We had performed preliminary 14-3-3 overlay experiments of HeLa cell extracts, which showed that similar patterns of phosphoproteins were detected when yeast or mammalian 14-3-3 isoforms were used as the probe, although there were marked differences in the intensities of binding signals for different proteins, indicating isoform selectivity (results not shown). Thus while using yeast 14-3-3 isoforms as the purification ligand in the present study may have had subtle effects on the selection of phosphoproteins isolated, it seems unlikely that the choice of ligand would mean that a substantial subset of the 14-3-3-binding proteins was missed. Another technical point is that different targets varied in their relative enrichment in the ARAApSAPA elution pool from the 14-3-3 column. For example, NuMA and the NMDA receptor subunit 2A were highly purified in the

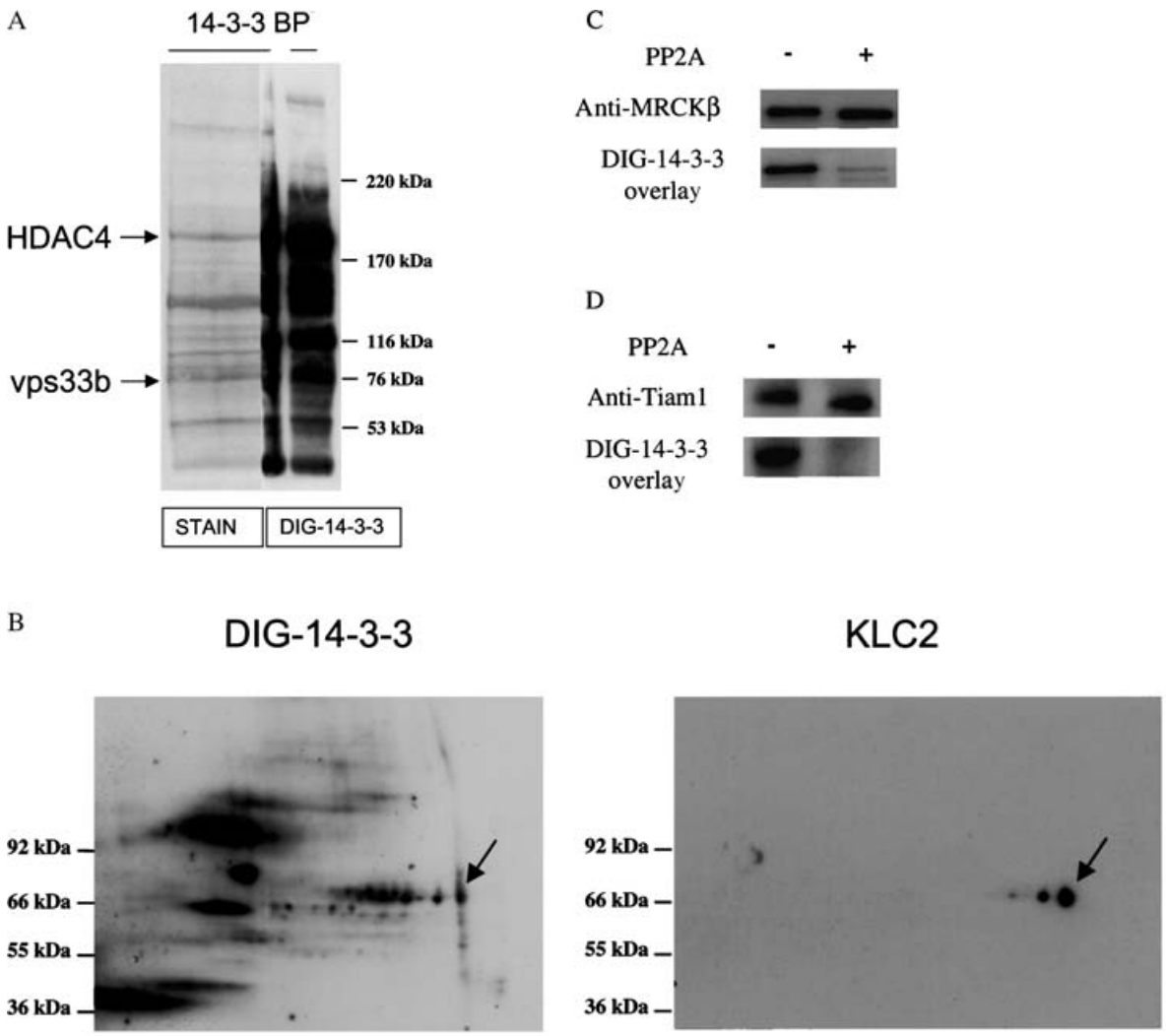

Figure 7 Identification of proteins that bind directly to 14-3-3s in a phosphorylation-dependent manner

(**A**) HeLa cell proteins were isolated by 14-3-3 affinity chromatography and a sample (200 μg) was fractionated further by Mono Q anion-exchange chromatography. Fractions that were eluted between 250 and 280 mM NaCl were run on a 3–8 % Bistris/SDS gel, blotted on to a Fluorotrans transfer membrane and stained with Sulphorhodamine B protein stain. A narrow slice of the stained lane was processed to identify DIG-14-3-3-binding proteins (14-3-3 BP) in an overlay (labelled DIG-14-3-3). Protein bands in the remainder of the lane were cut out, digested with trypsin and analysed by MALDI-TOF tryptic mass fingerprinting. Of all the proteins in this sample, only vps33b and HDAC4 could be assigned unambiguously, presumably because peptides were extracted less efficiently from blots than from the gels used to identify the proteins in Figure 5 and Table 1. (**B**) Duplicates of concentrated purified 14-3-3 fractions were resolved by two-dimensional gel electrophoresis using pH 4–7 strips and SDS/PAGE (10 % gels). Duplicate gels were transferred on to nitrocellulose and analysed by DIG-14-3-3 overlay and Western blotting with anti-KLC2. The series of spots that both bind directly to 14-3-3s (arrow in left-hand panel) and are recognized by Western blotting with anti-KLC2 (arrow in right-hand panel) are indicated. The identification of KLC2 in the indicated spots was confirmed by MS analysis (results not shown). (**C**) MRCKβ was immunoprecipitated from HeLa cell extracts. The purified protein was incubated in the presence or absence of PP2A, run on SDS/PAGE, transferred to nitrocellulose and probed for binding to anti-MRCKβ (MANDM1 antibody [14]; top panel) and DIG-14-3-3s in an overlay assay (bottom panel). (**D**) Tiam1 was immunoprecipitated from HeLa cell extracts, and the protein was dephosphorylated and examined for binding to DIG-14-3-3s, as shown in (**C**).

ARAApSAPA pool compared with the crude extract, whereas HDAC4 was not so highly purified (Figure 4). The enrichment of any particular proteins will depend on: (a) how much of the protein is phosphorylated under the physiological conditions when the cells were harvested; (b) the ability of the 14-3-3 column to compete with endogenous 14-3-3s for binding to the protein; and (c) the dissociation rate of the protein during the incubation with the ARAApSAPA phosphopeptide.

The overwhelming impression from the collective lists in Figure 5 and Table 1 is that we isolated many proteins with roles in generating precursors for cellular growth, and regulatory proteins involved in cellular growth, survival, proliferation and movement, including many proteins that have been implicated in cancers [41]. These include the *S*-adenosylmethionine synthetase (MAT2A gene product; [42]), nucleoside diphosphate kinase [43] and enzymes of *de novo* purine biosynthesis, a pathway that is needed for (and may limit) cell growth when the alternative 'salvage' pathway or the diet cannot provide the quantities of purines required for cells that are growing and dividing rapidly [44].

It is especially intriguing to find further enzymes of glycolysis, the oxidative pentose phosphate pathway and *de novo* purine biosynthesis among 14-3-3-binding phosphoproteins (Figure 5). The finding that 14-3-3s control the glycolytic-regulating enzyme PFK-2 in response to growth factors [13] had focused our attention on why glycolysis is very active in cancer cells, even though glycolysis generates less ATP per glucose molecule than respiration. One hypothesis is that intermediates of glycolysis feed

into the pentose phosphate pathway [45], which provides NADPH and the 5C sugar component of purines (Figure 5), and has also been found to have regulatory effects on *S*-adenosylmethionine and lipid metabolism [46,47]. Our findings (Figure 5) now suggest more extensive roles for reversible protein phosphorylation and 14-3-3s in directing flux through these pathways. Furthermore, recent discoveries have triggered a resurgence of interest in how glycolysis is integrated with cell-survival processes and cellular transformation. For example, the anti-apoptotic effect of protein kinase B depends on the availability of glucose in a mechanism that involves hexokinase and glucokinase [48]. Also, the pro-apoptotic protein, BAD, associates with glucokinase as part of an anti-apoptotic complex in response to survival signals that trigger the phosphorylation of BAD [49] and at sites that also promote the interaction of BAD with 14-3-3 proteins. When the many other indications that 14-3-3s are critical to cell survival are also taken into account [1,10,11], it seems likely that investigating how interactions between 14-3-3s and enzymes of metabolism are regulated will give new insights into links between cellular metabolism and cell-survival processes.

There are further reasons for focusing on the enzymes that synthesize biosynthetic precursors. The literature on C_1/folate/purine metabolism (Figure 5) is dominated by hundreds of papers implicating homocysteine as a risk factor in cardiovascular disease and other diseases [(reviewed in [50]). Moreover, anti-metabolite drugs that block the *de novo* synthesis of purines are amongst the most successful treatments for cancers and inflammatory diseases [51]. Given this intense clinical interest, it is astonishing that very little is known about either the mechanisms that regulate C_1/folate/purine metabolism in response to hormones and nutrients or the reasons for the wide variations in blood homocysteine levels in the human population. Our findings, therefore, indicate an exciting potential for dissecting how phosphorylation and 14-3-3s affect metabolic fluxes through these pathways.

At first sight, the other novel 14-3-3-interacting proteins identified here appeared to be quite a diverse group, but, actually, it is striking how many of these proteins perform energy-demanding cellular roles in chromatin remodelling, actin dynamics and trafficking of proteins and vesicles. Considerable genetic and biochemical evidence has already implicated 14-3-3s in the control of the actin cytoskeleton and cellular trafficking [34,35,52,53], and our findings indicate that interactions with a multiplicity of target proteins underlie the regulation of these cellular processes by 14-3-3s. Finding proteins involved in cellular trafficking that bind to 14-3-3s is interesting, because 14-3-3s are heavily implicated in regulating the translocation of a large number of proteins between the nucleus and cytoplasm [1,2], from the cytoplasm into mitochondria and chloroplasts [54], and from the ER (endoplasmic reticulum) to the plasma membrane [55]. In considering trafficking, we note that several mitochondrial and ER proteins, including possible cytoplasmic precursors of some of these proteins, were isolated (Table 1). Whether 14-3-3s interact with the precursors in the cytoplasm, and/or regulate these proteins within the organelles, is not known: both scenarios have been reported for different proteins in plants [8,54]. Another possibility is that 14-3-3s interact with these proteins indirectly via transmembrane proteins.

We also note that several of the 14-3-3 affinity-purified proteins have been linked to diseases other than cancer. Thus mutation of DJ-1 underlies PARK7, an autosomal recessive early-onset form of human Parkinsonism [56]. Recently, genetic defects in a 'cell-migration complex' comprising 14-3-3ε, Lis1 (related to the non-catalytic α-subunit of platelet glycoprotein 1b), NUDEL and dynein heavy chain were found to underlie the Miller–Dieker 'smooth brain' syndrome [57]. Alhough we did not identify NUDEL and Lis1 here, we did find a human protein related to the *Aspergillus nidulans nudC* (Table 1). In the fungus, *nudC* regulates levels of *nudF*, which is the fungal version of Lis1 ([57], and references therein). In addition, several of the proteins in Table 1 have received special attention because they are involved in processes that become deregulated in diabetes, including a fructosamine 3-kinase [58,59], a variant of insulin-receptor substrate-2 and insulin-degrading enzyme, which has also been linked to Alzheimer's disease [60]. These findings are particularly intriguing, because 14-3-3s have already been implicated in all of these diseases (e.g. see [61,62]).

The major implication of the present work is that one common molecular mechanism, i.e. binding of 14-3-3 proteins to phosphorylated sites on intracellular proteins, connects signalling pathways to the regulation of a much wider diversity of target proteins than had been realized previously. Here, we have identified >200 candidate targets, which at least trebles the known 14-3-3-interacting 'phosphoproteome'. Thus the question of which signals regulate which targets becomes more pressing, and with so many targets, also more challenging. We suggest that the 14-3-3 affinity procedures used here should provide a useful generic screen for exploring the cellular signals that regulate the dynamic interactions between 14-3-3s and their diverse targets. Proteins that are affinity-purified from cell extracts will have been phosphorylated on the 14-3-3-binding sites *in vivo* (Figures 2 and 3). This means that comparing the subsets of proteins that can be 14-3-3-affinity-purified from cells stimulated in different ways, treated with drugs, and so on, has the potential to provide a general screen to identify the signalling pathways that regulate different subsets of 14-3-3/target interactions. In the present study, the predominance of novel target proteins with potential roles in processes associated with biosynthesis and growth and proliferation of cells led us to perform a pilot study, which indicated that the proliferation status of the cells regulates the 14-3-3 binding of a subset of target proteins (Figure 6). Future work will aim to extend these studies to a wider range of target proteins, and to dissect out details of the signalling pathways, phosphorylation mechanisms and cellular consequences of these interactions.

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