REVIEW ARTICLE

Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes

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14-3-3 proteins exert an extraordinarily widespread influence on cellular processes in all eukaryotes. They operate by binding to specific phosphorylated sites on diverse target proteins, thereby forcing conformational changes or influencing interactions between their targets and other molecules. In these ways, 14-3-3s 'finish the job' when phosphorylation alone lacks the power to drive changes in the activities of intracellular proteins. By interacting dynamically with phosphorylated proteins, 14-3-3s often trigger events that promote cell survival – in situations from preventing metabolic imbalances caused by sudden darkness in leaves to mammalian cell-survival responses to growth factors. Recent work linking specific 14-3-3 isoforms to genetic disorders and cancers, and the cellular effects of 14-3-3 agonists and antagonists, indicate that the cellular complement of 14-3-3 proteins may integrate the specificity and strength of signalling through to different cellular responses.

Key words: 14-3-3 protein, cancer, cell signalling, neurodegenerative disorder, plant, protein kinase B (PKB).

INTRODUCTION

Signalling pathways regulate the dynamics of cell behaviour by tuning enzyme activities allosterically [1] and by directing the assembly of multicomponent complexes [2–4]. Very complicated structures can be built, demolished and remodelled rapidly and precisely. How can this be achieved within the crowded environment of a cell? The emerging answer is that many proteins have modular structures, comprising both catalytic domains and distinct 'adapter' domains that are specialized for binding to other proteins, phospholipids or nucleic acids ([3,4] and see http://www.mshri.on.ca/pawson/domains.html). Modular architecture has powerful features. First, regulation can be exerted when signalling pathways create or destroy docking sites for adapter domains. For example, SH2 (Src homology 2) domains bind to pTyr (phosphotyrosine) residues in specific sequence contexts [5], while FHA [6,7] and some WD40 domains bind to sites containing pThr (phosphothreonine) and pSer (phosphoserine) residues [8]. Secondly, modular structures are evolvable, because it is straightforward to copy and move domains by genetic duplications and recombinations. For example, >200 proteins contain FHA domains, and in these diverse settings there are FHA domains that bring enzymes and their substrates together, while others anchor signal proteins close to cytoskeletal structures or to receptors in membranes [6,7,9]. Thirdly, proteins often have multiple adapter domains that act combinatorially, which strengthens binding (because multiplying the number of interaction surfaces can increase affinity constants exponentially) and permits sequential recruitment of multiple proteins (scaffolding) so that very complex structures can be constructed in an orderly fashion [3,4].

14-3-3s ARE DIMERS, SO ARE THEY SIMPLE ADAPTERS?

The adapter concept sets the scene for a focus on 14-3-3 proteins. In common with FHA and WD40 domains, 14-3-3s bind to specific pSer and pThr motifs within target proteins [10]. Two optimal 14-3-3-binding phosphopeptide motifs, mode I (Arg-Ser-Xaa-pSer-Xaa-Pro) and mode II (Arg-Xaa-Tyr/Phe-Xaa-pSer-Xaa-Pro), have been isolated in oriented peptide library screens [11], although 14-3-3-binding sites in many proteins do not conform to these optimal motifs, presumably because other structural features contribute to the interactions.

However, 14-3-3s are not modular components of other proteins. They are discrete binding proteins, with no intrinsic enzyme activity. So what does this imply about their function?

Important clues came from the crystal structures (see the green structure in Figure 1), which showed that each 14-3-3 polypeptide contains nine helices, arranged to form an almost right-angled corner. Two 14-3-3 subunits associate strongly with each other to form saddle- or C-shaped dimers. Homo- or hetero-dimers can be made from the multiple 14-3-3 isoforms that are present in most eukaryotes, although only certain combinations may be possible, depending on steric compatibility [12]. There are seven closely related genes, encoding β, ε, η, γ, τ, ζ and σ isoforms that are conserved across mammalian species [13], whereas *Drosophila* and yeast contain two genes, and *Arabidopsis* plants have fifteen, including possible pseudogenes [14–16]. In the dimers, the N-terminal helices of two subunits contact one another and form the floor of a central groove, while the C-terminal helices form its walls [17,18]. In some 14-3-3 isoforms, a C-terminal loop that is disordered in the crystal structure may form a tenth helix

Abbreviations used: AANAT, arylalkamine/serotonin N-acetyltransferase; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; BSE, bovine spongiform encephalopathy; Cdc, cell-division cycle; CDK, cyclin-dependent kinase; chk, checkpoint kinase; CJD, Creutzfeldt–Jakob disease; COPI, coatamer I; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; FKHR, Forkhead in rhabdomyosarcoma; FOXO, Forkhead box, class O; HCV, hepatitis C virus; IRS-1, insulin receptor substrate-1; LR axis, left–right axis; MAPK, mitogen-activated protein kinase; MDS, Miller–Dieker syndrome; NLS, nuclear localization signal; NR, nitrate reductase; PDE3B, phosphodiesterase 3B; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; SCA, spinocerebellar ataxia; SH2, Src homology 2; SnRK, SNF1-related kinase; TSE, transmissible spongiform encephalopathy; WPK4, wheat protein kinase 4.

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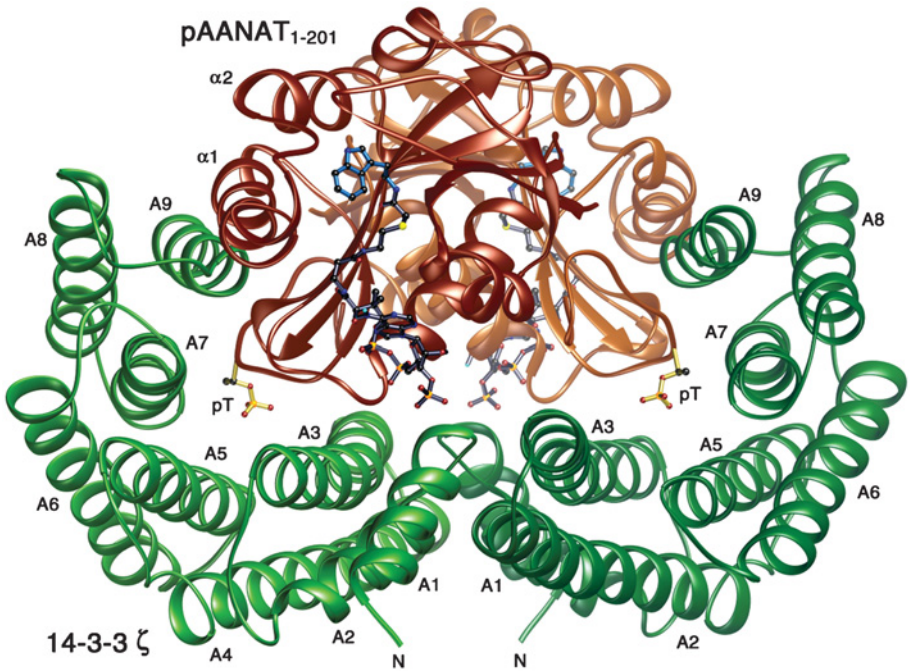

Figure 1 Crystal structure of a 14-3-3 dimer bound to phosphorylated AANAT

Structure of the 14-3-3–pAANAT complex, indicating the pThr[31] of AANAT (brown/yellow), the proline twist C-terminal to pThr[31], and the loop in AANAT ($\alpha 1/\alpha 2$ in brown) whose movement is restricted by the 14-3-3 dimer (green). Reprinted from *Cell* **105**, Obsil, T., Ghirlando, R., Klein, D. C., Ganguly, S. and Dyda, F., "Crystal structure of the 14-3-3ζ:serotonin N-acetyltransferase complex: a role for scaffolding in enzyme regulation", pp. 257–267, © 2001, with permission from Elsevier.

that regulates the access of binding partners into the central groove [19,20]. Phosphorylated sites on target peptides and proteins dock directly into either side of this inner groove, which contains the residues that are most invariant among the isoforms [21,22].

The dimeric structure suggested immediately that 14-3-3s might represent the simplest 'adapter' strategy, where two different target proteins bind simultaneously to each monomer of the same 14-3-3 dimer. However, while 14-3-3s are components of several multiprotein complexes (e.g. [23–25]), only rarely has a 14-3-3 dimer been reported to act as a bridge between two targets. Several pairings involving Raf-1 have been reported, namely Raf-1 and Bcr (B-cell receptor) [26], Raf-1 and A20 [27] and Raf-1 and PKC (protein kinase C) ζ [28]. Raf-1 is a protein kinase that links activated cell-surface receptors to the classical MAPK (mitogen-activated protein kinase) [ERK (extracellular-signal-regulated kinase) 1/2] cascade by MEK (MAPK/ERK kinase) phosphorylation [29], and may have other roles in mammalian cell transformation and differentiation [30,31]. Why, out of the >250 binding partners for 14-3-3s, Raf-1 has featured repeatedly in cases of '14-3-3 as adaptor' is unclear. Is the Raf-1 structure especially suited to fit into one side of the central groove and accommodate a second binding partner on the other side? Or is it simply that more researchers have tested Raf-1, which was one of the earlier established 14-3-3-binding phosphoproteins? While further examples of 14-3-3s mediating interactions between proteins are emerging, for example Tau and glycogen synthase kinase 3β [32], and the Ron receptor tyrosine kinase and $6\beta 4/3\beta 1$ integrin [33], the exact arrangement of these complexes is not fully defined. Do both partners bind to a single 14-3-3 dimer simultaneously, or does binding of one partner to 14-3-3s change its conformation, enabling it to interact with the second partner?

14-3-3s AS MULTIPURPOSE 'CONFORMATION CLAMPS'

Dimerization of 14-3-3s is important, because point mutations that disrupt 14-3-3 dimers impair the regulatory functions of 14-3-3s [34,35]. But why, when there is (so far) such limited evidence for 14-3-3s acting as intermolecular bridges?

Another possibility is that a 14-3-3 dimer binds two sites on the same target protein. A synthetic phosphopeptide with two tandem 14-3-3 consensus motifs binds over 30-fold more tightly than the same peptide containing only a single motif [11]. Moreover, several 14-3-3-binding proteins, including Raf-1 [10,36], c-Cbl [37], 3BP2 [38], tyrosine hydroxylase [39], FOXO (Forkhead box, class O) transcription factors [40–42], AANAT (arylalkamine/serotonin N-acetyltransferase) [43] and yeast forms of Cdc (cell-division cycle) 25C [44] contain two phosphorylated sites that are implicated in 14-3-3 binding, and are separated by polypeptides of various lengths. It has been postulated that one site called the 'gatekeeper' is indispensable for a stable 14-3-3 interaction, whereas a second site 'enhances' the interaction, but has too weak an affinity to bind 14-3-3 alone [45] (Figure 2). In this model, once the gatekeeper site is phosphorylated and bound to one monomer in the 14-3-3 dimer, proximity enhances the chances of the secondary site interacting with the other 14-3-3 subunit. Testing whether the gatekeeper/enhancer model is generally applicable to 14-3-3–target interactions may be difficult experimentally. For example, mapping the 14-3-3-binding sites by mutation analysis alone might not reveal putative enhancer sites on targets, since mutations at these sites might have only minor effects on overall 14-3-3–ligand binding [45]. Another complication is that mutations at sites that do not bind directly to 14-3-3s can sometimes affect phosphorylation at distal 14-3-3-binding sites [46]. Such technical difficulties

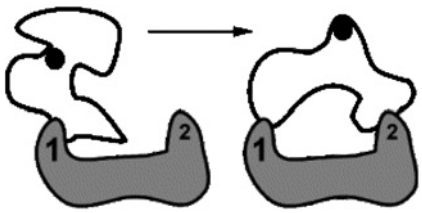

Figure 2 Model of 14-3-3-dependent conformational change upon two-site binding

14-3-3 binding relies initially upon interaction of a gatekeeper residue with one monomeric subunit (1). Binding of weaker secondary sites (2) facilitates a ligand conformation that is unfavourable in the unbound state, exposing one or more regions of the protein (shaded circle) that are inaccessible in the free or monomer-bound form. Reprinted by permission of the Federation of the European Biochemical Societies from "How do 14-3-3 proteins work? – Gatekeeper phosphorylation and the molecular anvil hypothesis", by Yaffe, M. B., FEBS Letters **513**, pp. 53–57, © 2002.

probably underlie several controversies in the 14-3-3 field, and emphasize the importance of performing direct phosphorylation analysis of target proteins.

The core of the 14-3-3 dimer is a rigid, unyielding structure [45,47,48]. So an important extension of the gatekeeper/enhancer hypothesis is that, unless the two molecules snap into place like puzzle pieces, the target is likely to undergo conformational deformations when its two phosphate groups are inserted into the 14-3-3-binding groove (Figure 2) [45]. Indeed, there is compelling evidence that 14-3-3s are important allosteric mediators of protein conformational changes, particularly with respect to regulating enzyme activity. Conformational changes are often energy-driven by nucleotide triphosphate hydrolysis and/or post-translational modifications, such as phosphorylation [1]. But sometimes protein phosphorylation is not enough to drive the shape changes that are needed to alter enzyme activities, and 14-3-3s appear to provide the extra structural support needed to stabilize target enzymes in one or other conformation.

In fact, the first structure of a 14-3-3 bound to a phosphoprotein has been a revelation, showing beautifully the 14-3-3 dimer as an 'allosteric clamp' that grips its phosphorylated target enzyme in an active conformation (Figure 1) [47]. AANAT activity regulates the synthesis of melatonin, the hormone that synchronizes our daily body rhythms to the day–night cycle. At night, AANAT is activated by PKA (cAMP-dependent protein kinase, also known as protein kinase A) phosphorylation at two sites, Thr^{31} and Ser^{205}, of which the N-terminal site is a canonical mode I 14-3-3-binding motif (Arg-Arg-His-pThr^{31}-Leu-Pro-Ala-Asn) [43]. The phosphorylated AANAT that gave crystal structures lacks the C-terminal pSer^{205} phosphorylation site, and two of these molecules interlock into the clasp of a 14-3-3 dimer. The pThr^{31} residues on each AANAT monomer are just the right distance apart to insert one into each of the phosphopeptide-binding sites at either side of the 14-3-3 central groove. In structures of mode I phosphopeptide–14-3-3 complexes, the proline in the +2 position from the phosphorylated residue is in a *cis* conformation and twists the peptide back out of the docking site [11], and this proline twist is particularly clear in the AANAT–14-3-3 structure [47]. The AANAT and 14-3-3 are also held together by extensive contacts between other regions of the 14-3-3 channel and N-terminal domain of AANAT, although these contacts must be insufficient to form a stable complex otherwise binding would not be regulated by phosphorylation [43,47,49].

The value of the AANAT–14-3-3 structure was enhanced by prior knowledge that the N-terminal domain of free AANAT undergoes rearrangements during its catalytic cycle [50,51]. By comparing the various structures, it becomes clear that the 14-3-3 dimer activates phosphoAANAT by restricting the movement of a floppy loop, forcing open the active site of the enzyme into a conformation that favours substrate binding [43,49].

14-3-3s seem to operate more widely as multipurpose 'allosteric clamps' that are recruited to hold enzymes in place wherever a docking site is created by a combination of inherent protein shape and phosphate added in response to a signalling pathway. For example, plant nitrate reductase [52] and mammalian ASK1 (apoptosis signal-regulating kinase 1) [53] are inhibited when they become phosphorylated and bind 14-3-3s, whereas 14-3-3s activate phosphorylated tryptophan and tyrosine hydroxylases [39].

14-3-3s COMPETE WITH OTHER BINDING PROTEINS TO CONTROL LOCATION AND ACTIVITY OF TARGETS

14-3-3s are heavily implicated in regulating the subcellular distribution of many phosphorylated target proteins. In dissecting their trafficking functions, a further action of 14-3-3s is emerging in which they influence their targets by blocking the access of other proteins.

For example, a novel role for 14-3-3s was recently defined in forward transport of ion channels and receptor complexes from the ER (endoplasmic reticulum). After synthesis, transmembrane proteins are retained in the ER for multimer assembly and quality control. In one retention mechanism, dibasic motifs on the target proteins bind to cytosolic COPI (coatamer I)-coated vesicles, which return the protein to the ER if it ever escapes to the Golgi. Once properly assembled, however, proteins destined for the plasma membrane must evade this ER retention system. Recently, 14-3-3s were found to bind to phosphorylated 'ER escape motifs' on potassium channels, an acetylcholine receptor and an immune system complex. Binding of 14-3-3s to the escape motif prevented COPI binding to the dibasic motif, and promoted the recruitment of the protein to the cell surface [54–56].

14-3-3s are also associated with dynamic nucleocytoplasmic shuttling. Phosphorylation-dependent binding of 14-3-3s to telomerase promotes its nuclear localization [57]. But more often, the reverse has been reported, in that many nuclear proteins can become phosphorylated, bind to 14-3-3s and accumulate in the cytoplasm. An example in plants is the bZIP (basic leucine zipper) transcription factor RSG (repression of shoot growth), which induces expression of a gibberellin biosynthetic enzyme, and hence regulates stem elongation and development [58]. In *Saccharomyces cerevisiae*, the transcription factors MSN2 and MSN4 are phosphorylated, bind 14-3-3s and exit the nucleus in response to nutrients and TOR (target of rapamycin) signalling [59]. In animal cells, 14-3-3 binding and nuclear exit has been found for the cell cycle protein phosphatase Cdc25C [60], insulin-regulated FOXO transcription factors [40–42], the glucocorticoid receptor [61] and its co-repressor RIP140 (receptor-interacting protein 140) [62], the CDK (cyclin-dependent kinase) inhibitor p27(Kip1) [63,64], the *Xenopus* Hat1 acetyltransferase and/or its binding partner RbAp48 [65] and HDAC (histone deacetylase) 4 and 5 [66,67].

One general proposal is that 14-3-3s promote nuclear export and/or reduce rates of nuclear import by masking NLSs (nuclear localization signals) on their targets. Thus binding of an NLS on the CDK inhibitor p27(Kip1) (p27) to importins promotes translocation of p27 into the nucleus where it regulates cyclin–CDK complexes. However, when growth factors stimulate the cell cycle, p27 becomes phosphorylated and binds 14-3-3s. Phosphorylation alone has no effect on the association of importin

with p27, but binding of 14-3-3s to the phosphorylated NLS of p27 blocks importin binding, which promotes cytoplasmic localization of NLS-phosphorylated p27 [63,64].

In other cases, whether 14-3-3s actually cause the nuclear exclusion, or merely escort the exiting protein is a tricky and controversial question. In several cases, mutant targets that cannot bind 14-3-3s do not get translocated. However, such mutations can have complicated effects. For example, mutations in the FOXO transcription factor FKHR (Forkhead in rhabdomyosarcoma) that block its binding to 14-3-3s have been found to restrict nuclear exclusion at least in part by suppressing phosphorylation of other residues [41]. For FKHR at least, 14-3-3 binding is just one of several nuclear export mechanisms [40,46]. Perhaps 14-3-3s also prevent these 'nuclear' proteins from making deleterious interactions with cytoplasmic components.

Regulated competition between 14-3-3s and other proteins for binding to targets may operate more widely in cells. Thus a swap between 14-3-3s and other binding partners is part of the mechanism by which nuclear-encoded precursor proteins are translocated into chloroplasts and mitochondria [68]. Phosphorylation-dependent binding of 14-3-3s to the pro-apoptotic protein BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) promotes cell survival by preventing BAD from being able to bind to and inactivate the anti-apoptotic protein Bcl-X_L (reviewed in [69]). In another case, 14-3-3 binding to the IRS-1 (insulin receptor substrate-1) may interfere with the ability of the IRS-1 to recruit and activate PI3K (phosphoinositide 3-kinase) [70]. 14-3-3 binding has also been reported to protect target proteins from modifications such as dephosphorylation [71] and proteolysis [72].

Combinations of the 'adapter', 'allosteric clamp' and 'mutually-exclusive binding' types of 14-3-3 action may occur together, as has been noted for the aforementioned protein kinase Raf-1. Thus in allosteric clamp mode, 14-3-3s may hold Raf-1 in a partially active conformation before full activation (e.g. [34]). In addition, when the N-terminal 14-3-3-binding site of Raf-1 is mutated, Raf-1 can still activate the MAPK pathway, but cannot induce cell transformation and differentiation [31]. Perhaps these other functions of Raf-1 are mediated by 14-3-3s in adapter mode, directing Raf-1 to interact with other signalling proteins (see above; [26–28]). Finally, in mutually-exclusive binding mode, 14-3-3 binding to PKA-phosphorylated Raf-1 blocks the translocation of Raf-1 to the plasma membrane for interaction with its upstream activator Ras [36].

DEFINING THE 14-3-3-INTERACTING PHOSPHOPROTEOME – CLUSTERS AND THEMES

What is really so phenomenal about 14-3-3s is the number and diversity of their phosphoprotein-binding partners – with more than 300 target proteins (and rising) in intracellular signalling and trafficking, cell cycle control, metabolism and ion channel physiology.

Equally fascinating is that the lists of known 14-3-3 targets from the different eukaryotic phyla were, until recently, highly skewed. Interactions with 14-3-3s were identified mainly when someone's favourite protein pulled out a 14-3-3 in a yeast two-hybrid screen. Thus the intensity of cancer research explains why 14-3-3s have became famous for interacting with mammalian oncogenic signalling pathways, whereas in plants, most known 14-3-3 targets are biosynthetic enzymes, ion channels and regulators of plant growth, reflecting interest in improving crop yields. Attempts to use two-hybrid screens to search systematically for 14-3-3-interacting proteins have rarely been successful, possibly because 14-3-3s bind preferentially to 14-3-3s from the target library (although trihybrid screening for proteins that inhibit the 14-3-3–Raf-1 interaction has been useful [73]). 14-3-3s will not bind proteins from cDNA libraries expressed in *Escherichia coli*, because the targets will not be phosphorylated. 14-3-3-binding motifs are too small and degenerate to be able to search genomes for likely binding proteins; for example, the *Arabidopsis* genome encodes approx. 6000 proteins with potential mode I motifs [74]. Thus 'hot' research areas and technical difficulties conspired to a situation where our lists of 14-3-3 targets represented only a biased subset of the full 14-3-3-interacting phosphoproteome.

To redress the sampling bias, several groups are using tagged 14-3-3s or 14-3-3–Sepharose to pull out binding partners from cell extracts [75–77], eluting specifically bound targets by competition with 14-3-3-binding phosphopeptides [76,77]. This is an attractive strategy because many proteins can be isolated in a single experiment, and improvements in MS are facilitating their identification. One large experiment identified > 200 human proteins that can bind specifically to the phosphopeptide-binding site of 14-3-3s, either directly or as components of multiprotein complexes [77]. The isolated proteins included enzymes of metabolism, intracellular signalling and trafficking, and regulators of the proteasome and actin dynamics. The inference is that the binding of 14-3-3s to phosphorylated sites on intracellular proteins is an extremely common mechanism, connecting signalling pathways to the regulation of a much wider diversity of target proteins than had been realized previously.

Even though the current lists of 14-3-3-binding partners may still be distorted, there are indications that 14-3-3 targets may not be just a random array of cellular proteins. There are striking clusters of 14-3-3 targets in common cellular processes, including melatonin biosynthesis [78], primary metabolism, anti-apoptotic pathways, signalling to cellular proliferation, cellular trafficking and actin dynamics [77]. For example, in actin dynamics, 14-3-3s interact with LIM kinase, cofilin, the Cdc42-activated protein kinase MRCKβ (myotonic dystrophy kinase-related Cdc42-binding kinase β), VASP (vasodilator-stimulated phosphoprotein) and possibly other actin-binding proteins [77,79,80]. These findings are consistent with considerable genetic evidence implicating 14-3-3s in the control of actin cytoskeleton and cellular trafficking [79,80]. At the moment, we can only theorize about why multiple 14-3-3 interactions might be important in the control of actin function. Does a single signal trigger the phosphorylation and binding of 14-3-3 to all its actin-regulating targets simultaneously to generate a full-blown change in actin structure? Or do multiple waves of signalling and 14-3-3 binding impinge on actin regulators?

COMMON SIGNALS REGULATE SUBSETS OF 14-3-3-INTERACTING PROTEINS

As more and more phosphoproteins are identified as 14-3-3 targets, the question of how all these interactions are regulated at the cellular level becomes more critical. Most regulatory studies focus on one target protein, but, in principle, the 14-3-3-affinity purification methods mentioned in the previous section could be adapted to tackle the global responses of the entire 14-3-3-interacting proteome to any cellular stimulus [77,81]. 14-3-3-affinity purified proteins have been phosphorylated on their 14-3-3-binding sites *in vivo*. This means that comparing the subsets of proteins that can be 14-3-3-affinity purified from cells stimulated in different ways, from different stages of the cell cycle, and so on, may provide a generic way to sort out which 14-3-3–target interactions are regulated by which signalling pathways.

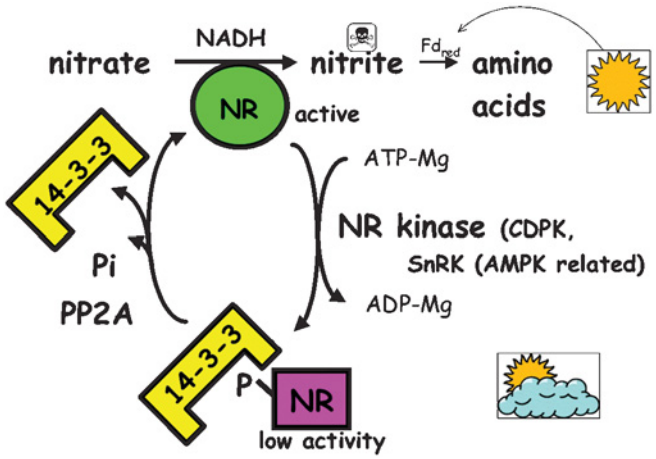

Figure 3 14-3-3s inhibit phosphorylated NR in leaves in the dark

When leaves are actively photosynthesizing, NR is in an active, dephosphorylated state. When photosynthesis is blocked (depicted here as a cloud blocking the sun), NR becomes phosphorylated (on Ser^{543} on the spinach enzyme), which creates a phosphopeptide motif that binds directly to 14-3-3s in the presence of bivalent metal ions. 14-3-3 binding inhibits NR activity. AMPK, AMP-activated kinase; Fd_{red}, reduced ferredoxin.

The emerging picture is that distinct subsets of 14-3-3 targets become phosphorylated and bind to 14-3-3s in response to different signals. For the relatively few targets that have been investigated in detail to date, it is striking that signalling pathways that inhibit apoptosis and/or prevent dangerous cellular events have particularly come to the fore in promoting the phosphorylation of 14-3-3-binding sites [69,82–85]. In the present review, two very different cell-survival roles for 14-3-3s will be highlighted, namely 'how plant leaves cope with sudden darkness' and 'human cell responses to growth factors'.

14-3-3s OPERATE TO COUNTERACT THE DANGERS OF SUDDEN DARKNESS IN LEAVES

A plant growing in a sunny climate receives constant illumination that drives the photosynthesis of all its organic compounds from CO_2, water and simple minerals such as nitrate. In contrast, the fickle Scottish summer exposes plants to huge variations in sunlight, often on a minute-to-minute basis, and, of course, there can be total blackout at night. Sudden darkness is potentially dangerous for leaves because levels of photosynthetically generated reductant fall, ATP is depleted, and toxins such as nitrite start to accumulate. However, it appears that these metabolic imbalances do not spiral to lethal levels because they are rapidly counteracted by a 'dark-induced' signalling pathway that regulates the phosphorylation, 14-3-3 binding and activity of several key enzymes, including the cytosolic enzyme NR (nitrate reductase).

Regulation of NR is connected intimately to photosynthetic events in the chloroplast (Figure 3; reviewed in [86]). Thus, when photosynthesis is active, cytosolic NR is also active in reducing nitrate into nitrite, which enters the chloroplast where it is reduced further to ammonium and is incorporated into amino acids. When photosynthesis is inhibited, the chloroplast is no longer able to process the nitrite. However, a build-up of this toxic ion is averted because a 'photosynthesis-inhibited' signal is sent from the chloroplast to the cytosol, which leads to the phosphorylation of NR. The phosphorylated NR binds to 14-3-3s, and 14-3-3 binding inhibits the enzyme's activity, thus preventing the generation of nitrite. In addition to co-ordinating NR activity with photosynthesis, NR is cycled between active and inactive forms by (de)phosphorylation/14-3-3s in response to other environmental factors and in co-ordination with sugar supplies (which provide the carbon portion of amino acids) [87].

Knowledge of the signalling pathways that integrate chloroplast and cytosolic metabolism is rudimentary, although two types of protein kinase that can phosphorylate the 14-3-3-binding site of NR have been identified, namely the plant SnRKs (SNF1-related kinase; related to the mammalian AMP-activated protein kinase) and CDPKs (calcium-activated protein kinases that are unique to plants and protozoa). Intriguingly, members of both kinase families, namely the wheat SnRK1 homologue, WPK4 (wheat protein kinase 4) [88] and the *Arabidopsis* CPK1 isoform [72,76,89] have themselves been identified as 14-3-3-interacting phosphoproteins. Ikeda et al. [88] speculated that WPK4 might deliver 14-3-3s to NR to promote their interaction after phosphorylation has taken place.

In addition to driving amino acid biosynthesis, photosynthesis produces sugars and several enzymes of cytoplasmic sugar metabolism have been identified as binding partners for 14-3-3 proteins, including sucrose-phosphate synthase [76,90], F2KP (fructose-6-phosphate 2-kinase/phosphatase), which regulates levels of the sugar signalling metabolite fructose 2,6-bisphosphate [91], a trehalose-6-phosphate synthetase [76] and enzymes in chloroplastic starch metabolism [92]. The plasma membrane (H^+)-ATPase, which supports the import of nitrate and sugars into plant cells, is also regulated by phosphorylation and 14-3-3s ([93]; see discussion below on fusicoccin). One universal precursor for all these biosynthetic pathways is ATP, made by the ATP synthases that use transmembrane electrochemical proton gradients generated by the light reactions of photosynthesis in the chloroplast, or by respiration in mitochondria. Although there is no obvious mechanism for 14-3-3s to enter these organelles, it has been reported that 14-3-3s bind directly to the phosphorylated β subunit of the ATP synthase in the chloroplast when the electrochemical proton gradient fails, for example, in the dark. The binding of 14-3-3s appears to prevent the ATP synthase from operating in reverse and hydrolysing ATP under these conditions [94].

Overall, phosphorylation and 14-3-3s play important roles in regulating complicated daily rhythms in sugar metabolism in co-ordination with photosynthesis, ATP production and nitrate reduction [95].

A BREAKTHROUGH IN IDENTIFYING PKB (PROTEIN KINASE B) SUBSTRATES IN MAMMALIAN CELLS?

In mammalian cells, PKB (also known as Akt) is activated downstream of PI3K when cells are stimulated by insulin, growth factors, certain G-protein-coupled receptor agonists, and integrin binding. Depending on the cellular context, active PKB promotes a variety of responses, including cell growth, inhibition of apoptosis, cell proliferation, differentiation, some secretory events, T-cell activation, changes in cell shape (lamellipodia formation/membrane ruffling) and cell movement [96,97]. Finding such all-pervasive effects of PKB on cell behaviour has motivated intensive searching for the substrates that connect PKB to so many cellular processes.

As new PKB targets are discovered, it is emerging that one common molecular mechanism – binding of 14-3-3 proteins to sites that have been phosphorylated by PKB – regulates several (although not all) of the diverse functions that are associated with PKB activation (Figure 4). Thus the ability of insulin-like growth factor-1 to stimulate glycolysis can, in part, be explained by PKB

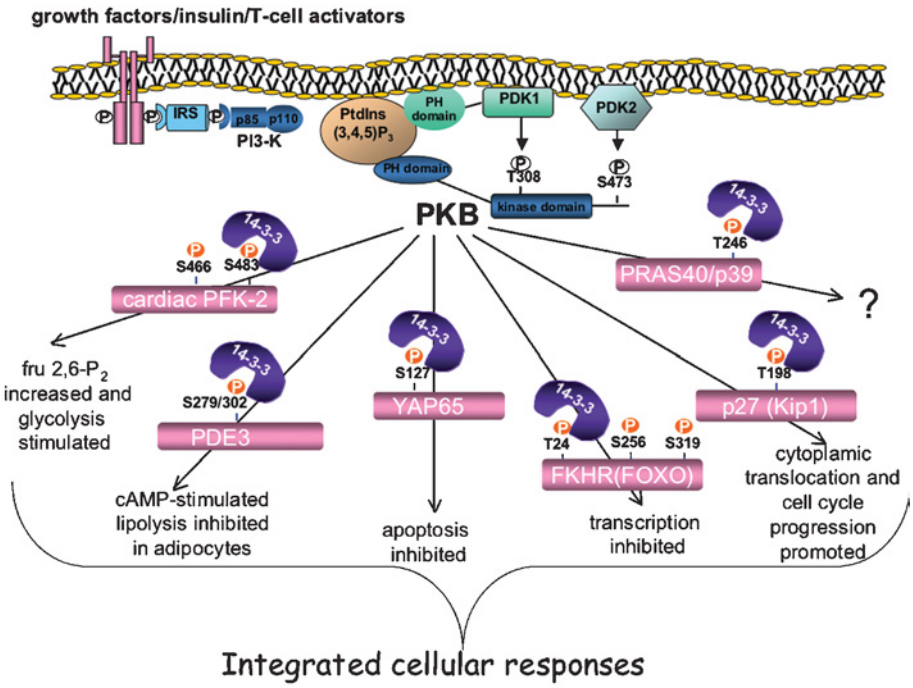

Figure 4 Many proteins bind to 14-3-3s after phosphorylation by the growth-factor- and insulin-stimulated PKB/Akt

Activation of receptor tyrosine kinases (or other receptors, such as G-protein-coupled receptors, not shown) recruits PI3K family lipid kinases to the plasma membrane where they convert PtdIns(4,5)P_2 into PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 recruits PH (pleckstrin homology) domain-containing proteins to the lipid bilayer, including PKB and PDK (phosphoinositide-dependent kinase) 1, and PKB becomes activated. Proteins that bind to 14-3-3s after phosphorylation by PKB are shown. These interactions contribute to the anti-apoptotic and metabolic effects of PKB. fru 2,6-P_2, fructose 2,6-bisphosphate.

phosphorylation and the subsequent binding of 14-3-3s to PFK-2 (phosphofructokinase-2), an enzyme that generates fructose 2,6-bisphosphate, which in turn stimulates glycolysis [98]. The major mechanism whereby insulin antagonizes catecholamine-induced increases in cAMP, activation of PKA, and phosphorylation/activation of hormone-sensitive lipase, is believed to be via PKB phosphorylation and activation of PDE3B (phosphodiesterase 3B). Recently, the PKB-phosphorylated PDE3B was found to bind to 14-3-3s [99], although the role of 14-3-3s in the insulin regulation of lipolysis is not yet known. Moreover, PKB-dependent binding to 14-3-3s of at least three proteins has been implicated in inhibiting early steps of apoptotic cell death, namely YAP65 (Yes-associated protein) [100,101], mammalian and *Caenorhabditis elegans* versions of FOXO transcription factors (contributing to the growth-factor-induced nuclear exit of these transcription factors; see above) [40–42]. In addition, there is a proline-rich protein, termed PRAS40 (proline-rich Akt substrate), that gives a very strong signal in 14-3-3 overlay assays of growth-factor-stimulated cell extracts [102], and whose binding to 14-3-3s after PKB phosphorylation in NGF (nerve growth factor)-stimulated neuronal cells has been implicated as anti-apoptotic [103]. PRAS40 is probably the same protein as p39, whose binding to 14-3-3s depends on both PKB and amino acid signalling [81]. PKB can also positively regulate G_1/S cell-cycle progression through a number of mechanisms, including phosphorylation of the CDK inhibitor p27(Kip1), which then binds 14-3-3s and becomes localized in the cytoplasm and degraded [63,104] (see above).

Looking at the array of 14-3-3 interactions in the PKB signalling Figure (Figure 4), it is interesting to speculate that the relative strength of signalling through to different downstream targets of PKB might be influenced by the spectrum of 14-3-3 isoforms present in the cell (see the discussion on isoforms below). Perhaps 14-3-3 concentrations limit and/or somehow co-ordinate the overall cellular responses to PKB activation.

14-3-3s IN CARCINOGENESIS AND CANCER THERAPY

The rampant proliferation of cancer cells is often independent of growth factors. Indeed, the PI3K/PKB pathway is constitutively activated in many cancers (reviewed in [105]). In the clinic, activation of PKB correlates with advanced tumour progression (e.g. [105,106]). So their roles in PKB-mediated responses to growth factors immediately thrusts 14-3-3s into the cancer arena.

But there are further reasons for linking 14-3-3s with cancer [107]. Several virulence proteins from tumour-promoting viruses target 14-3-3s directly. For example, HCV (hepatitis C virus) is a common cause of liver failure and is linked to human hepatic carcinoma. Recently, the HCV core protein that has been implicated in cell-growth regulation was found to become phosphorylated on Ser^{53}, bind to 14-3-3s in its host cell and activate Raf-1 by a mechanism that depended on 14-3-3 binding [108]. The middle T antigen of murine polyomavirus binds to a number of cellular proteins involved in the regulation of cell growth and proliferation, including 14-3-3s [109]. Binding to 14-3-3s depends on the Ser^{257} phosphorylation site of middle T, and mice infected with polyomavirus expressing $Ser^{257} \rightarrow$ Ala mutant middle T antigen showed a striking deficiency in the induction of salivary gland tumours [110].

In addition, 14-3-3s interact with a long list of oncogene products, tumour suppressor proteins and regulators of cell survival, proliferation and growth, including the insulin-like growth factor-1 receptor, p53, PI3K and other components of growth factor signalling pathways (further examples in [13,111,112]). While cellular regulation and roles of most of these interactions are not

well understood, a few cases highlight the involvement of 14-3-3s in signalling pathways that are altered in cancers.

14-3-3s ACTIVATE p53 AND 14-3-3σ IS A p53 TARGET GENE

The p53 tumour suppressor has a master role in cellular responses to stresses, such as DNA damage, hypoxia, growth factor depletion and heat shock. In response to these stimuli, p53 is stabilized and activated by multiple mechanisms (including phosphorylation, dephosphorylation and acetylation), yielding a potent sequence-specific DNA-binding transcription factor. Once activated, p53 either induces cell-cycle arrest at G_1 or G_2, giving time to repair damaged DNA before cell division is resumed and/or initiate apoptosis, which protects the organism by eliminating the defective cell [113].

One modification induced by DNA damage (caused by ionizing radiation) is dephosphorylation of Ser^{376} of p53, which creates a binding site for (unknown isoforms of) 14-3-3s at the nearby $pSer^{378}$. The doubly phosphorylated $pSer^{376}$/$pSer^{378}$ in non-irradiated cells cannot bind to 14-3-3s [114]. In cells, mutant forms of p53 that do not interact with 14-3-3 proteins retained sequence-specific DNA binding activity, but were compromised in their ability to activate transcription of the p53-induced genes p21, waf1 and cip1, and did not induce G_1 arrest [115].

In addition, one specific isoform, 14-3-3σ, is a p53-induced gene in epithelial cells [116]. Other proteins, including BRCA1 (breast cancer gene 1), a tumour suppressor for breast and ovarian cancers, co-operate with p53 to direct the expression of the 14-3-3σ isoform [117]. Also, certain phosphorylated splice variants of p63, a homologue of p53 in the basal layer of several stratified epithelial tissues, bind to the promoter of the 14-3-3σ gene [118], which may correlate with the fact that 14-3-3σ expression is normally restricted to keratinocyte stem cells in stratified epithelia [119–121].

14-3-3σ can induce cell-cycle arrest at G_2 by a mechanism that involves 14-3-3σ binding to and sequestering CDK1–cyclin B1 complexes in the cytoplasm [107,122,123]. At the same time as it induces cell-cycle arrest, 14-3-3σ also seems to delay the alternative pathway to apoptosis by affecting localization of the pro-apoptotic protein Bax [124] and interacting with telomerase [125]. Details such as the kinases that phosphorylate the 14-3-3σ-binding sites on these proteins are still sketchy.

Other 14-3-3s (but apparently not 14-3-3σ) are also involved in p53-independent mechanisms to initiate the G_2/M checkpoint in DNA-damaged cells, including phosphorylation of and binding of 14-3-3s to Cdc25C phosphatase by the chk1 and chk2 checkpoint kinases and cytoplasmic sequestration. The sequestered Cdc25C cannot activate CDK1, and entry into mitosis is thereby prevented. If mitosis has already started, this stalling mechanism cannot operate (e.g. [124]).

THE 14-3-3σ PARADOX OPENS A CHINK IN THE ARMOUR OF CANCERS

Elevated levels of several 14-3-3 isoforms have been found in cancers and cell lines derived from tumours [126–128]. Furthermore, when primitive neuroectodermal brain tumours were treated with 2-methyloestradiol, this drug inhibited tumour growth and induced apoptosis, accompanied by a decrease in the levels of 14-3-3 proteins [129], and antisense expression of 14-3-3β suppressed growth of tumour cells in culture and *in vivo* [130]. Together, these findings enhance our perceptions of 14-3-3s as multitasking cell-survival proteins.

In contrast, 14-3-3σ is selectively lost by epigenetic hypermethylation of the gene in breast cancer [131,132] and other cancers [133–137]. In fact, loss of 14-3-3σ by gene methylation is reportedly the most consistent molecular alteration so far discovered in breast cancer [138]. A second mechanism for the loss of 14-3-3σ protein is its targeting for ubiquitin-mediated proteolysis by Efp, an oestrogen receptor-responsive RING-finger-containing protein implicated in promoting breast cancer [132,139]. As a corollary, 14-3-3σ expression was increased in a benign mucosal disease that is characterized by hyperdifferentiation and apoptosis [140]. Why should the 14-3-3σ protein be selectively eliminated in cancers and induced in a disease that involves apoptosis? This seems paradoxical because 14-3-3σ has anti-apoptotic functions ([124,125]; see previous section). Presumably, loss of 14-3-3σ enhances some other aspect of the transformation process, related to its roles in cell-cycle arrest at G_2 [107,122,123] and/or in cell senescence [141]. But many questions remain. Do other 14-3-3 isoforms take over roles of the eliminated 14-3-3σ? If 14-3-3σ is not lost, can transformation occur? At what stage in tumour progression is 14-3-3σ lost?

Whatever the answer to the 14-3-3σ paradox, an important clinical implication is that 14-3-3σ-null cancers may be sensitive to killing by DNA-damaging drugs [124,132]. This is one example of how genomics is starting to contribute in matching drugs to patients who can benefit the most. These findings also focus our attention on developing 14-3-3 agonists and antagonists, both as cancer therapies and for experimental use to enhance our basic understanding of 14-3-3 functions in cells.

EXPERIMENTAL 14-3-3 ANTAGONISTS

The obvious starting point for designing 14-3-3 antagonists is with the mode I (Arg-Ser-Xaa-pSer-Xaa-Pro) and mode II (Arg-Xaa-Tyr/Phe-Xaa-pSer-Xaa-Pro) phosphopeptides [11], and 14-3-3-binding phosphopeptide motifs derived from natural target proteins. An enhancement was described whereby tandem coupling of two 14-3-3 binding sequences via a flexible linker led to an approx. 30-fold increase in affinity for 14-3-3s [11]. Unphosphorylated peptide 14-3-3 antagonists have also been identified from phage display libraries, including R18 (PHCVPRDLSWLDLEANMCLP; [21,142]), whose affinity for 14-3-3s is as high as the best 14-3-3-binding phosphopeptides (K_d values of approx. 70–90 nM). Because they share a common binding site in the conserved amphipathic groove of 14-3-3s, R18 and the phosphopeptides are broadly capable of abolishing the interactions of any 14-3-3 with any target protein *in vitro*, and are useful as competitors for defining the kinetics, specificity and functional effects of 14-3-3 binding to specific targets. For example, if a 14-3-3-binding peptide activates NR in a leaf extract, we know that the enzyme had originally been inhibited by 14-3-3s [91]. These peptides are also useful for specifically eluting proteins from 14-3-3-afffinity columns [76,77].

The 14-3-3-binding phosphopeptides and R18 have been developed into reagents that can be expressed in cells and animals, or transduced through the plasma membrane. Thus when a 14-3-3-binding phosphopeptide was rendered cell-permeable by linking to penetratin, this reagent helped to show that 14-3-3s are essential for the activation of glycolysis by growth factors [98]. When GST (glutathione S-transferase)–R18 was transfected into *Xenopus* embryos, the resulting phenotypes demonstrated that 14-3-3s have multiple roles in both axial patterning and expression of mesodermal marker genes [143]. Two tandem R18 sequences have been incorporated into an artificial protein construct named difopein [144]. When expressed in cells, difopein promoted apoptosis, accompanied by decreased mitochondrial transmembrane potential, activation of caspase 3 and DNA fragmentation. Difopein-induced apoptosis was blocked by caspase

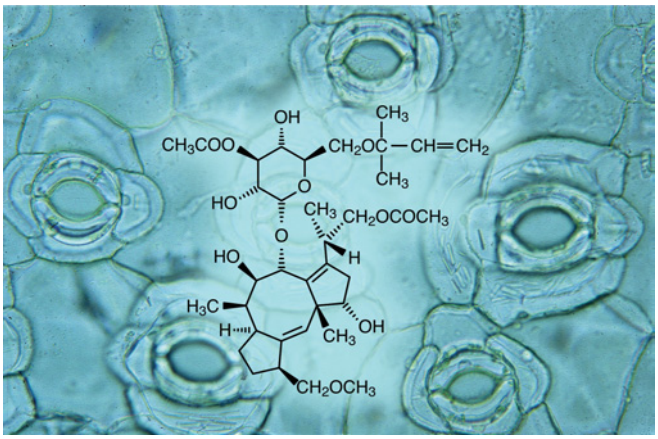

Figure 5 Structure of the fungal toxin fusicoccin

Fusicoccin permanently activates the plant plasma membrane (H^+)-ATPase by filling a cavity that forms when a 14-3-3 dimer binds to the C-terminus of the proton pump [93]. Pathologically, activation of the plant plasma membrane (H^+)-ATPase promotes permanent opening of guard cells. This Figure was kindly supplied by Claudia Oecking.

inhibitors, but not by expression of constitutively active PKB [144]. The simplest interpretation of these findings is that 14-3-3s restrain apoptosis by binding to proteins that are phosphorylated downstream of PKB in growth factor signalling, but upstream of caspase and mitochondrial dysfunction in the anti-apoptotic pathway. Recall that there is a growing list of candidates, because 14-3-3s have been repeatedly implicated in mediating cellular effects downstream of PKB [81,98,100].

Of course, a major limitation in using these 14-3-3 antagonists inside cells is that they disrupt many or all 14-3-3–target interactions, making dissection of cellular functions for each 14-3-3–target interaction difficult, although not impossible [98,144]. Actually, for medical intervention in cancers, lack of intracellular specificity might be an advantage – disabling all 14-3-3 functions provides a multi-pronged attack on cell-survival processes. But the frustrations of 14-3-3 biologists would be greatly soothed by having more specific compounds. A second need is for 14-3-3-binding molecules that are more stable and chemically versatile than peptides. Three leads indicate the feasibility of attaining both these goals. One is the precedent set by the cell-permeable non-peptide compounds that modulate SH2 binding to phosphorylated tyrosine residues [145]. Secondly, target interactions of certain isoforms have been reported to be sensitive to small molecules and ions, including metal ions and polyamines [146], and mizoribine, an imidazole nucleoside with immunosuppressive activity [147]. Further hopes come from Nature, which got there first by evolving the fungal toxin fusicoccin, which is an exquisitely specific agonist of one particular 14-3-3–target interaction in plants.

14-3-3s AS TARGETS OF PATHOGENIC PROTEINS AND TOXINS

Because the 14-3-3s are ancient, conserved throughout millennia of eukaryotic evolution and vital to so many cellular processes, perhaps it is not too surprising that tumour-promoting viruses (discussed above) and other microbial pathogens have evolved toxins that target 14-3-3s.

Fusicoccum amygdali is a fungal plant pathogen that secretes fusicoccin (Figure 5), a terpenoid that specifically enhances binding of 14-3-3s to the plant plasma membrane proton pump [(H^+)-ATPase]. Plant 14-3-3s bind to the plasma membrane (H^+)-ATPase in an unusual way by interacting with a Tyr-pThr-Val (YpTV) motif at the extreme C-terminal end of the protein [93]. Stimuli such as blue light trigger phosphorylation and binding of 14-3-3s, which activates the proton pump generating an electrochemical proton gradient that drives nutrient transporters and regulates cell turgor (e.g. [148]). In disease, fusicoccin chronically activates the proton pump by stabilizing its binding to 14-3-3s. The resulting loss of turgor control causes infected plants to lose water and wilt. At low concentrations, fusicoccin can also stimulate the elongation of plant cells in a phenomenon that is studied within the 'acid growth theory' of plant growth. This property has even been exploited in Russian agriculture where crops have been sprayed with fusicoccin to stimulate germination and plant growth.

The many studies of fusicoccin action have culminated in a structural account of how fusicoccin forms a specific and stable glue between the proton pump and 14-3-3s [93]. The YpTV–14-3-3 interaction is weak, because being at the extreme C-terminal end, the YpTV motif of the proton pump just enters the binding groove of 14-3-3 and stops, leaving free the space that is occupied by the proline twist when mode I and II phosphopeptides bind. However, fusicoccin can tuck precisely into this niche, and bonds with both the end of the proton pump and residues in the 14-3-3s, increasing the affinity between the two proteins by approx. 100-fold. In contrast, fusicoccin binding would clash with the binding of 14-3-3 consensus motifs that involve residues C-terminal to the phosphorylated residue, and indeed high concentrations of fusicoccin disrupt 14-3-3 binding to other proteins *in vitro* (S. E. Meek and C. MacKintosh, unpublished work).

While fusicoccin has long been believed not to enter mammalian cells, it was found recently that exposing frog embryos to fusicoccin during early development randomized the asymmetry of the LR (left–right) axis. This led to the further discovery that the 14-3-3ε mRNA and proteins display LR asymmetry at the first cell division, implicating 14-3-3ε in an extremely early aspect of LR patterning [149]. In addition, bean plants contain a terpenoid, cotylenin, which closely resembles fusicoccin and is a potent inducer of differentiation in several myeloid leukaemia cell lines [150]. But whether or not cotylenin also targets 14-3-3 interactions is unknown.

Pseudomonas aeruginosa is a Gram-negative bacterium that often infects immunocompromised patients. It changes the morphology of host cells by injecting virulence factors, including ExoS that redirects endocytosis and rearranges the actin cytoskeleton by ADP-ribosylating small GTPases, such as Ras and Rab5. This ADP-ribosylating activity depends absolutely on binding of ExoS to cellular 14-3-3s. In contrast with most physiological targets, ExoS does not have to be phosphorylated to bind to 14-3-3s. The site in ExoS that binds to 14-3-3s is QSGHSQGLLDALDLASKP [151], and mutagenesis and kinetic studies suggest that this sequence binds to the same binding site on 14-3-3s as physiological phosphoproteins, but depends on contacts with common residues to different extents. For example, a $Lys^{49} \rightarrow Glu$ mutation of 14-3-3ζ disrupted binding to both Raf-1 and ExoS [152], while mutation of Val^{176} prevented its interaction with Raf-1, but did not affect the binding and activation of ExoS by 14-3-3 [153]. The bacterium injects a substantial amount of ExoS protein into cells, raising the question of whether ExoS displaces endogenous targets from the cellular 14-3-3s.

More recently, the RolB protein from the plant pathogenic bacterium *Agrobacterium rhizogenes* has been found to bind to plant 14-3-3s and be translocated into the nucleus as part of the mechanism by which the pathogen transforms plants and induces hairy root growth [154].

14-3-3s IN BRAIN DEVELOPMENT AND NEURODEGENERATIVE DISEASES

14-3-3s are abundant in brain tissues, from where they were originally identified and given their numerical name based on column fractionation and electrophoretic mobility on starch gel electrophoresis [155]. Although few of their many binding partners in brain (J. E. Harthill, F. C. Milne and C. MacKintosh, unpublished work) have been identified, 14-3-3s are clearly critical for brain development, memory and learning, and are implicated in several neurological disorders.

Lissencephaly is a brain malformation associated with profound mental retardation, in which the brain surface is smooth because cells migrate aberrantly in the cortical layers during development. In children with ILS (isolated lissencephaly), there is heterozygous deletion of the *LIS1* gene at locus 17p13.3 [156], while in the more severe MDS (Miller–Dieker syndrome), both *LIS1* and the nearby 14-3-3ε gene (and other genes) are always deleted on one chromosome [157,158]. Recently, exciting work with knockout mice has revealed molecular links between 14-3-3ε and Lis1 that may underlie the cell migration abnormality of these diseases [158]. Mice deficient in 14-3-3ε or heterozygous for the *LIS1* gene have similar defects in brain development and neuronal migration, while mice heterozygous for both genes have more severe migration defects [158]. Previously, Lis1 was found to interact with the protein, NUDEL, which also binds to the cytoplasmic dynein motor protein [159]. Now it has been discovered that 14-3-3ε binds to NUDEL phosphorylated by Cdk5/p35, and in mice deficient in either Lis1 or 14-3-3ε, NUDEL and Lis1 are mislocalized [158], consistent with reduced cytoplasmic dynein function, and providing a potential explanation for the human neuronal migration defects associated with 17p13.3 deletions.

SCAs (spinocerebellar ataxias) are neurodegenerative diseases caused by CAG repeat expansions that insert polyglutamine tracts into various proteins. In SCA1, neurodegeneration is caused by accumulation of a polyCAG form of ataxin-1 [160]. Recently, PKB was found to phosphorylate polyCAG-ataxin-1 and promote its binding to 14-3-3s, thereby stabilizing the protein [161]. At first sight, promoting neurodegeneration in this way seems contrary to the wider cell-survival functions of PKB/14-3-3s. However, this ataxin-1 is a pathological mutant protein, and PKB/14-3-3 regulation of normal ataxin-1 (if such regulation occurs) might have quite different cellular consequences.

The two *Drosophila* 14-3-3 isoforms, 14-3-3ζ (Leonardo) and 14-3-3η, positively regulate Ras-mediated signalling in the development of the compound eye [162], and Leonardo has been found to be important for learning [163–166], being preferentially expressed in the neurons that mediate learning, termed mushroom body cells [166]. Flies with single allelic mutations in *Leonardo* are poor learners, but their mushroom bodies have no obvious anatomical changes, suggesting that the behavioural phenotype may reflect signalling defects, for example in the Ras/Raf pathway [163,164]. The high conservation of 14-3-3ζ and similarly elevated neuronal expression, indicates that these findings in flies may be relevant for understanding behavioural neuroplasticity in vertebrates [163].

CJD (Creutzfeldt–Jakob disease), one of the TSEs (transmissible spongiform encephalopathies), is a rapidly fatal neurodegenerative disease. Despite its extreme rarity, CJD has the highest interest factor (publications per case) of any disease [167]. Scientifically, the fascination stems from the elusive nature of the causative infective agent [168], but awareness was really heightened by the huge impact of BSE (bovine spongiform encephalopathy), which is thought to have been transmitted to 146 people in the U.K. and elsewhere as a new variant form of CJD. The BSE crisis triggered an urgent need to develop diagnostic tests for BSE and CJD, and remarkably certain isoforms of 14-3-3s were identified in the CSF (cerebrospinal fluid) in BSE and CJD cases. Although other illnesses can give a positive 14-3-3 (e.g. [169]) these can normally be excluded by other criteria. Therefore, in an appropriate clinical context, a positive 14-3-3 test is strongly supportive of a diagnosis of CJD (although less linked to the new variant CJD) and a negative result is unusual [170]. 14-3-3 immunoassays have helped agencies such as the National CJD Surveillance Unit in the U.K. to track the course of the disease, and have probably allayed fears of an imminent CJD epidemic. Whether or not 14-3-3s play any active biochemical role in the development of TSEs is uncertain, although, if the 14-3-3s are simply released non-specifically from damaged brain tissue, why are only certain isoforms present in the CSF?

REDUNDANCY, SPECIFICITY AND REGULATION OF 14-3-3 ISOFORMS

The present review has reflected the fact that studies of individual target proteins have dominated the 14-3-3 field, while information about the regulation of the 14-3-3 isoforms is patchy. However, the new findings that 14-3-3 isoforms are differentially regulated in cancers [126–128,131,133,134], the redundant and isoform-specific functions revealed by 14-3-3 gene knockouts in *Drosophila*, yeast and mice [162–166,171], and the compelling new work linking 14-3-3ε to specific molecular defects in MDS [157,158] will surely inspire a new focus on the 14-3-3 proteins themselves.

The inner binding grooves of all 14-3-3 homo- or heterodimers are much the same [13], and all 14-3-3 isoforms can bind to all phosphorylated targets that have been tested *in vitro*. However, there can be marked isoform-specific differences in binding affinities [13]. Perhaps isoform-specific residues at the outer 'lip' of the binding groove affect target access [19,20]. If 14-3-3 isoforms do favour certain phosphorylated targets over others, this means that cells could give kinetically different responses to signals depending on the cellular complement of 14-3-3 isoforms. Moreover, even though 14-3-3s are abundant in many cell types, there are indications that targets within a cell can sometimes compete for binding to 14-3-3s [172,173], and the 'stay green for longer' phenotypes of plants overexpressing 14-3-3s indicate that the concentrations of 14-3-3s can be limiting under some situations [95].

It is also clear that different 14-3-3s isoforms have distinct subcellular and tissue distributions in plants and animals [60,174–183], which has been most thoroughly studied in the brain [184]. 14-3-3s have been identified within mitochondria and chloroplasts [94,185], and even in the extracellular wall of *Chlamydomonas* [186]. How 14-3-3s reach these locations needs to be explored further, since 14-3-3s have no obvious signal sequences.

There are temporal changes in 14-3-3 isoform expression during development and in acute responses to extracellular signals and drugs [187–189]. The 14-3-3s can also be phosphorylated. For example, phosphorylation of Ser^{58} of 14-3-3ζ (or equivalent residues in 14-3-3β or η) by MAPKAPK-2 (MAPK-activated protein kinase 2), or by a sphingosine-activated truncated form of PKCδ prevented dimerization [190–194]. In addition, 14-3-3ζ that has been phosphorylated by casein kinase I cannot bind to phospho-Raf-1 [195].

The existence of multiple 14-3-3 isoforms, which are spatially and temporally regulated, may provide cellular specificity in

responses to the signalling cascades that trigger the phosphorylation of 14-3-3-binding sites on target proteins.

I thank members of the MRC Protein Phosphorylation Unit, and attendees at the Gordon Research Conference on Biology of 14-3-3 Proteins for their helpful corrections and discussion. Thanks to Fred Dyda, Michael Yaffe and Claudia Oecking for Figures 1, 2 and 5 respectively.

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Received 2 September 2003/14 April 2004; accepted 28 May 2004
Published as BJ Immediate Publication 28 May 2004, DOI 10.1042/BJ20031332