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REVIEW ARTICLE

Akt/PKB: one kinase, many modifications

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Akt/PKB, a serine/threonine kinase member of the AGC family of proteins, is involved in the regulation of a plethora of cellular processes triggered by a wide diversity of extracellular signals and is thus considered a key signalling molecule in higher eukaryotes. Deregulation of Akt signalling is associated with a variety of human diseases, revealing Akt-dependent pathways as an attractive target for therapeutic intervention. Since its discovery in the early 1990s, a large body of work has focused on Akt phosphorylation of two residues, Thr^{308} and Ser^{473}, and modification of these two sites has been established as being equivalent to Akt activation. More recently, Akt has been identified as a substrate for many different post-translational modifications, including not only phosphorylation of other residues, but also acetylation, glycosylation, oxidation, ubiquitination and SUMOylation. These modifications could provide additional regulatory steps for fine-tuning Akt function, Akt trafficking within the cell and/or for determining the substrate specificity of this signalling molecule. In the present review, we provide an overview of these different post-translational modifications identified for Akt, focusing on their consequences for this kinase activity.

Key words: Akt, post-translational modification, protein function, protein kinase B, signal transduction.

INTRODUCTION

The serine/threonine protein kinase Akt (also known as protein kinase B or PKB) is a member of the AGC family of protein kinases, conserved from primitive metazoans to humans. Akt is involved in the regulation of many different cellular processes, such as cell growth, survival, proliferation, apoptosis, metabolism and angiogenesis. These processes are mediated by both overlapping as well as specific functions of the three Akt isoforms Akt1, Akt2 and Akt3, which are closely related and widely expressed in mammals [1]. Remarkably, deregulation of Akt signalling is associated with a diversity of human diseases including cardiac hypertrophy, diabetes, neuronal degeneration, vascular disorders and cancer [2–4]. Therefore, Akt-dependent pathways are considered an attractive target for therapeutic intervention. Consequently, a deep understanding of the molecular mechanisms underlying the regulation of this kinase activity becomes of paramount importance [5].

Akt activation downstream of mitogens, cytokines, integrins and other extracellular cues consists of recruitment of Akt to the plasma membrane through its binding to PI3K (phosphoinositide 3-kinase)-generated PtdIns(3,4,5)P_3, imposing a conformational change on Akt and allowing the subsequent phosphorylation of two different residues within the molecule. PDK1 (phosphoinositide-dependent protein kinase 1) phosphorylates Akt at a threonine residue within the kinase domain also known as the activation loop site (positions 308, 309 and 305 in Akt1, Akt2 and Akt3 respectively), whereas mTORC2 [mTOR (mammalian target of rapamycin) complex 2] phosphorylates Akt at a serine residue within the C-terminal regulatory domain referred to as the HM (hydrophobic motif) site (positions 473, 474 and 472 in Akt1, Akt2 and Akt3 respectively), stabilizing its active conformation. Upon full activation by these two phosphorylation events, Akt isoforms exert their action by phosphorylating a wide variety of downstream targets at different subcellular compartments [6,7]. It is worth mentioning that other kinases have been described that can phosphorylate Akt at these two mentioned residues, even in a PI3K-independent manner (reviewed in [8]).

The three Akt isoforms present in mammals are encoded by different genes and share the structural feature of three functional domains: an N-terminal PH (pleckstrin homology) domain which is essential for binding to lipids such as PtdIns(3,4,5)P_3, a central catalytic domain related to protein kinases A and C, and a C-terminal regulatory tail containing the HM phosphorylation site [FXXF(S/T)Y] [9]. The PH and catalytic domains are largely conserved among the three Akt isoforms, whereas the linker region that connects these two domains has the lowest sequence identity among them.

As important as the molecules that stimulate Akt activity are the molecules that shut it off. Among them is the well-known

Abbreviations: Ack1, activated Cdc42 kinase 1; Cdk, cyclin-dependent kinase; CYLD, cylindromatosis; DUB, deubiquitinating enzyme; EGF, epidermal growth factor; GSK3, glycogen synthase kinase 3; HDAC, histone deacetylase; HM, hydrophobic motif; IGF-1, insulin growth factor 1; IR, insulin receptor; IRS1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; mTORC2, mTOR complex 2; MULAN, mitochondrial ubiquitin ligase activator of NF-κB (nuclear factor κB) 1; Myr-Akt, myristoylated Akt; NEDD, neural-precursor-cell-expressed developmentally down-regulated; OGT, O-GlcNAc transferase or UDP-*N*-acetylglucosamine:peptide β-*N*-acetylglucosaminyltransferase; PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent protein kinase 1; PH, pleckstrin homology; PIAS1, protein inhibitor of activated STAT (signal transducer and activator of transcription) 1; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTM, post-translational modification; rictor, rapamycin-insensitive companion of mTOR; ROS, reactive oxygen species; SAE, SUMO-activating enzyme; SENP1, SUMO1/sentrin/SMT3 (suppressor of mif two 3 homologue 1)-specific peptidase 2; SUMO, small ubiquitin-related modifier; TM, turn motif; TRAF6, tumour-necrosis-factor-receptor-associated factor 6; Ub, ubiquitin; Ubl, ubiquitin-like protein.

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tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), which catalyses the conversion of PtdIns(3,4,5)P_3 into PtdIns(4,5)P_2, thus antagonizing PI3K/Akt signalling. PI3K activity is also counteracted by the SHIP [SH2 (Src homology 2)-domain-containing inositol phosphatase] that converts PtdIns(3,4,5)P_3 into PtdIns(3,4)P_2 [10,11]. Although the regulation of Akt by these two lipid phosphatases is indirect, other protein phosphatases such as PP2A (protein phosphatase 2A) and PHLPPs (PH-domain leucine-rich repeat protein phosphatases) exert a direct effect by dephosphorylating Akt Thr^{308} and Ser^{473} respectively [12,13].

Despite a large body of data dealing with the regulation of Akt activity by its specific phosphorylation on Thr^{308} and Ser^{473}, information about the influence of other PTMs (post-translational modifications) on Akt function is less abundant. We are currently used to considering these two phosphorylations as equivalent to Akt activation. Nevertheless, we should be aware that phosphorylation in other residues as well as other types of PTM could add complexity to this equation, by fine-tuning the activity, affecting trafficking within the cell and/or determining the substrate specificity of this signalling molecule.

In the present review, we discuss recent data on different PTMs undergone by Akt, and, when possible, both their regulation of and their consequences for Akt activity, stability and subcellular localization. In general, each of these PTMs has been reported to exert either a positive or a negative impact on Akt function. However, considering that each piece of the described data has been obtained using different cell culture and/or animal models, and also in the context of diverse cellular processes, it is possible to envisage that future research may unravel unforeseen consequences for a given PTM depending on the cellular context and/or the physiological setting, as well as depending on its combination with other PTMs occurring on the same molecule.

Taking into account that each type of modification entails a particular enzymatic mechanism and a different set of experimental tools for its study, each modification is described separately. Nevertheless, for the sake of simplicity, we provide a Table in which the modifications have been grouped by their so-far defined positive or negative consequence for Akt activation and/or activity (Table 1).

AKT PHOSPHORYLATION: BEYOND Thr^{308} AND Ser^{473}

As already mentioned, phosphorylation of Thr^{308} and Ser^{473} is required for full activation of Akt and there is extensive literature dealing with these two PTMs [13–19]. Phosphorylation of other residues, although less explored, has been also proved to be important for Akt folding, stability and activity. Below we describe other less well-known Akt phosphorylations together with the reported impact of these modifications in Akt function (Figure 1).

SERINE/THREONINE PHOSPHORYLATION

Thr^{92}

The Hung laboratory found that the peptidylprolyl *cis–trans* isomerase Pin1, which catalyses pSer/Thr-Pro *cis–trans* isomerization regulating the stability of its substrate proteins, interacts with Akt. They found that depletion of Pin1 reduces Akt protein levels. Furthermore, they defined that phosphorylation of Thr^{92}-Pro and Thr^{450}-Pro on human Akt1 is required for its full interaction with Pin1, allowing conformational changes and proper folding of Akt. Consistently, a double mutation of Akt on these two threonine residues gives rise to an unstable Akt, whereas single mutation of either Thr^{92} or Thr^{450} only marginally affects expression and activation of this kinase [17,20]. Although a lot of information has become available regarding phosphorylation of Thr^{450} (see below) in recent years, the regulatory mechanisms as well as the responsible kinase for Thr^{92} phosphorylation remain elusive.

Ser^{124}

Akt has been reported to be phosphorylated at Ser^{124} in a serum- and PI3K-independent manner [19]. The mutation S124A only slightly affected the activation of Akt by growth factors. Tsichlis's group proposed that phosphorylation of Akt at Ser^{124} renders the protein responsive to subsequent growth factor-induced and PI3K-dependent activation of this kinase [19]. So far, there is no identified regulatory stimulus or kinase involved in this phosphorylation. However, high phosphorylation levels on this residue have been detected in different cell lines [16,21] raising intriguing questions regarding the functional implications of this modification.

Ser^{129}

The Ruzzene laboratory described that Akt Ser^{129} can be phosphorylated by protein kinase CK2 both *in vitro* and *in vivo* and that this phosphorylation is associated with an increase in the catalytic activity of Akt [22]. Consistently, they showed that down-regulation of CK2 catalytic subunit or a mutation of the CK2 target site in Akt correlates with decreased Akt activity. They proposed a mechanism in which maximal activation of Akt would depend on its constitutive phosphorylation by CK2, which gives rise to a pool of Akt molecules susceptible to become fully active upon phosphorylation of Thr^{308} by PDK1 [22]. Further studies from this group showed that this phosphorylation is involved in the interaction of Akt with the chaperone Hsp90 (heat-shock protein 90), preventing Akt dephosphorylation of Thr^{308} [23].

Thr^{312}

Recent work from Gulen et al. [24] has demonstrated that Akt Thr^{312} is modified by phosphorylation, with GSK3α (glycogen synthase kinase 3α) being the kinase responsible for this modification. Considering that this residue is located within the substrate-binding site of Akt, they postulated that phosphorylation of this residue could interfere with the interaction of Akt with its substrates, therefore preventing Akt kinase function.

Thr^{450}

Phosphorylation of Akt at Thr^{450}, also named the TM (turn motif) site, was reported in 1996 [16]. Similar to phosphorylation at Ser^{124}, Akt was reported to be phosphorylated at Thr^{450} in a PI3K- and serum-independent fashion [19]. Also, as mentioned for Ser^{124}, the T450A mutation only slightly inhibits the activation of Akt by growth factors, so this phosphorylation was proposed to be constitutive and to take part in an initial step before Akt full activation [21,25–27].

In 2008, two independent laboratories reported that phosphorylation of Akt at Thr^{450} controls Akt protein folding and maturation [28,29]. Since Thr^{450} is followed by a proline residue, a screening of ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase), p38, Cdk

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Table 1 Different post-translational modifications of Akt, their consequence for Akt activation and/or activity and the identified modifier enzymes

In the Consequence column, A refers to a positive (activating) effect of the specific PTM on Akt function, I refers to a negative (inhibitory) effect of the specific PTM on Akt function, S refers to a defined stabilizing effect of the specific PTM on Akt, and D refers to a defined destabilizing effect of the specific PTM on Akt. The Akt2; Akt3 column refers to the corresponding residues in the different Akt isoforms. An asterisk in this column indicates that, whereas the residue is conserved, the consensus required for the proposed modification is not.

Amino acid	Residue (Akt1)	PTM	Residue (Akt2; Akt3)	Consequence	Enzyme(s) involved
Lysine	8	Lys[63] ubiquitination	8; 8	A	TRAF6
Lysine	14	Lys[63] ubiquitination	14; 14	A	TRAF6
Serine	124	Phosphorylation	126; 123*	A	Unknown
Serine	129	Phosphorylation	131; –	A	CK2
Tyrosine	176	Phosphorylation	178; 174	A	ACK1
Lysine	276	SUMOylation	277; 273	A	PIAS1; SENP1
Threonine	308	Phosphorylation	309; 305	A	PDK1
Tyrosine	315	Phosphorylation	316; 312	A	Src; PTK6
Tyrosine	326	Phosphorylation	327; 323	A	Src; PTK7
Threonine	430	O-GlcNAc	431; 427	A	OGT; OGA
Serine	473	Phosphorylation	474; 472	A	mTORC2
Tyrosine	474	Phosphorylation	475; 473	A	Unknown
Serine	477	Phosphorylation	478; 476	A	Cdk/cyclin A
Threonine	479	Phosphorylation	–; –	A	Cdk/cyclin A
Threonine	479	O-GlcNAc	–; –	A	OGT; OGA
Threonine	92	Phosphorylation	Ser[92]; 91	S	Unknown
Threonine	450	Phosphorylation	451; 447	S	mTORC2
Lysine	14	Acetylation	14; 14	I	SIRT1
Lysine	20	Acetylation	20; 20	I	SIRT1
Threonine	305	O-GlcNAc	306; 302	I	OGT
Threonine	312	Phosphorylation	313; 309	I	GSK3α
Threonine	312	O-GlcNAc	313; 309	I	OGT
Serine	473	O-GlcNAc	474; 472	I	OGT; OGA
Cysteine	–	Oxidation	124; –	I	Unknown
Lysine	284	Lys[48] ubiquitination	285; 281	D	MULAN

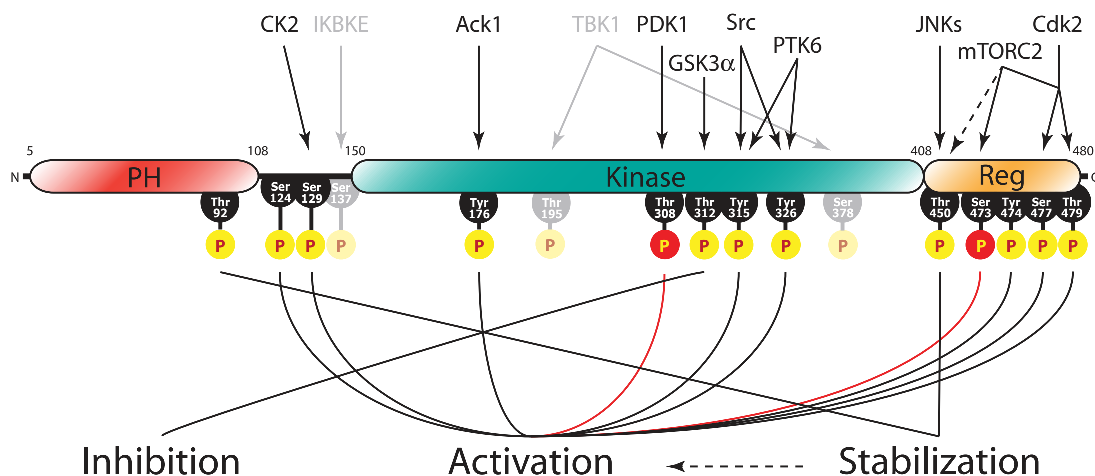

Figure 1 Less studied Akt phosphorylations

The scheme shows the structure of Akt containing the three functional domains: PH domain (PH), catalytic domain (Kinase) and C-terminal regulatory domain (Reg), and different phosphorylation target residues. The kinases responsible for these modifications as well as the known consequences for Akt stability and activity are also depicted (see the text for further details). The two phosphorylations that are usually considered equivalent to Akt activation are shown in red (Thr[308] and Ser[473]), whereas a few identified phosphorylations that are not described in the text are shown in grey.

(cyclin-dependent kinase), GSK3, CK2 and other proline-directed protein kinases was conducted, but the results indicated that such kinases had no significant effect on Akt phosphorylation at this motif. Furthermore, both groups demonstrated that depletion of mTORC2 constituents [rictor (rapamycin-insensitive companion of mTOR), sin1, mTOR] affected Akt phosphorylation at Ser[473] and Thr[450]. Using *in vitro* kinase assays, Ikenoue et al. [28] demonstrated that mTORC2 phosphorylates Ser[473], but not Thr[450], in Akt; suggesting that mTORC2 may not be the kinase directly responsible for the TM phosphorylation [28]. On the other hand, the Jacinto laboratory suggested that mTORC2 phosphorylates Akt directly at Thr[450], coupling translation with protein folding and maturation [29]. They showed that mTORC2 co-localizes with polysomes, stably interacting with a large ribosomal subunit protein present at the tunnel exit. At this location, mTORC2 phosphorylates Akt polypeptides

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as they emerge from the ribosome tunnel, specifically on the TM Thr[450]. This constitutive phosphorylation inhibits co-translational ubiquitination and subsequent degradation. In this way, mTORC2, which is mainly known to mediate Akt activation by phosphorylating the HM site (Ser[473] on Akt1), also controls the co-translational folding of nascent cytosolic Akt polypeptides. It is worth noting that phosphorylation of the HM site upon signalling-triggered membrane recruitment of Akt occurs only post-translationally, due to the fact that this residue remains buried inside the ribosomal tunnel until the polypeptide is fully released [30]. This may in part explain how mTORC2 independently controls both phosphorylation sites.

Despite the fact that the above described studies have indicated pThr[450] to be a constitutive modification of Akt, Shao et al. [31] and Wei et al. [32] reported that phosphorylation of Akt at this residue can actually be regulated by JNKs upon hypoxia, and suggested that this phosphorylation primes Akt for subsequent phosphorylation by PDK1.

Ser[477] and Thr[479]

Very recently, Wei's laboratory found that Akt activation, as evaluated by Ser[473] phosphorylation, fluctuates across the cell cycle, mirroring the expression pattern of cyclin A2, the predominant mammalian cyclin A isoform. These results, together with the observation that depletion of either cyclin A or Cdk2, reduced Akt phosphorylation and cellular activity, led them to examine Akt as a Cdk/cyclin A target. They identified four cyclin A-binding motifs within Akt and unravelled two new phosphorylation events occurring at Akt1's extreme C-terminal residues Ser[477] and Thr[479]. Phosphorylation of these two residues triggers Akt1 activation either through enhancing its association with mTORC2 to promote pSer[473] or by functionally compensating for pSer[473] to lock Akt1 in its active conformation. With respect to the clinical relevance of these findings, they found a positive correlation between Akt1-pSer[477]/pThr[479] and Akt1-pSer[473] in breast cancer samples as well as in breast-cancer-derived cell lines. These and other results led the authors to suggest a physiological role for cyclin A2 in controlling Akt activation and thus its pro-survival and oncogenic functions [33,34].

TYROSINE PHOSPHORYLATION

Tyr[176]

Work from the Mahajan laboratory described how growth factor binding to RTK (receptor tyrosine kinase) could lead to activation of Ack1 [activated Cdc42 kinase 1; also known as ACK or TNK2 (tyrosine kinase, non-receptor 2)] [35]. This kinase can directly phosphorylate Akt kinase domain at Tyr[176], leading to PtdIns(3,4,5)P_3-independent recruitment of Akt to the plasma membrane and enhancing further phosphorylation at Thr[308] and Ser[473], even upon PI3K inhibition. Interestingly, transgenic mice expressing activated Ack1 in the prostate display increased levels of Akt Tyr[176] phosphorylation and develop prostatic intraepithelial neoplasia. Finally, by analysing annotated breast tumour samples, they found a positive correlation between Ack1 activation, Tyr[176] Akt phosphorylation and the severity of the disease. In this line, Ack1 activation and Akt pTyr[176] inversely correlate with survival of breast cancer patients [35].

Tyr[315] and Tyr[326]

Since PI3K and Akt activity had been proved to be regulated by Src and other tyrosine kinases [36,37], Qiu's laboratory analysed whether Akt activity can be modulated by tyrosine phosphorylation [38]. They found that Akt is a direct substrate of Src both *in vivo* and *in vitro* and that Tyr phosphorylation of Akt is critical for its full activation by growth factors. Interestingly, they identified two residues near the activation loop of Akt which are fundamental for its activation: Tyr[315] and Tyr[326]. Replacing these residues with phenylalanine blocked Akt kinase activity upon growth factor stimulation [38]. Later on, they showed that interaction of Akt and Src, phosphorylation of Akt Tyr[315] by Src, as well as Akt full activation all depend on a conserved proline-rich motif (PXXP) in the C-terminal regulatory region of Akt [39]. In 2009, Luan et al. [40] described that, upon insulin stimulation, a signalling complex is formed by the crucial role of β-arrestin-2 that scaffolds Akt and Src to the activated IR (insulin receptor). Proper expression of β-arrestin-2 and formation of this complex is required for accurate insulin signalling as well as whole-body insulin action [40]. Finally, Tyner's group found that PTK6 (protein tyrosine kinase 6), a non-myristoylated Src-related intracellular tyrosine kinase, is also capable of phosphorylating Akt at Tyr[315] and Tyr[326] [41].

Tyr[474]

In addition to the findings described above, Pearson's group also proved that Akt tyrosine phosphorylation is an important step for Akt regulation [21]. They showed that activation of Akt by either pervanadate, a tyrosine phosphatase inhibitor, or serum is linked to phosphorylation of Akt at Tyr[474]. On the basis of direct chemical identification of phosphorylation sites by *in vivo* labelling followed by HPLC profiling of tryptic digestions, they point to this residue as the major site of tyrosine phosphorylation within Akt. Replacing Tyr[474] with phenylalanine drastically inhibited Akt activity, even to the same extent as replacing Ser[473] with alanine. Nevertheless, the Y474F mutant showed only marginally lowered Ser[473] phosphorylation. In contrast, mutating Ser[473] to alanine not only severely decreased phosphorylation of Thr[308], as is already known, but also of Tyr[474]. Thus phosphorylation of both residues, Ser[473] and Tyr[474], is required for Akt full activation and may co-operate in the regulation of Akt's kinase domain [21]. Considering the close proximity between these two residues, it is worth mentioning that the authors were not able to find any peptide doubly phosphorylated. It is possible to speculate that phosphorylation on these two sites may be finely regulated in order to allow a sequential phosphorylation/dephosphorylation cycle. Interestingly, the authors highlighted the fact that corresponding tyrosine residues are also present in other members of the AGC kinase family. Furthermore, it has also been reported that this residue is important for PRK2 (protein kinase C-related protein kinase 2) activity [42], suggesting its involvement in a common regulatory mechanism for this family of mitogen-activated protein kinases.

Proteomics and Akt phosphorylation

Several Akt residues, such as Ser[122], Ser[137], Thr[146], Thr[195] and Ser[378] among others, have been identified as phosphorylation sites by proteomic approaches. However, the occurrence of these phosphorylation events in intact cells and/or their physiological function remain to be elucidated (see PhosphoSitePlus at http://www.phosphosite.org for more information) [43,44].

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BEYOND AKT PHOSPHORYLATION

Acetylation

Reversible acetylation of lysine residues by the action of HATs (histone acetyltransferases) and HDACs (histone deacetylases) is a PTM that neutralizes the positive charge of this amino acid and regulates the activity of histones as well as of many other nuclear and cytoplasmic proteins. Proteome-wide analysis ('lysine acetylome') has been a powerful tool to identify that lysine acetylation targets large macromolecular complexes involved in diverse cellular processes such as chromatin remodelling, cell cycle, splicing, nucleocytoplasmic transport and cytoskeletal remodelling [45]. The physiopathological relevance of this PTM is demonstrated by the fact that HDACs are important drug targets in cancer and neurodegenerative disease, and a large number of clinical trials are currently being conducted.

Considering that: (i) SIRT1, a member of the sirtuin family of type III HDACs is present in the nucleus and in the cytoplasm, (ii) SIRT1 regulates many of the cellular process that are known to be regulated by Akt, and (iii) SIRT1 activators have been used as therapeutic agents for metabolic disorders characterized by defective Akt signalling such as Type 2 diabetes [46], the Gupta laboratory explored Akt acetylation, finding not only that this kinase is indeed reversibly acetylated but also that its deacetylation, mediated by SIRT1, is required for Akt binding to PtdIns(3,4,5)P_3 and its translocation to the plasma membrane, as the initial step during Akt activation [47]. It is also of remarkable interest that PDK1 recruitment to the plasma membrane is regulated by SIRT1-mediated deacetylation in a similar manner, co-ordinating the translocation of Akt and one of its main activators to the cellular location where activation takes place [47]. For both proteins, Akt and PDK1, the acetylated lysine residues were mapped within the PH domains (Figure 2). Furthermore, the authors took advantage of the prostate cancer cell line PC3, in which proliferation and survival are Akt-dependent, to demonstrate that impairing Akt deacetylation by depleting endogenous SIRT-1, reduces Akt phosphorylation and increases cell death, whereas overexpressing SIRT-1 stimulates cell proliferation. As evidence for the pathobiological relevance of Akt acetylation, they showed that, whereas HeLa cells stably transfected with wild-type Akt promote large tumour formation when injected into nude mice, HeLa cells stably transfected with an Akt mutant that mimics constitutive acetylation (AktK20Q) were unable to do so.

Oxidation

ROS (reactive oxygen species), historically considered as damaging agents, are currently known as important physiological regulators of intracellular signalling pathways. Specific effects of ROS are mediated by the covalent modification of particular cysteine residues within redox-sensitive proteins. Oxidative PTM of cysteine residues regulates protein structure and function. Consistently, alteration of redox signalling contributes to a variety of human pathological conditions [48,49].

The Furdui laboratory has made interesting contributions with respect to isoform-specific PTMs of Akt, finding not only that this kinase is modified by oxidation, but also that this modification occurs exclusively on Akt2 [50]. This reversible event takes place upon induction of ROS by different extracellular cues such as PDGF (platelet-derived growth factor) and involves Cys^{124} located within the linker region between the PH and kinase domains of Akt2, which are not conserved in either Akt1 or Akt3. Besides the linker region, the PH and kinase domains each present two redox-sensitive sites, Cys^{60} and Cys^{77}, and Cys^{297} and Cys^{311} respectively (Figure 2). PDGF-induced oxidation triggers the formation of Cys^{124}–Cys^{297}/Cys^{311} intramolecular disulfide bonds inhibiting the activity of Akt2 [50]. Akt2-knockout MEFs (mouse embryonic fibroblasts) transduced with wild-type or C124S mutant Akt2 were used as a model system to show the involvement of Akt2 oxidation in cell migration and cell cycle progression [51]. Whether disulfide bond formation alters Akt subcellular localization, triggers Akt2 degradation or is subsequently reversed to recycle Akt for further activation cycles are still open questions. In any case, the authors propose that Akt oxidation may fine-tune the timing and/or amplitude of Akt2-dependent signalling [51].

Glycosylation

Protein O-GlcNAcylation is a dynamic and inducible PTM consisting of the attachment of a single O-GlcNAc (β-D-*N*-acetylglucosamine) monosaccharide to serine or threonine residues of nuclear and cytoplasmic proteins. Thus O-GlcNAcylation can compete with phosphorylation at the same site, and the interplay between O-GlcNAc and O-phosphate has been implicated in the regulation of protein function as well as in the aetiology of several human diseases, such as diabetes and cancer. In other cases, a competitive occupancy by either O-GlcNAc or phosphate at proximal sites has been attributed to the bulky size of the O-GlcNAc residue, the negative charge of the phosphate moiety or the induction of conformational changes in the protein by either modification. On other proteins, O-GlcNAcylation and phosphorylation occur at distant sites, coexisting on the same molecule or even taking place on different subpopulations of molecules (reviewed in [52]).

The addition of O-GlcNAc to proteins is catalysed by OGT (O-GlcNAc transferase or UDP-*N*-acetylglucosamine:peptide β-*N*-acetylglucosaminyltransferase), which is enriched in the nucleus, but also present in the cytosol. Conversely, the removal of O-GlcNAc from proteins is catalysed by OGA (O-GlcNAcase or β-*N*-acetylglucosaminidase) that is highly concentrated in the cytosol, but also present in the nucleus of every cell type (for reviews, see [52,53]).

In addition to phosphorylation and other PTMs, O-GlcNAcylation of both Akt1 and Akt2 was reported several years ago [54,55]. This modification also affects other components of the PI3K/Akt signalling axis.

Upon insulin binding to its receptor, activated PI3K drives the translocation of OGT from the nucleus to the plasma membrane through its binding to PtdIns(3,4,5)P_3. This PtdIns(3,4,5)P_3-mediated recruitment allows OGT association with the IR, leading to OGT phosphorylation at tyrosine residues and enhancing OGT activity [56]. This redistribution and activation of OGT allows for the modification by O-GlcNAcylation of downstream targets in the pathway, including IR-β, IRS1 (insulin receptor substrate 1) [57,58], Akt and PDK1 [56,59,60]. OGT activity attenuates insulin signalling by inhibiting phosphorylation at Thr^{308} of Akt and promoting IRS1 phosphorylation at Ser^{307} and Ser^{632}/Ser^{635} [60]. Recent work from Wang et al. [61] employed an antibody-based strategy to detect glycosylation and site-specific phosphorylation of endogenous Akt combined with mass spectrometry, molecular dynamics simulation and site-directed mutagenesis to confirm that Akt undergoes O-GlcNAcylation at Thr^{305} and Thr^{312} (Figure 2). Furthermore, they postulated that these modifications are likely to inhibit Akt phosphorylation at Thr^{308} by disrupting the interaction between Akt and PDK1, in turn compromising Akt cellular functions [61]. These authors also identified Akt GlcNAcylation at Ser^{126} and Ser^{129} by mass

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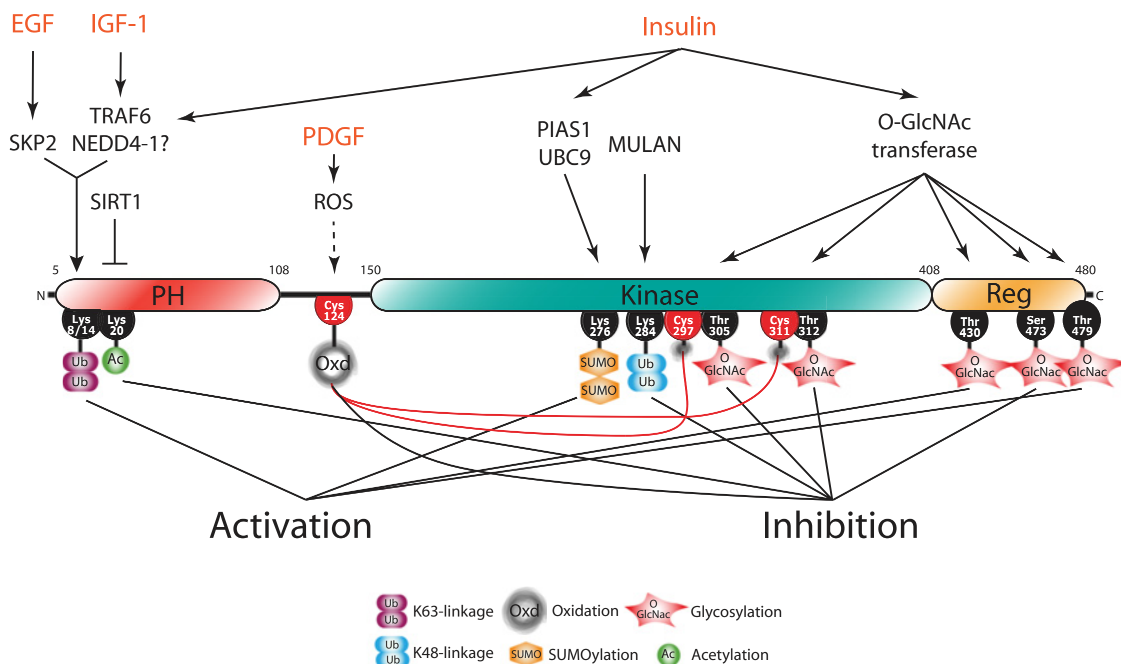

Figure 2 Akt is subject to different PTMs

The scheme shows Akt structure containing the three functional domains, as indicated in Figure 1, and the target residues for glycosylation (O-GlcNAc), acetylation (Ac), oxidation (Oxd), which is exclusive to Akt2, ubiquitination (Ub) and SUMOylation (SUMO). The extracellular stimuli, the enzymes identified and the consequences for Akt activity are also depicted (see the text for further details).

spectrometry. However, they did not analyse this modification further at these two sites.

It is worth noting that, according to Gulen et al. [24], described above in the 'Akt phosphorylation' section, Akt Thr^{312} is also a phosphorylation target. Whether this phosphorylation could also disrupt Akt interaction with PDK1 inhibiting Akt phosphorylation at Thr^{308}, as suggested for Thr^{312} GlcNAcylation, remains to be studied. Thus Akt can undergo either phosphorylation or O-GlcNAcylation at the same residue (Thr^{312}), and both modifications exert a similar effect on Akt activity. Additional experimental work would be necessary to validate the postulated mechanisms by which these two PTMs diminish Akt function.

Remarkably, OGT, together with two of its substrates, PDK1 and Akt, are all recruited to the plasma membrane in a signalling-induced manner. At this cellular compartment, both activation and attenuation of the signalling cascade takes place. How exactly this complex interplay is timely and precisely regulated to assure proper activation and termination of the pathway is still an open question.

An example of O-GlcNAcylation and phosphorylation occurring at the same site but exerting opposite effects on protein function has also been described for Akt. In particular, work from Kang et al. [62] demonstrated that Akt1 undergoes glycosylation on Ser^{473}. Treatment of murine pancreatic β-cells with glucosamine diminishes Akt phosphorylation on Ser^{473} and increases Akt O-GlcNAcylation at the same residue, correlating with enhanced apoptosis of these cells [62].

Another example of Akt O-GlcNAcylation has been described recently by Heath et al. [63]. This work showed higher levels of O-GlcNAcylation in a constitutively active version of Akt than in the wild-type kinase. After predicting putative O-GlcNAc modification sites by bioinformatic tools, the authors found that replacing either Thr^{430} or Thr^{479} with alanine within the constitutively active Akt decreases O-GlcNAcylation, and this correlates with reduced phosphorylation levels at Ser^{473} but not at Thr^{308}. These results led them to propose a positive correlation between Akt O-GlcNAcylation at Thr^{430}/Thr^{479} and its phosphorylation at Ser^{473}. Both mutants (T430A or T479A) showed a decreased binding to rictor, a component of mTORC2, providing a mechanistic explanation for the reduced phosphorylation at Ser^{473}. As discussed above, Thr^{479} has been described also as a phosphorylation site crucial for Akt activation [33]. It is worth mentioning that the sole use of the Thr→Ala mutant tool is not sufficient to conclude about the direct involvement of a given PTM in protein function. This is particularly evident in this case, in which Thr^{479} has been identified as a target for different modifications. It is not possible to distinguish whether O-GlcNAcylation is required for Akt activation or whether the lack of phosphorylation on Thr^{479} is affecting Akt activation and consequently its O-GlcNAcylation.

Modification by peptide conjugation

So far, we focused on the PTM of Akt by the addition of different small chemical substituents. However, substrate proteins may also be modified by the covalent and reversible attachment of another polypeptide such as Ub (ubiquitin) or different members of the Ub family referred to as Ubls (ubiquitin-like proteins). The eukaryotic Ub family comprises approximately 20 proteins, the most studied besides Ub being SUMO (small ubiquitin-related modifier) and NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) that regulate a wide variety of cellular

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processes, including chromatin organization, transcription, nuclear transport, translation, proteolysis, autophagy, trafficking and signal transduction, among others.

Indeed, Akt is modified by Ub and Ubl conjugation and different laboratories have begun to propose that these modifications could play an important role determining the specificity of the cellular response to different Akt-activating extracellular cues.

Ubiquitination

The ubiquitination pathway involves three main steps, activation, conjugation and ligation, performed by Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s) and Ub ligases (E3s) respectively. The outcome of this stepwise reaction is the covalent attachment of the Ub C-terminal end to a lysine residue on the target protein by an isopeptide bond. DUBs (deubiquitinating enzymes) cleave this bond removing Ub from the substrate, adding another step of control and flexibility to this PTM.

Broadly speaking, there are two types of ubiquitination: degradative and non-degradative. It is well established that Lys^{48}-linked Ub chains can target proteins for degradation by the 26S proteasome, the major cytosolic proteolytic system in eukaryotes [64]. However, it has become increasingly clear that conjugation of Ub to proteins can alter their properties and function without labelling them for degradation. Lys^{63}-linked Ub chains are the best characterized in this latter group and have been involved in different processes such as signalling activation and protein trafficking.

In the last few years, several reports have described both types of ubiquitination on Akt and have explored the implication of these modifications at different steps along the activation/deactivation cycle of this kinase. In this section, we aim to summarize the various postulated mechanisms that propose ubiquitination as a crucial PTM for the regulation of Akt activity (Figure 3).

Degradative ubiquitination. For many years, protein stability has been considered an important regulatory step for Akt activity [65]. However, only recently have the mechanisms of Akt ubiquitination received closer attention.

Different Ub E3 ligases have been described as responsible for incorporating Lys^{48}-linked polyUb chains into Akt protein. First the E3 ligases BRCA1 (breast cancer early-onset 1) [66] and CHIP [C-terminus of the Hsc (heat-shock cognate) 70-interacting protein] [67,68] and later on TTC3 (tetratricopeptide repeat domain 3) [69] and MULAN [mitochondrial ubiquitin ligase activator of NF-κB (nuclear factor κB) 1] [70] have been reported. Each one of these E3 ligases has been associated with Akt ubiquitination in different cell lines or in response to different stimuli. Nevertheless, they all function through very similar mechanisms. Overexpression of these E3 ligases increases Akt ubiquitination and Akt proteasome-dependent degradation, consequently decreasing Akt protein levels. In contrast, knockdown of any of these E3 ligases prevents Akt ubiquitination and degradation, increasing Akt stability. E3 ligases preferentially bind to pAkt (phosphorylated at Thr^{308} and Ser^{473}) promoting specific degradation of activated Akt, most likely within the nucleus. A very elegant study from the An laboratory suggested that Lys^{284} in Akt is a specific residue for MULAN-mediated Akt ubiquitination [70]. This work also showed that MULAN E3 ligase overexpression suppresses Akt-induced cell growth and cell migration more efficiently when triggered by wild-type Akt than by a mutant Akt in which Lys^{284} has been replaced by arginine (K284R mutant). Altogether, these results point to stimuli-triggered Akt Lys^{48}-linked ubiquitination as a way for attenuating endogenous Akt signalling or for terminating its activation. Suppression of E3 ligase expression and/or the proteasomal degradation pathway leads to an excess of active Akt with consequences for cell growth, migration and proliferation and a strong implication in cancer disease.

As mentioned above, Ub-dependent Akt degradation is prevented by the translation-coupled phosphorylation event at Thr^{450} mediated by mTORC2 in a growth-factor-independent manner [29]. Lack of this phosphorylation renders Akt unstable due to an improperly folded C-terminal domain.

Non-degradative ubiquitination. In the last few years, a role for non-degradative Lys^{63}-linked polyubiquitination in Akt activation has been established. Pioneering work from the laboratory of Lin Hui-Kuan demonstrated that an increase in Akt ubiquitination occurs in response to different Akt-activating stimuli, such as growth factors or cytokines [71]. Interestingly this polyubiquitination is non-degradative, affecting its activation but not the stability of this kinase. Furthermore, they found TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) as a direct E3 Ub ligase acting on Akt in response to these stimuli, promoting Akt recruitment to the plasma membrane and thus its subsequent activation. Although the requirement of the interaction of the PH domain with PtdIns(3,4,5)P_3 for the stabilization of Akt at the plasma membrane has been known for a long time, the mechanism whereby Akt was recruited to this cellular compartment has remained elusive. Taking into account several results together with the fact that the E3 ligase TRAF6 is unable to further activate a modified Akt that has already been localized to the membrane (Myr-Akt or myristoylated Akt), the authors conclude that the ubiquitination of the Akt PH domain is necessary for its transport from the cytoplasm to the plasma membrane, and stimulates Akt phosphorylation at Thr^{308} and Ser^{473} [71].

This same laboratory subsequently reported that different E3 Ub ligases could mediate Akt ubiquitination in response to different extracellular stimuli known to activate the Akt signalling pathway [72]. Whereas, as demonstrated above, the E3 ligase TRAF6 is required for complete activation of Akt upon stimulation with IGF-1 (insulin growth factor 1), the E3 ligase complex Skp2-SCF mediates Akt ubiquitination in response to EGF (epidermal growth factor) through ErbB receptors. In the latter scenario, deficiencies in SKP2 cause inhibition of Akt activation, glucose uptake and glycolysis, and, more importantly, breast cancer progression in several tumour models tested [72].

More recently, the Fan laboratory thoroughly studied how Akt ubiquitination by the E3 ligase NEDD4-1 could regulate at least two different steps along the activation of this kinase [73]. They showed that NEDD4-1$^{-/-}$ MEFs are severely impaired to activate Akt upon IGF-1 or insulin stimulation, whereas activation of this kinase is unaffected upon EGF or serum stimulation. These results suggest that NEDD4-1 is specifically needed for the activation of the pathway in response to certain extracellular signals.

Taking into account that: (i) blocking Akt recruitment to the plasma membrane with an specific inhibitor (MK2206) prevented Akt ubiquitination by NEDD4-1, and (ii) Myr-Akt, which is directed to the plasma membrane by the addition of a myristoyl group, is more efficiently ubiquitinated by NEDD4-1, the authors concluded that Akt ubiquitination by NEDD4-1 depends on membrane recruitment of Akt and speculated that this modification may stabilize Akt at this location for its consequent phosphorylation/activation [73]. On the other hand, using cell fractionation and microscopy-based approaches the authors showed that NEDD4-1-mediated Akt ubiquitination regulates its trafficking to nuclear/perinuclear compartments along its activation [73]. These results provide new insights into

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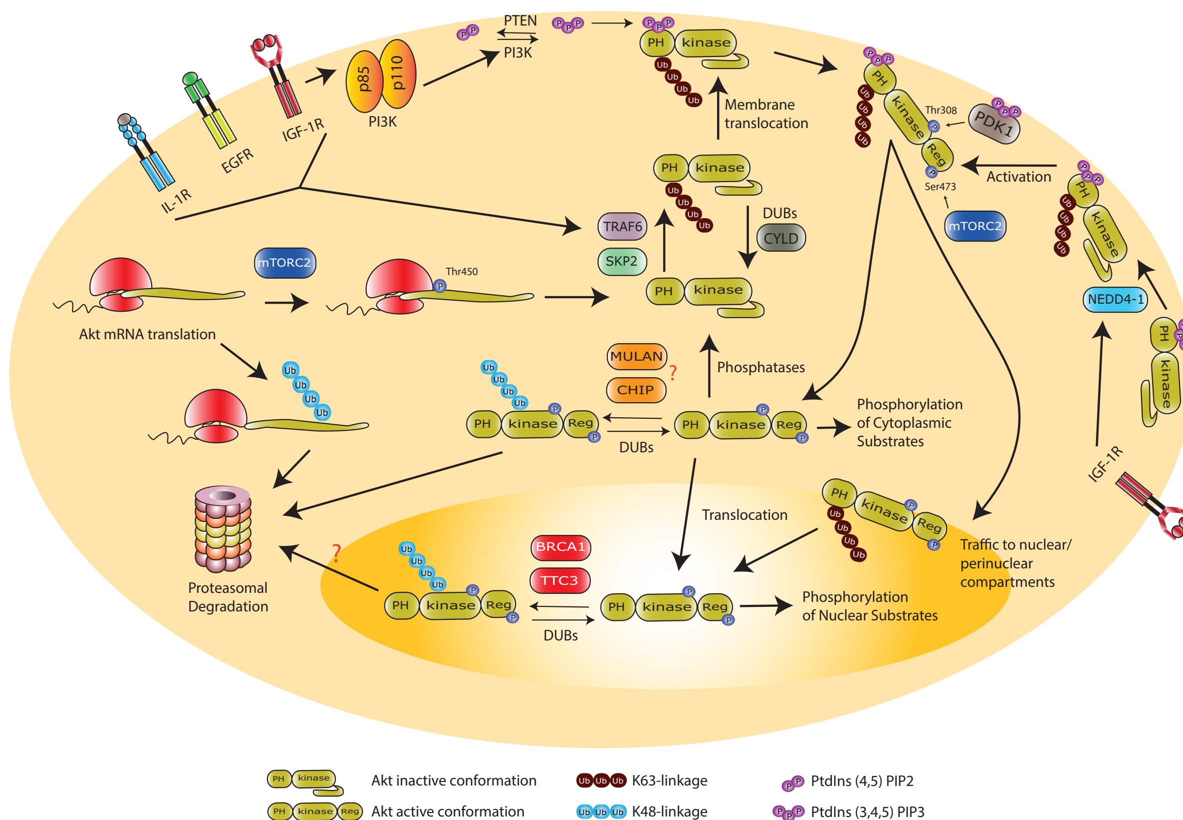

Figure 3 Akt regulation by ubiquitin conjugation

Akt is phosphorylated on Thr^{450} by mTORC2 in a co-translational manner. This phosphorylation stabilizes the nascent protein, preventing its further Ub-mediated proteasomal degradation. Different mechanisms have been proposed for the ubiquitination-mediated Akt activation. On one hand, under different specific stimuli, E3 Ub ligases TRAF6 or SKP2 catalyse Akt Lys^{63}-linked polyubiquitination, promoting Akt recruitment to the plasma membrane. Akt is stabilized at this location by PH-domain-mediated PtdIns(3,4,5)P_3 binding. This results in the activation of Akt through its phosphorylation at Thr^{308} and Ser^{473} by PDK1 and mTORC2 respectively. On the other hand, upon certain stimuli, Akt is recruited to the plasma membrane in a PH-dependent manner. There, NEDD4 catalyses Akt Lys^{63}-linked polyubiquitination, stabilizing Akt at this location and leading to Akt activation as mentioned above. Activated Akt translocates to perinuclear/nuclear compartments, a process that has been proposed to be enhanced by Akt Lys^{63} ubiquitination. Either in the nucleus or in the cytoplasm, Akt phosphorylates specific substrates. Different Ub-mediated mechanisms have been proposed to attenuate or shut off Akt signalling. DUBs such as CYLD prevent Akt Lys^{63}-linked ubiquitination impairing its activation. Additionally, E3 Ub ligases specifically recognize active Akt (pAkt), catalysing Lys^{48}-linked poly-ubiquitination and labelling this kinase for proteasomal degradation. Red question marks indicate that the subcellular location where these processes take place is still uncertain.

this poorly explored regulatory step of Akt and may become relevant for the critical role of active nuclear Akt in tumorigenesis.

Deubiquitinating enzymes (DUBs). As described above, there is a growing body of data about Akt ubiquitination and its implications for Akt activity. In comparison, little is known about the reverse reaction, the deubiquitination process. Lim et al. [74] identified CYLD (cylindromatosis) as a DUB that interacts directly with and deubiquitinates Akt. They showed that this protease specifically cleaves Lys^{63}-linked polyUb chains on Akt, reducing Akt activation upon extracellular stimulation. Data from this work [74] and from the Lin Hui-Kuan laboratory [75] show a higher level of Akt basal activity in CYLD-deficient cells, suggesting that, in wild-type cells, CYLD interacts with Akt, maintaining low levels of ubiquitinated Akt isoforms and therefore low levels of Akt activity. Studying the relevance of this interaction in cancer disease, they found an inverse correlation between CYLD protein levels and Akt activation, evaluated by Ser^{473} phosphorylation, in human prostate cancer samples.

Altogether, these results propose the processes of ubiquitination/deubiquitination of Akt as a fine-tuning regulation of this kinase that affects cellular homoeostasis and tumorigenesis.

SUMOylation

The steps involved in the SUMO conjugation pathway resemble those of the Ub pathway involving an activating enzyme [the heterodimer SAE (SUMO-activating enzyme) I–SAE II in mammals], a conjugating enzyme (Ubc9) and different ligases [76]. In the Ub pathway, substrate specificity is usually provided by E3 ligases, which typically contain substrate-binding sites [77,78]. In the SUMO pathway, the sole E2 enzyme (Ubc9) usually binds the substrate directly, but the SUMO E3 ligases seem to contribute to substrate specificity.

In 2013, Wang's group and our laboratory almost simultaneously reported SUMO conjugation as a novel PTM of Akt [79,80]. Performing point mutations of putative SUMOylation consensus motifs, we both defined Lys^{276} as a major SUMOylation target site within this protein. These results were recently confirmed by mass spectrometry analysis carried out by the Vertegaal laboratory [81]. We show further that either an Akt mutant at the acceptor lysine residue (K276R) or at the SUMO consensus motif (D274N, E278Q) completely abrogates Akt activity. Data from the Wang laboratory [79] and from ours (Guillermo Risso and Anabella Srebrow, unpublished work) identified PIAS1 [protein inhibitor of activated STAT (signal transducer and activator of transcription) 1] as an E3 SUMO ligase

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that specifically interacts with Akt enhancing its SUMOylation, and SENP1 [SUMO1/sentrin/SMT3 (suppressor of mif two 3 homologue 1)-specific peptidase 2] as the key protease for Akt SUMO deconjugation. In agreement with these results, Wang and colleagues found that overexpression of PIAS1 and SUMO1 increase Akt activity, whereas overexpression of SENP1 reduces it [79].

Moreover, both studies described high levels of SUMOylation in a hyperactive variant of this kinase (Akt E17K) found in several human tumours. This hyperactivity of Akt E17K is compromised by the decrease in its SUMOylation levels. Furthermore, Wang's group demonstrated that decreasing Akt E17K SUMO conjugation levels impairs its tumorigenic activity in a xenograft model in nude mice [79].

Despite the fact these results show that changes in Akt SUMOylation levels severely affect Akt activity, further investigation will be required before we completely understand the precise mechanism involved in this regulatory step, including its response to different stimuli, the subcellular location where it takes place and the exact stage during the life of this kinase that is being modulated by SUMO conjugation. The best established parameter of Akt activation within living cells has been its phosphorylation levels at Thr^{308} and Ser^{473}. As for every member of the AGC kinase family, phosphorylation of these two residues is necessary and sufficient for complete activation of Akt1. Most of the PTMs discussed in the present review display a clear connection with these two Akt phosphorylations, consequently altering the activation/inactivation balance of this kinase. So far, SUMO conjugation does not seem to fit into the same category. Although more evidence is necessary, it is worth summarizing some of the experiments performed by our laboratory that tackled this question [80], suggesting that Thr^{308} and Ser^{473} phosphorylation and SUMO conjugation to Akt could be considered as independent events: (i) a double mutant Akt1 (K276R/K301R) displays lower SUMOylation, but equal phosphorylation levels, compared to wild type Akt, at least under steady-state conditions; (ii) pharmacological blockade of the PI3K/Akt pathway does not alter Akt SUMO conjugation levels; (iii) stimulation of the SUMO pathway by Ubc9 overexpression does not alter Akt phosphorylation; (iv) point mutations that abolish or mimic Akt phosphorylation do not interfere with its SUMOylation; and (v) hyperphosphorylation detected on the mutant Akt E17K, which is also hyper-SUMOylated, is not affected by a mutation that dramatically decreases its SUMOylation levels. In agreement, work from Wang's group showed that the knockdown of Ubc9 affects Akt activity in response to insulin stimulation without altering its phosphorylation enhancement [79]. These results together with those showing that downstream activities of Akt are drastically impaired in the Akt SUMO-deficient mutant, indicate that, whereas SUMO conjugation does not seem to regulate the activation of this kinase, it might regulate subsequent processes such as its cellular trafficking or its interaction with downstream targets.

More recently, another paper was published also indicating that the modification of Akt by SUMO is important for its activity. In discordance with the two studies discussed, the authors claimed that SUMOylation of Akt regulates its activation and they proposed two different residues as the SUMO acceptor sites on Akt: Lys^{182} and Lys^{189} [82].

As mentioned in the Introduction, activation of Akt is opposed by the action of PTEN, which antagonizes the action of PI3K hydrolysing PtdIns(3,4,5)P_3. Different laboratories have described that SUMO conjugation to PTEN regulates its subcellular distribution and activity [83–85]. In this context, it is tempting to speculate that the concept of 'group SUMOylation', which refers to the requirement for simultaneous modification of multiple targets involved in the same biological process [86,87], could apply to the regulation of the PI3K/Akt signalling axis.

CONCLUSIONS AND PERSPECTIVES

Despite extensive research conducted on Akt regulation over the last two decades, just recently a broader picture of different Akt PTMs has started to emerge.

The orchestration of the whole set of modifications undergone by this key signalling molecule as well as the consequences of a putatively complex interplay between them in both health and disease are still unclear and will demand our attention for many years to come.

It is tempting to envisage that growing knowledge about this wide variety of modifications will provide clues for still unsolved questions regarding isoform-specific regulation, substrate specificity and differential responses to extracellular cues, among others.

In general, individual studies have mainly dealt with a single extracellular stimulus that, through the canonical activation of Akt (membrane recruitment followed by phosphorylation of Thr^{308} and Ser^{473}), leads to the regulation of a limited number of Akt substrates, consequently affecting a given cellular process. Thus we are far from understanding how activation of a sole kinase may lead to different or even opposing cellular responses following different extracellular cues. In this regard, it is also puzzling how a single stimulus can trigger diverse and antagonistic biological responses. An interesting example is provided by the action of NGF (nerve growth factor), which triggers a decision between proliferation and neuronal differentiation in PC12 cells, through a molecular switch involving Akt [88].

Regarding spatial signalling dynamics, it is interesting that Akt not only localizes to the plasma membrane, nucleus and cytosol, but also has been found to be recruited to the membranes of cellular organelles such as mitochondria and endoplasmic reticulum [7,89]. Non-conventional intracellular stimuli for the Akt pathway and non-canonical mechanisms of Akt up- and down-regulation, together with novel Akt substrates, have been reported to be associated with these newly described Akt subcellular destinations [90–93].

The scenario becomes even more complex if we take into account the contributions being made during the last decade by molecular systems biology studies, which have been exceptionally valuable in shedding light on to the field by incorporating temporal quantitative aspects of signalling dynamics, by moving from average measurements of cell populations into real-time single-cell measurements, as well as by tackling cell-to-cell variation (also known as phenotypic heterogeneity). In this respect, it has been shown that phenotypic diversity in the activation patterns of signalling molecules associated with cancer, including Akt, can explain differences in the susceptibility of genetically identical cells to chemotherapy drugs [94].

Regarding temporal dynamics, recent work has demonstrated that the curves describing changes over time of parameters such as activity, modification state, concentration or localization of different signalling molecules, among them Akt, encode information about the amplitude, duration and frequency of the signal which is also relevant to fully understand how cells communicate and perceive their environment (reviewed in [95]).

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Clearly, regulatory mechanisms must operate within the cell so as to ensure the interaction of Akt, and other signalling molecules, with the appropriate set of substrates in order to generate a specific response. These control mechanisms might depend, among other things, on the cellular context, on the cross-talk with other signalling pathways that are also activated by a given stimulus, on temporal and spatial dynamics and, possibly, on a still undeciphered 'Akt PTM code', which may serve as a link between different stimuli and the subcellular localization of Akt and its substrates, and ultimately, with the execution of particular cellular programmes. PTMs may directly affect the contact surface between Akt and its different substrates or, alternatively, regulate their subcellular trafficking allowing or impairing their encounters within the cell. Undoubtedly, deciphering and understanding this PTM code could provide hitherto unidentified targets for therapeutic intervention in different human malignancies.

Note added in proof

During the production process of this article, a new report on Akt SUMOylation appeared online [96]. We apologize for not being able to comment on this work.

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