Membrane Proteins as 14-3-3 Clients in Functional Regulation and Intracellular Transport

14-3-3 proteins regulate the function and subcellular sorting of membrane proteins. Often, 14-3-3 binding to client proteins requires phosphorylation of the client, but the relevant kinase is unknown in most cases. We summarize current progress in identifying kinases that target membrane proteins with 14-3-3 binding sites and discuss the molecular mechanisms of 14-3-3 action. One of the kinases involved is Akt/PKB, which has recently been shown to activate the 14-3-3-dependent switch in a number of client membrane proteins.

To function correctly and to act in a coordinated manner with its neighbors, a cell must be acutely aware of its surroundings and must react to changes in the environment. Several classes of membrane proteins enable this sensing and any subsequent adaptations, which include cell surface receptors, channels, transporters, and structural proteins important for maintaining cellular integrity. Recently, the modulation of cell-surface expression of such key membrane proteins has been recognized as a physiologically important regulatory mechanism. Protein-protein interactions regulate vesicular transport along the biosynthetic pathway and can thereby tailor specific sorting steps to the needs of each individual protein. One intensively studied mechanism controlling the sorting of membrane proteins is protein phosphorylation and subsequent recruitment of a phospho-binding protein from the cytosol. This review will summarize recent progress in understanding how interactions with a key class of phospho-binding proteins, the 14-3-3s, regulate the biosynthetic delivery of membrane proteins to the cell surface. Particular attention is given to a discussion of the kinases involved in phosphorylating 14-3-3 binding sites and the emerging role of Akt/PKB as one such relevant kinase.

14-3-3 Proteins and 14-3-3 Binding Motifs

The 14-3-3 proteins are an evolutionarily conserved family of proteins implicated in a diverse range of functions (24, 60). They were first identified in 1967 as an abundant protein in brain lysates but have since been shown to be highly expressed in all cells of eukaryotic organisms. Mammals have seven homologs, often called isoforms (β, γ, ε, ζ, η, τ, σ), plants 15, and yeast two (Bmph1 and Bmph2) (24, 60). 14-3-3 proteins have a molecular mass of ~30 kDa, exist as either homo- or hetero-dimers, and are rigid in structure (45, 92). Structural analysis reveals that each monomer contains an amphipathic ligand-binding groove allowing each 14-3-3 dimer to bind to two separate binding sites. These two binding sites are either in the same protein or in two independent client proteins, which are then linked by the interaction with 14-3-3 (26, 45, 92). Phosphorylation of client proteins drastically increases the affinity of 14-3-3 binding to these sites, although prior phosphorylation of the substrate is not required in all cases (95, 96). An unbiased selection of 14-3-3 binding peptides and comparison with known 14-3-3 binding proteins revealed consensus sequences capable of mediating interactions with all 14-3-3 isoforms (95). Mode I binding sites have the consensus sequence R-S-X-pS/pT-X-P, and mode II binding sites have the consensus sequence R-X-F/Y-X-pS/pT-X-P, where pS/pT represents the phospho-serine or phospho-threonine and X can be any amino acid (52, 70, 95). It should be noted that the proline (P) located at position 2 of the phosphorylation site occurs in only about one-half of known 14-3-3 binding motifs (35). In addition to these two canonical binding motifs, 14-3-3 can bind to the extreme COOH terminus of several proteins, recently defined as a mode III binding site (12, 80). The motif is R-X-X-pS/pT-X-COOH, with serine or threonine at position –2 being absolutely required, whereas an arginine (R) residue at position –5 increases binding affinity (68, 80).

Clamping, Masking, and Scaffolding

Although 14-3-3 proteins have no intrinsic enzymatic activities or particular subcellular localization of their
own, their physical interactions with client proteins allow for the broad spectrum of activities with which they are associated. 14-3-3 proteins are thought to modify the properties (and the function) of client proteins by one of three principal mechanisms (FIGURE 1) (18, 47, 51). First, due to its inherent rigidity, the binding of 14-3-3 is thought to stabilize certain conformations of client proteins (clamping) (FIGURE 1A) (58, 94). Second, 14-3-3 binding may physically occlude specific sorting signals or structural motifs in target proteins (masking) (FIGURE 1B) (54, 55, 96). Third, 14-3-3 binding may facilitate interactions between the client protein and other proteins, either by providing a platform for protein recruitment or by anchoring client proteins in specific cellular locations (scaffolding) (FIGURE 1C) (51). As far as membrane proteins are concerned, very few examples exist where the interactions of 14-3-3 with client proteins are sufficiently well understood to ascribe a particular mode of action. Nevertheless, 14-3-3s may be considered as general switch proteins whose function depends on the client protein. In most cases, phosphorylation at a serine or threo-
nine residue activates the switch, and the subsequent binding of 14-3-3 proteins prevents rapid dephosphorylation.

Among membrane proteins, the best-characterized example of clamping is the plant plasma membrane H⁺-ATPase (PMA) (FIGURE 1A) (8, 19). The distal COOH terminus of the PMAs acts as an auto-inhibitory domain (62). On phosphorylation of a threonine residue at the penultimate position of the COOH terminus, 14-3-3 can bind. X-ray diffraction studies using crystals of 14-3-3 in complex with the last 52 amino acids of the PMA2 have shown that each 14-3-3 dimer binds to two H⁺-ATPase molecules. Structural modelling suggests that the linkage of neighboring PMA molecules by 14-3-3 leads to the assembly of a hexagonal structure consisting of six PMA subunits and six 14-3-3 proteins (19). This dodecameric complex is stabilized by the relatively rigid 14-3-3 “clamps.” In addition, the binding of 14-3-3 is thought to displace the auto-inhibitory R-domain of PMA; in this way, the dodecameric co-assembly of 14-3-3 and the PMA causes activation of the ATPase activity (39, 61).

14-3-3 binding also brings about functional changes in the activity of several mammalian potassium channels, but these effects are mechanistically much less well understood. For example, 14-3-3 proteins bind to hERG potassium channels in a phosphorylation-dependent manner and regulate their biophysical properties (37). In vivo, the binding of 14-3-3 proteins is promoted by sympathetic stimulation, which leads to activation of β1-receptors and subsequent elevation of cAMP and activation of PKA in cardiac muscle cells. Phosphorylation of two serine residues on hERG channels via PKA promotes binding of 14-3-3 and leads to an augmentation of the potassium currents carried by these channels. This is associated with a decrease in action potential duration. Thus 14-3-3 proteins may play a role in the regulation of cardiac action potential duration during sympathetic stimulation. Interestingly, the binding of 14-3-3 proteins was found to retard dephosphorylation of the channel by phosphatases (37), which represents a typical example of masking.

The functional characteristics of the two-pore potassium channel TRESK (TWIK-related spinal cord potassium channel) are also affected by 14-3-3 binding (16). The open probability of TRESK channels is regulated in a unique way by Ca²⁺ ions via the calcium-calmodulin-dependent protein phosphatase calcineurin (15). Calcineurin binds to the channel and mediates dephosphorylation of serine residue in the M2-M3 linker of the channel (Ser276 in mouse TRESK), causing a strong increase in its open probability. It has been speculated that the binding of 14-3-3 proteins to an adjacent mode I motif (phosphorylated at residue Ser263 by PKA in mouse TRESK) interferes with the re-phosphorylation of Ser276 and thus prolongs the calcium-calmodulin-dependent activation of the channel (16, 21). Thus the effect of 14-3-3 proteins on TRESK channels may be attributable to masking of a phosphorylatable motif.

In hERG and TRESK channels, 14-3-3 proteins are involved in the regulation of the open probability of the channels. In several other ion channels, interaction with 14-3-3 is required for efficient surface expression, i.e., 14-3-3 proteins modulate intracellular traffic. In these cases, too, the underlying mechanism is most likely masking of a short-sequence motif, namely an arginine-based (RXR) retention/retrieval signal. Arginine-based ER-localization signals, which bind to components of the COPI complex (49), were first identified in ATP-sensitive potassium channels (K₅ᵥ channels) (98). These channels assemble as octameric complexes consisting of four Kir6 channel subunits and four sulphonylurea receptor (SUR) subunits, and each of these subunits carries an RXR signal. It is thought that the ER localization effected by these motifs is due to retrieval of substrate proteins from post-ER compartments (50). Efficient cell surface transport of proteins harboring RXR motifs requires that the motif becomes inaccessible, and 14-3-3 proteins have been implicated in the masking of RXR motifs in several instances (6, 29, 31, 55, 56, 68, 89, 100). Low-affinity binding of 14-3-3 to RXR motifs in a phosphorylation-independent manner has been demonstrated using a multimer of the distal COOH terminus of Kir6.2 in pull-down assays from cytosolic cellular extracts (96). This interaction was sufficient to allow the exit of a multimeric reporter protein from the ER and promototion of the subsequent transport to the cell surface, demonstrating how 14-3-3 binding might overcome ER localization by competing with COPI components for binding at these motifs (masking). A similar conclusion was drawn from experiments with the two-pore-domain potassium (K₅ᵥ) channels TASK-1 and TASK-3 (55, 68). These channels carry a mode III 14-3-3 binding motif at their extreme COOH terminus. The minimal motif required for interaction of 14-3-3 with these channels was found to be R-R-X-S-X (68). ER localization is mediated by a COOH-terminal tri-basic (KRR) ER-retention/retrieval motif that differs in behavior from the similar RXR motif and overlaps with the two arginine residues of the mode III 14-3-3 binding site (56, 100). 14-3-3 binds to this site with high affinity when the penultimate serine residue is phosphorylated (55, 68). The binding of 14-3-3 to the distal COOH terminus of the TASK subunits is mutually exclusive with the binding of COPI components. Thus 14-3-3 binding can overcome ER-
retention by COPI (55, 100). TASK-1 and TASK-3 channels also possess an NH$_2$-terminal ER-retention/retrieval signal that affects cell surface expression independently of 14-3-3 binding to the COOH terminus (100), in contrast to the original model where 14-3-3 binding to the COOH terminus was proposed to interfere with COPI binding to the NH$_2$ terminus of the polytopic membrane protein (55, 100). A similar antagonistic mechanism based on an overlapping binding site for COPI and 14-3-3 regulates the ER export of lip35 isoform of MHC class II-associated invariant chain. In this case, too, an RXR-type motif prevents efficient ER export through interactions with COPI (55, 77); following phosphorylation by PKC at Ser8, 14-3-3 is recruited, COPI is displaced, and ER export is promoted (3, 42).

The mode III binding motif for 14-3-3 was further characterized by a cell sorting-based screen of random sequence motifs attached to a reporter construct (80). The construct consisted of a truncated Kir2.1 channel, an RXR motif (from Kir6.2) inserted at the COOH terminus of Kir2.1, and an artificial COOH terminus generated by using a peptide library. With the use of this approach, mode III 14-3-3 binding motifs (located at the extreme COOH terminus) were shown to override an RXR motif located upstream (FIGURE 1B) (12, 80). In agreement with other work (55, 56, 68), the serine or threonine residue at the penultimate position was found to be absolutely required for efficient surface expression (80). Interestingly, the constructs showing the highest surface expression had an arginine residue at position –3 relative to the essential serine/threonine residue, which is also the case in TASK-1 and TASK-3. The authors concluded that interaction of 14-3-3 with the mode III binding domain could “override” the RXR retention signal (80), which is compatible with masking.

Potential scaffolding roles of 14-3-3 proteins in the context of membrane protein regulation or trafficking are less well defined. Interactions between the α3 subunit of the nicotinic acetylcholine receptor (nAChR) and a multi-subunit cytoskeletal-anchoring complex currently provide the best evidence (FIGURE 1C) (71). The cytoskeletal-anchoring complex comprises the microtubule plus-end binding protein, EB1, macrophilin, IQGAPI and APC (adenomatous polyposis coli), and 14-3-3 (71). As a constituent of the cytoskeletal-anchoring complex, 14-3-3 is believed to act as a tether to allow interaction with α3-containing receptors (71). Thus 14-3-3 proteins may provide for a mechanism by which nAChRs containing only specific subunits are recruited to postsynaptic clusters and may stabilize them there. In addition, 14-3-3s are important in actively promoting the forward transport of N-cadherin/β-catenin complexes from the ER via an interaction with the Rho-GTPase activating protein PX-RICS (53). The N-cadherin/β-catenin/PX-RICS complex recruits 14-3-3ζ/θ via a mode I binding motif, and the association with 14-3-3 is thought to enable coupling of the complex to the microtubule motor dynein/dynactin, thus leading to forward transport (53).

### Protein Phosphorylation and 14-3-3 Binding

A number of proteomic screens yielded over 200 proteins, many of them membrane proteins, which interact with 14-3-3 in a phosphorylation-dependent manner (67). Unfortunately, in the majority of cases the identity of the physiologically relevant kinase(s) that phosphorylate the mode I, II, or III 14-3-3 binding motifs is unknown. The information governing kinase recognition and 14-3-3 binding is largely shared, often leading to overlapping consensus sites for protein kinase recognition and 14-3-3 binding. A comparison of canonical protein kinase and 14-3-3 binding sites (FIGURE 2) reveals several common features in addition to the phospho-serine or threonine, particularly a basic residue (preferentially arginine) at position –3 but to a lesser extent also at position –5 relative to the essential serine/threonine. These features are common among the consensus sites for many kinases (FIGURE 2) including protein kinase A (PKA) (101), protein kinase G (PKG) (28), protein kinase C (PKC) (30), protein kinase B (PKB/Akt) (2), and Ca$^{2+}$-calmodulin-dependent kinase (CamK) family (14, 65). Interestingly, bioinformatic analyses of known 14-3-3 client proteins suggest that mode I 14-3-3 binding sites, which share substantial homology with many kinase recognition sites, are far more prevalent than mode II sites (35). High-resolution structures of 14-3-3 proteins bound to client phosphoproteins have further clarified the mechanistic basis for such interactions (58, 61, 76, 95). The structural relationship between client protein phosphorylation and the binding of 14-3-3 to mode III sites is less clear (FIGURE 2). Certainly, given the evidence from studies on TASK channels and other mode III motif-containing client proteins, phosphorylation of precisely the penultimate residue appears to be vitally important for the interaction with 14-3-3 (55, 68, 80). In fact, adding one residue to the carboxy terminus containing a mode III motif interferes with 14-3-3 binding (80). The atomic details of 14-3-3 binding to these motifs remain unclear due to the lack of high-resolution structures of 14-3-3 bound to such client proteins.
Subcellular Site of 14-3-3 Recruitment and Action

Which vesicular transport step does 14-3-3 binding to a client membrane protein affect? Studies on the intracellular traffic of different membrane proteins suggest that 14-3-3 binding is important in regulating the movement of target proteins early in the biosynthetic pathway, between the ER and the Golgi, or within early Golgi compartments (3, 6, 42, 55, 68, 80, 89). The cell surface targeting of ADAM22 (29) is a particularly informative case (FIGURE 3): ADAM22 is exported from the ER as an inactive precursor and is subsequently activated through proteolytic processing in late Golgi compartments, making pre- and post-Golgi populations of the protein readily distinguishable (46). ADAM22 contains two RXR-type ER-retention/-retrieval signals that overlap with adjacent 14-3-3 binding sites. The precursor form of ADAM22 is serine phosphorylated and binds to 14-3-3, whereas the mature form of ADAM22 is less serine phosphorylated and hence binds less 14-3-3 (29). Mutation of the 14-3-3 binding sites leads to accumulation of unprocessed ADAM22 in ER and in Golgi compartments (29), consistent with the hypothesis that 14-3-3 binding is important in promoting the passage of ADAM22 through the early biosynthetic pathway. Similarly, TASK channel subunits unable to bind 14-3-3 accumulate in the early secretory pathway, and their subcellular localization largely overlaps with the distribution of COPI (100). The results obtained with ADAM22, TASK channels, and some other membrane proteins (3, 6) suggest that an antagonism between COPI and 14-3-3 binding is the major mechanism underlying the 14-3-3-regulated transport of membrane proteins to the cell surface.

Which Kinases are Implicated in 14-3-3-Regulated Cell Surface Expression?

Unfortunately, the similarities between different protein kinase recognition sites are extensive, making a prediction of which kinase may be functionally significant difficult. For example, a bioinformatic analysis (93) of the TASK-3 COOH terminus suggests that >20 different mammalian kinases have a high probability of being able to phosphorylate the penultimate serine residue known to be important for 14-3-3 binding (FIGURE 2). This list includes members of the AGC and CamK families as well as many other ubiquitous kinases such as casein kinase, Wnk kinases, and members of the MAP-kinase signaling cascade. Given that these kinases are likely to have redundant activities (albeit with differing spatial and temporal regulation), it is hardly surprising that the identification of the physiologically relevant kinases remains one of the greatest challenges in the field.

Despite these obstacles, a number of studies have begun to highlight specific kinases that may have more important roles than others. Among these, a kinase with several emerging roles in regulating 14-3-3 interactions with client membrane proteins is Akt/PKB. Many other protein kinases do of course play very important roles; for example, PKA (37) and CaMKII phosphorylation (99) of 14-3-3 client proteins has been well documented. As discussed above (and in FIGURE 2), the similarity between 14-3-3 binding motifs and many kinase recognition sites suggests a diverse cohort of kinases is likely to be important. However, less information is available on the role of other kinases in promoting the binding of 14-3-3, particularly in the context of membrane protein trafficking. Thus, in the interests of space, we have chosen to highlight the emerging roles of Akt because of its relevance to several fields including metabolic regulation, developmental biology, cellular signaling, and cancer research. The emphasis placed on the role of Akt/PKB in this review should in no way be taken to indicate that other kinases are less important. We hope that highlighting current developments delineating the role of Akt/PKB in 14-3-3 recruitment will lead to new approaches to tackling the role of other kinases in the near future.

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cellular processes, including the regulation of metabolism, cellular growth, differentiation, and survival (5, 81). Activation of Akt is associated with cellular adaptations that promote cell division and survival and suppress pro-apoptotic signals.

Akt is conserved in eukaryotes having homologues in yeast (SCH9) (86), plants, Drosophila (dAkt) (23), C. elegans (63), and animals (Akt1–3) (41, 81, 90). It is a key component of signal transduction by ligand-activated receptor tyrosine kinases (RTKs) (87) (FIGURE 4), including growth factors and insulin receptors, and acts downstream of PI3K (phosphatidylinositol-3-kinase). On ligand binding, the RTK auto-phosphorylates, which leads to the recruitment of scaffolding proteins such as insulin receptor substrate 1 (IRS1) (17, 36). IRS1 recruits the p85 regulatory-subunit of the bipartite, class 1 phosphatidylinositol 3-kinase (PI3K), and activates its p110 catalytic-subunit (27, 32). Activated PI3K catalyses the conversion of PIP(4,5) to PIP(3,4,5), allowing the recruitment of Akt and phosphoinositide-dependent kinase 1 (PDK1) to the membrane via their plekstrin homology (PH) domains (4, 22). PDK1 then phosphorylates Akt at Thr308. RTK activation also stimulates mammalian target of rapamycin-complex 2 (mTORC2) by a hitherto unknown mechanism (75), and mTORC2 phosphorylates Akt at residue Ser473. Phosphorylation of Akt at both Thr308 and Ser473 is required for complete activation of its kinase activity and the subsequent phosphorylation of downstream substrates (1). The activity of Akt is negatively regulated by dephosphorylation of Ser473 by PH-domain and leucin-rich repeat protein phosphatase (PHLPP) (7, 25) and by the conversion of PIP(3,4,5) to PIP(4,5) by phosphatase and tensin homolog (PTEN) or by the inositol 5’ phosphatase SHIP (25, 38).

The canonical phosphorylation site targeted by Akt was deciphered (2) using a peptide library based on the Akt target site in GSK3β, the first known substrate of Akt (2, 13). Akt consensus sites conform to R-X-R-X-X-pS/pT-h, where pS/pT represents the phospho-serine or phospho-threonine, h is any bulky hydrophobic residue, and X can be any amino acid (2, 57). This consensus motif closely resembles the one responsible for mode I and mode III 14-3-3 binding (FIGURE 2), suggesting that Akt substrates are strong candidates for...
being 14-3-3 client proteins. The similarity is particularly pronounced when the canonical mode I motif is compared with an optimized Akt phosphorylation motif as defined by a peptide and protein library screen (R-K-R-X-R-Y-pS-F-G) (FIGURE 2) (57).

**Akt-Regulated 14-3-3-Dependent Membrane Protein Trafficking**

The binding of 14-3-3 in an Akt-phosphorylation-dependent manner can alter the trafficking of membrane proteins either directly or indirectly, depending on whether the site of phosphorylation, and thus 14-3-3 binding, is on the membrane protein itself or on an accessory protein.

**Direct Modulation of Trafficking by Akt/14-3-3**

Phosphorylation by Akt can directly affect the trafficking of target proteins by providing a phospho-binding site for interactions with 14-3-3, as is the case for the NR2C subunit of the NMDA receptor (NMDAR) (9). NMDARs are hetero-multimers composed of NR1, NR2, and NR3 subunits that assemble in the early biosynthetic pathway (48, 66, 84). Each subunit isoform contains trafficking information directing discrete steps of intracellular transport of the channel (79, 84). Following the binding of growth factors to their cognate receptors, Akt is activated, resulting in phosphorylation of Ser1096 of NR2C. The Akt phosphorylation site overlaps with a mode I 14-3-3 binding motif (9). 14-3-3 binding leads to increased cell surface expression of NR2C-containing NMDARs. Interestingly, co-assembly of NR2C with NR1 was required to observe recruitment of 14-3-3 to the NMDAR (9). NR1 subunits have previously been shown to contain RXR ER-localization motifs (78). Phosphorylation sites for PKA and PKC flank the RXR motifs, and phosphorylation of the corresponding serines can overcome their ER localization activity in chimeric reporter constructs containing the isolated COOH terminus of NR1 (78, 79) but not in full-length channels (33). Hence, it may be speculated that phosphorylation on the Akt target site may enable binding of 14-3-3 to NR1/NR2C heteromers in a way that suppresses the COPI-dependent activity of signals present on NR1.

14-3-3 binding in an Akt-dependent manner is known to be important in the regulation of the subcellular localization of the gap junction protein, connexin 43 (Cx43) (64). EGF stimulation of fibroblasts leads to increased Akt activity and phosphorylation of Cx43 at Ser373 and Ser369. This promotes association with 14-3-3 and subsequent translocation of a complex composed of Akt, Cx43, and 14-3-3 to the edges of gap junction plaques (64).

The function of some mode III 14-3-3 binding motifs in forward transport may also depend on Akt-dependent recruitment of 14-3-3 proteins (11). For example, GPR15, a G-protein-coupled receptor

![FIGURE 4. Activation pathway for Akt]

Activation and the subsequent auto-phosphorylation of receptor tyrosine kinases (RTKs) lead to the recruitment of second messengers such as insulin receptor substrate 1 (IRS1). These in turn lead to the recruitment and activation of class 1 phosphatidylinositol 3-kinase (PI3K), the activation of which leads to the conversion of PIP2 to PIP3 in the plasma membrane. PIP3 recruits both Akt and PDK to the membrane, where PDK phosphorylates Akt at threonine 308. Akt is also phosphorylated at serine 473 by mTORC2, another downstream target of RTK activation. When phosphorylated at both sites, Akt becomes active and is able to phosphorylate downstream targets, generally leading to increased cellular survival/proliferation and decreased apoptosis. The activity of Akt is inhibited by dephosphorylation of serine 473 by PLHPP and by the conversion of PIP3 back to PIP2 by SHIP and PTEN.
associated with HIV invasion, was shown to possess a mode III 14-3-3 binding motif. It has since been demonstrated that Akt phosphorylation of the threonine contained within the mode III motif can trigger 14-3-3 recruitment in response to growth factor stimulation, leading to increased surface expression of GPR15 (11).

Indirect Modulation of Trafficking by Akt/14-3-3

In addition to modulating cellular trafficking through facilitating a direct interaction between 14-3-3 and a client membrane protein, Akt can also modify trafficking by regulating interactions between a membrane protein and other proteins in an indirect manner. In this case, the 14-3-3 client protein is an accessory protein that modulates the trafficking of the membrane protein. The best-characterized mechanism of this type involves the interaction of 14-3-3 with Akt substrate of 160 kDa (AS160) (40). AS160 is a Rab-GTPase activating protein (Rab-GAP) involved most notably in the recruitment of GLUT4 glucose transporters from an intracellular compartment to the cell surface following stimulation of adipocytes and skeletal muscle cells with insulin (73, 97). At rest, >90% of GLUT4 is located in an intracellular endosomal/trans-Golgi derived compartment due the balance between exocytosis and endocytosis. The binding of insulin to its cell surface receptor stimulates the signaling cascade outlined above (FIGURE 4), eventually leading to the activation of Akt (20, 72, 91). Activated Akt phosphorylates AS160 at Ser341 and Thr642, leading to the recruitment of 14-3-3 (10, 40, 69). Once bound to 14-3-3, the Rab-GAP-activity of AS160 is inhibited, shifting the equilibrium of Rab8A,10, 13, 14 to the active GTP-bound form, enabling them to promote GLUT4 forward trafficking by relieving the effectiveness of retention mechanisms and promoting cell-surface expression (34, 74, 85).

The surface expression of the epithelial sodium channel (ENaC) may be regulated in a similar manner by an AS160/14-3-3/Akt-dependent mechanism (44). Furthermore, 14-3-3 controls a key regulator of ENaC surface expression, the ubiquitin-E3 ligase, Nedd4 (82, 83). Ubiquitination of ENaC leads to an increased rate of endocytosis from the cell surface and protein degradation. The activation of Akt via insulin receptor signaling leads to the phosphorylation of Nedd4, the recruitment of 14-3-3, and the inhibition of the ubiquitin-ligase activity of Nedd4. In the absence of ubiquitination, the rate of endocytosis of ENaC is slow and the surface expression of ENaC remains high (43).

Concluding Remarks

Progress has been made in recent years toward understanding the prerequisites for and functional consequences of 14-3-3/client protein interactions. Despite this, several key questions remain unanswered. More than 200 proteins have been identified as 14-3-3 interaction partners, but the underlying molecular basis and the precise purpose of these interactions have been elucidated only in a small number of cases. To better understand the significance of these interactions we must address the following questions.

First, which kinases are physiologically significant for each substrate? The relevant kinase is not known for the vast majority of client proteins, and the substantial overlap existing between the recognition sites of numerous different kinases and 14-3-3 binding sites make their identification very difficult. In an in vivo setting, the access of any given kinase to its substrate is tightly regulated. By moving the system to an in vitro setting, this level of regulation may be lost, leading to the identification of a kinase that might not normally be able to phosphorylate the substrate. To identify the physiologically relevant kinase(s) while retaining this aspect of regulation is probably the toughest challenge currently faced by the field. Second, what role does 14-3-3 isoform specificity play in mediating the downstream functional consequences? Many reports of 14-3-3/client protein interactions consider 14-3-3 in general terms with little attention to the precise composition of the relevant 14-3-3 dimers. The propensity of the different 14-3-3 isoforms to form homo- or hetero-dimers may enable specific 14-3-3 dimers to fulfill specific roles. The precise subunit composition of 14-3-3 dimers may have important functional implications. Since the COOH termini of 14-3-3 proteins are most divergent and hence most likely to contain isoform-specific structural determinants (59, 88), COOH-terminal tagging might obscure 14-3-3 isoform-specific behaviors. Third, does 14-3-3 binding promote the generation of supramolecular complexes? In most cases, complexes arising from 14-3-3 binding remain elusive. Many reports combine several observed binary interactions into hypotheses involving complexes that contain all identified proteins. We must probe the existence of such 14-3-3-dependent supramolecular complexes more rigorously. Fourth, how exactly does 14-3-3 exert its role as a switch protein in the interplay between phosphorylation, 14-3-3 binding, and downstream functions of client membrane proteins? This is a particularly challenging objective for multimeric membrane proteins that present more than one 14-3-3 binding site. The presence of multiple binding
sites, including those of low affinity, can significantly increase the apparent binding affinity of 14-3-3 to a membrane protein complex. Hence, not all relevant sites may have been detected by the commonly used yeast two-hybrid and pull-down approaches. The above questions highlight how little we truly know about the structural, temporal, and spatial regulation of 14-3-3-client protein interactions and their regulation by protein kinases. Only through answering these questions can we fully unravel the intricacies of the regulation afforded by this enigmatic class of proteins.

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