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Spatial determinants of specificity in insulin action

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Abstract

Insulin is a potent stimulator of intermediary metabolism, however the basis for the remarkable specificity of insulin's stimulation of these pathways remains largely unknown. This review focuses on the role compartmentalization plays in insulin action, both in signal initiation and in signal reception. Two examples are discussed: (1) a novel signalling pathway leading to the phosphorylation of the caveolar coat protein caveolin, and (2) a recently identified scaffolding protein, PTG, involved directly in the regulation of enzymes controlling glycogen metabolism. (Mol Cell Biochem **182**: 65–71, 1998)

Key words: caveolae, caveolin, DARPP32, glycogen, protein phosphorylation, *P*rotein *T*argeting to *G*lycogen (PTG), type I protein serine/threonine phosphatase (PP1)

Introduction

Insulin is the most potent physiological anabolic agent known, promoting the synthesis and storage of carbohydrates, lipids, and proteins, and preventing their degradation and release back into the circulation. Despite years of intense investigation, there remains considerable uncertainty regarding the precise intracellular events that mediate the actions of this hormone. One confounding factor has been the variety of actions of insulin, which depend upon cell type, time of exposure, and the presence of other hormones [1]. Another is the fact that insulin can act as a growth factor for cells in culture, and shares many of the mitogenic signalling pathways elicited by other growth factors. However, the metabolic effects of insulin are unique, and cannot be reproduced with other cellular stimuli [2–5]. Taken together, these findings indicate that signalling mechanisms exist that respond uniquely to insulin, which allow for the specialized effects of insulin on metabolism.

Like receptors for many growth factors, the insulin receptor is a tyrosine kinase that undergoes activation upon insulin binding, leading to the tyrosine phosphorylation of a specific collection of intracellular proteins [6, 7]. Receptor activation leads to a variety of signalling pathways that diverge at or near the insulin receptor itself. While receptor autophosphorylation and the subsequent substrate phosphorylations are clearly required for the full expression of insulin's actions, many of these substrates and subsequent downstream pathways are shared by other receptors that do not mimic insulin's effects on metabolism. A number of hypotheses have been proposed to account for the signalling specificity underlying insulin action, including differences in the strength or duration of signals, combinatorial diversity of signals, or other unique features of these pathways in insulin responsive cells. Another potential mechanism to explain insulin's unique actions may lie in the spatial compartmentalization of signal transduction [8, 9], both regarding signal initiation at the plasma membrane, as well as signal reception inside the cell. In this review we provide two examples of how the subcellular targeting of signalling molecules may contribute a key ingredient in determining the specificity of insulin action.

Specificity in signal initiation: the role of caveolae

One clue to understanding how insulin can elicit its unique actions may lie in the compartmentalization of the signalling molecules themselves. Recent studies have suggested that signal initiation may he functionally segregated into distinct domains of the plasma membrane. Caveolae may represent one type of specialized region of the plasma membrane that

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is crucial for specificity in signal transduction. Caveolae are small invaginations of the plasma membrane with unique protein and lipid compositions [10–13]. These structures are clearly distinguished from clathrin-coated pits by their characteristic striated coat, which is made up largely of the caveolins [14, 15]. Caveolins are members of a multigene family with three known members [16–20]. In many cell types, caveolin copurifies with or binds to signalling molecules [21–35], suggesting that one potential function of caveolae lies in signal transduction.

Although caveolae have generated much recent interest, their exact function and protein composition remain controversial [36–39]. Other membrane domains with biophysical properties similar to caveolae, such as clathrin coated pits, can be contaminants in purified caveolar fractions [40, 41], so that the precise molecular composition of these structures is uncertain. Moreover, cellular membranes contain sub-domains termed lipid ordered domains or glycolipid rafts [42, 43]. These sub-domains, characterized by their Triton-insolubility, are highly enriched in cholesterol, glycolipids and sphingolipids, and de-enriched in phospholipids. Many proteins are localized to the lipid ordered domains by virtue of post-translational modifications. GPI anchors have been shown to be necessary and sufficient for the localization of proteins to these domains, as has tandem acylation of the Src-family kinases and G-proteins [44–47]. Caveolin itself is also acylated [48]. Although the extent of overlap between caveolae and glycolipid rafts is unknown, it is clear that caveolae form within these lipid ordered domains, perhaps due to the fact that caveolin is a cholesterol binding protein [49]. While glycolipid rafts form in the absence of caveolin [50, 51], expression of caveolins is both necessary and sufficient to induce the formation of caveolae [52–54]. Independently of whether all of the proteins which co-purify with caveolins are actually found within the caveolar structures, the membrane-bound proteins in the lipid ordered domains are found in close proximity to the caveolae, which form within these domains [38].

Caveolae are abundant in adipocytes and muscle, covering a significant fraction of the inner surface of the plasma membrane of adipocytes [55]. Of the four known forms of caveolin, caveolins-1 (α and β) and -2 are most highly expressed in adipocytes, followed by lung and muscle [17, 19, 56], while caveolin-3 appears to be a muscle specific isoform [20, 57]. In addition, caveolin-1 expression increases upon adipocyte differentiation [56, 59]. The abundance of caveolae and caveolins in adipocytes and muscle, together with their potential role in signalling, suggested a possible role in insulin signal transduction. Consistent with an important role for caveolae in insulin action, insulin specifically stimulates the tyrosine phosphorylation of the two forms of caveolin (22 and 24 kD) and an additional unidentified protein of 29 kD that coimmunoprecipitates with caveolin after disruption of the caveolar complexes with octylglucoside [37]. This phosphorylation does not occur in response to other growth factors in adipocytes. In addition, caveolin phosphorylation occurs only in cells in which metabolism is highly responsive to insulin. For example, it occurs only in the fully differentiated 3T3-L1 adipocytes, not in the preadipocytes, despite the expression of both caveolin and the insulin receptor in both cell types [59].

Although insulin specifically increases the tyrosine phosphorylation of caveolin, several lines of evidence indicate that caveolin is not a direct substrate of the insulin receptor. Analysis of caveolin-enriched fractions from 3T3-L1 adipocytes revealed that the caveolin kinase is the Src-family kinase Fyn [37, 59]. Fyn colocalizes with caveolin in low density, Triton-insoluble complexes in both preadipocytes and adipocytes, and it is the only detectable tyrosine kinase in these fractions. Moreover, overexpression of wild type Fyn leads to an increase in the basal level of tyrosine phosphorylation of caveolin, and hyperphosphorylation of caveolin in response to insulin [59]. A dominant negative Fyn construct blocked caveolin phosphorylation, although this might reflect an indirect effect on differentiation. Interestingly, the caveolin kinase in primary adipocytes appears to be the Src-family kinase Lyn, rather than Fyn. Lyn is expressed at high levels in primary adipocytes, and is colocalized with caveolin in low density, Triton-insoluble complexes prepared from these cells (unpublished observations).

How is the caveolar Fyn activated in response to insulin? Fyn and other Src-family kinases can be regulated either through the dephosphorylation of a regulatory tyrosine in the carboxy terminus of the protein [60], or through the occupancy of their SH2 domains by tyrosine phosphorylated proteins [61]. The autophosphorylation of an additional tyrosine is required for full activation of these kinases. No net change in the phosphorylation of Fyn has been observed in response to insulin in adipocytes, although there is a significant increase in constitutive phosphorylation of Fyn after adipocyte differentiation [59]. In addition, tyrosine phosphorylated peptides which bind specifically to the SH2 domains of Fyn stimulate the caveolin kinase activity in caveolar fractions, suggesting that the insulin receptor phosphorylates a specific substrate protein, which in turn activates the caveolar Fyn.

The unique conditions under which caveolin phosphorylation is observed suggest that the insulin receptor substrate responsible for the activation of caveolar Fyn would have several unique properties: (1) phosphorylation showing specificity for insulin in adipocytes; (2) association with Fyn (through an SH2 domain interaction) in response to tyrosine phosphorylation; (3) translocation of the phosphorylated protein into caveolae in response to insulin; and (4) phosphorylation in 3T3-L1 adipocytes, but not in the preadipocytes. While the well characterized insulin receptor

substrates IRS-1/-2 and Shc emerged as potential candidates [62, 63], none fulfilled all of these requirements. In contrast, the proto-oncogene product c-Cbl shares many properties with this presumed substrate protein. Insulin specifically stimulates the phosphorylation of c-Cbl in adipocytes [64]. As has been observed in lymphocytes, Cbl constitutively binds to Fyn in unstimulated adipocytes through an SH3 domain-mediated interaction, and insulin-stimulated tyrosine phosphorylation of Cbl increases this association. The Cbl which is phosphorylated in response to insulin binds specifically to fusion proteins containing the SH2 domain of Fyn, but surprisingly not SHP-2 or PI3 kinase. Cbl is translocated into caveolin-enriched Triton-insoluble complexes after insulin stimulation, and the Cbl in these complexes is tyrosine phosphorylated [59]. Most interestingly, unlike the other known substrates of the insulin receptor such as IRS-1/-2 and Shc, Cbl phosphorylation is specific for the differentiated adipocyte phenotype. In addition, tyrosine phosphorylation of Cbl is not observed in tissue culture cell lines which have been engineered to express high levels of the insulin receptor, while the other substrate proteins are readily phosphorylated in these cells [64].

The basis for the cell-type specificity of Cbl phosphorylation is currently unknown. Cbl is expressed at comparable levels in both the preadipocytes and adipocytes. Unlike IRS-1/-2 and Shc, Cbl does not contain a PTB domain, which has been identified in substrates that interact directly with the insulin receptor [65–67]. In addition, in contrast to IRS-1 and Shc, Cbl does not interact with the insulin receptor in the yeast two hybrid system. We hypothesize that tyrosine phosphorylation of Cbl may require a specific adaptor protein which allows for the interaction of Cbl with the insulin receptor, and that it is the regulation of the expression of this protein that accounts for the coordinate regulation of the phosphorylations of Cbl and caveolin in response to insulin in adipocytes. Although the model linking phosphorylation of Cbl to the phosphorylation of caveolin through the activation of Fyn is compelling (Fig. 1), the exact relationship between these three proteins is likely to be complex [68]. For example, Cbl may undergo processive phosphorylation after the activation of Fyn [69, 70]. However, the insulin-dependent association of Cbl and Fyn in caveolae leads to the intriguing possibility that the Fyn/Cbl pathway may have a unique function in adipocytes (phosphorylation of caveolin) due to the localization of these proteins to specialized domains of the plasma membrane.

The precise role of caveolin phosphorylation in insulin action remains uncertain. Preliminary attempts to identify proteins which interact with caveolin in a phosphorylation-dependent manner have thus far proven unsuccessful. However, the correlation between caveolin phosphorylation and the metabolic activities of insulin [37, 59], the lack of phosphorylation with other tyrosine kinase receptors [37, 59], and a number of reports describing numerous signalling molecules in caveolae or related membrane domains [21–35] suggest that this phosphorylation event may represent an important mechanism for the segregation of early signalling events in insulin action, perhaps as a way to ensure signalling specificity.

Spatial compartmentalization in signal reception: the regulation of glycogen metabolism

It has long been recognized that protein dephosphorylation plays an essential role in regulating the metabolic effects of insulin [71]. Indeed, many of the rate-limiting enzymes involved in glycogen metabolism, such as glycogen synthase, glycogen phosphorylase and phosphorylase kinase are regulated by their phosphorylation state. Insulin causes the dephosphorylation of these enzymes, which results in the activation of glycogen synthase and the inactivation of phosphorylase and phosphorylase kinase. This leads to a significant increase in the rate of glycogen synthesis. The dephosphorylation of these three enzymes is mediated through the activation of the type I serine/threonine phosphatase PP1 in response to insulin. However, insulin promotes the net dephosphorylation of only a specific subset of proteins, while PP1 and additional substrates for this enzyme are found ubiquitously in nearly all cellular compartments. Therefore, a mechanism must exist for the stimulation of discrete PP1 activities by insulin.

The specificity with which insulin stimulates the dephosphorylation of only selected substrates of PP1 suggests that there are distinct functional pools of PP1 in cells. Tissue specific targeting subunits have been identified that localize the catalytic subunit of PP1 (PP1C) to specific sites, conferring both insulin sensitivity to the enzyme, as well as substrate specificity. Several glycogen targeting proteins have been described, including G_m which is expressed in both heart and skeletal muscle [72], and G_L, the hepatic subunit [73]. While the precise functions of these proteins remains uncertain, recent experiments on a related glycogen localizing subunit of PP1C may help to explain how insulin can promote the rapid dephosphorylation of the specific group of proteins involved in glycogen metabolism. This protein, called PTG for *P*rotein *T*argeting to *G*lycogen, was identified in a two-hybrid screen of a 3T3-L1 adipocyte library using PP1C as the bait (74; also called R5, 75). PTG encodes a 33 kD protein which is found in all insulin responsive tissues. In addition to localizing PP1C to the glycogen pellet, PTG forms specific complexes with the PP1-regulated enzymes that control glycogen metabolism, including glycogen synthase, phosphorylase, and phosphorylase kinase. PTG thus increases PP1 specific activity

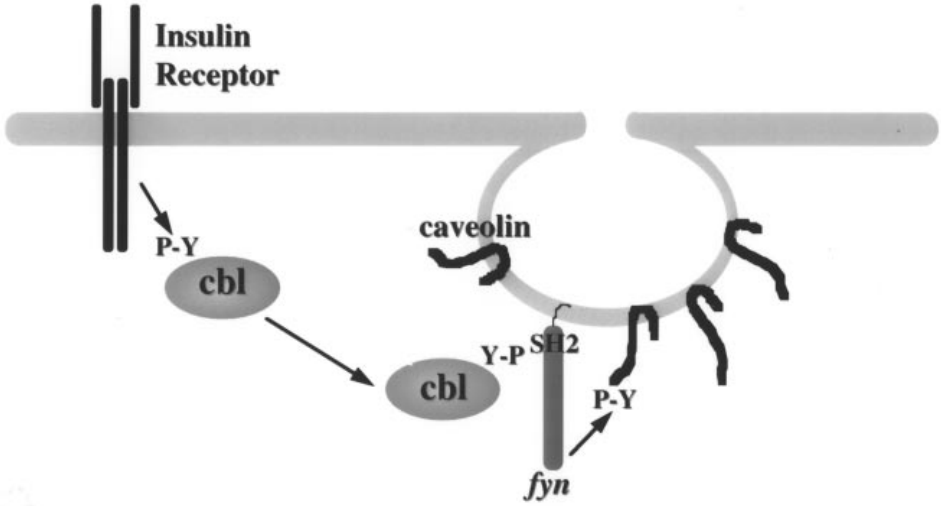

Fig. 1. Stimulation of the tyrosine phosphorylation of caveolin by insulin. Activation of the insulin receptor tyrosine kinase leads to the tyrosine phosphorylation of Cbl. Phosphorylated Cbl binds to the SH2 phosphotyrosine binding domain of Fyn, which is resident in the caveolae. This activates the kinase, resulting in the tyrosine phosphorylation of substrate proteins in these complexes such as caveolin.

against glycogen-bound substrates not only by targeting the PP1 to glycogen, but also by directly interacting with PP1 substrate proteins. This protein, therefore, may assemble glycogen synthase, phosphorylase, phosphorylase kinase, and PP1C onto the glycogen particle, generating a metabolic module for the localized reception of the appropriate intracellular signals [74] (Fig. 2). It is still unclear, however, whether all of the glycogen metabolizing enzymes are bound to a single PTG molecule simultaneously, or if the binding sites are shared between one or more proteins. It is expected that the PP1 at this compartmentalized site is sensitive to activation by insulin, while the non-responsive PP1 in other subcellular compartments is not.

The mechanisms by which insulin specifically activates glycogen-targeted PP1 are still unclear. At one time speculation centered around the phosphorylation of two closely spaced serine residues in the amino terminus of G_m, by the MAP kinase cascade [76 , 77]. However, it is now clear that this model has shortcomings. MAP kinase-activation is neither necessary nor sufficient for the activation of glycogen synthase or PP1 by insulin [2–5, 78]. In addition, the phosphorylation sites in G_m are not conserved in the other glycogen targeting subunits [74]. While the precise mechanisms involved in PP1 activation by insulin remain uncertain, one attractive hypothesis involves dis-inhibition of PP1 by the regulated dissociation of an inhibitory subunit. Studies suggest that regulation of PP1 basal activity contributes to insulin responsiveness, and that the low activity in the basal state is maintained by phosphorylated inhibitory peptides such as inhibitor-1 and DARPP-32 [79, 80]. For example, we have observed that adipocyte differentiation leads to a decrease in basal PP1 activity, and a significant increase in the stimulation of PP1 activity by insulin [81]. PP1 levels do not change during differentiation, while expression of the PP1 inhibitor peptide DARPP-32 is dramatically increased during adipogenesis [81], as is the PTG targeting subunit [82]. The DARPP-32 in 3T3-L1 adipocytes is exclusively localized to the particulate fraction [81], which includes the glycogen pellet. We are currently testing the hypothesis that insulin treatment leads to the dissociation of DARPP-32 from glycogen targeted, PTG-bound PP1 (Fig. 3). In addition,

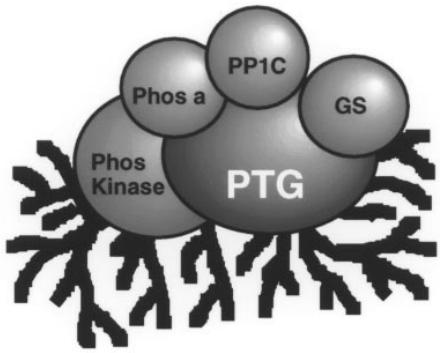

Fig. 2. PTG may act as a molecular scaffold for the hormonal control of glycogen synthesis. The catalytic subunit of PP1 (PP1C) is targeted to glycogen by PTG in 3T3-L1 adipocytes. PTG also serves as a glycogen scaffolding protein, co-localizing PP1 with its substrate enzymes which control glycogen metabolism, including glycogen synthase (GS), phosphorylase a (Phos a), and phosphorylase kinase (Phos Kinase). This may serve as a metabolic module for the localized reception of intracellular signals which regulate glycogen metabolism.

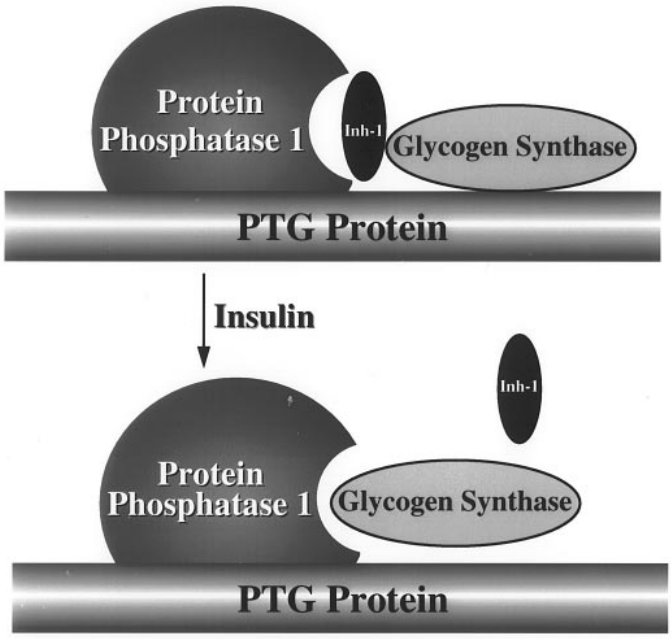

Fig. 3. Activation of glycogen-targeted PP1 by insulin. Glycogen-targeted PP1 activity may be regulated by the binding of inhibitory peptides, such as inhibitor 1 (inh-1) or DARPP-32. Insulin stimulation may cause the dephosphorylation and/or disassociation of bound inhibitor. Dis-inhibition of PP1 would result in the dephosphorylation and activation of glycogen synthase, mediating the insulin-stimulated increase in glucose storage as glycogen.

attempts are underway to determine whether dissociation of DARPP-32 is sufficient to account for the specific insulin-induced dephosphorylation of enzymes involved in glycogen metabolism. It is expected that lipid metabolism is similarly regulated through additional, lipid-specific PP1 scaffolding proteins.

Conclusions

We have focused much of our recent work on trying to understand the mechanisms that account for the remarkable specificity of insulin's regulation of intermediary metabolism. This specificity may be the result of the compartmentalization of both signal initiation at the cell surface as well as signal reception at the glycogen pellet or lipid droplet. We have identified a novel, insulin-stimulated signalling pathway which results in the tyrosine phosphorylation of caveolin, a structural component of unique plasma membrane domains termed caveolae. In addition, we have identified a novel targeting protein, PTG, which forms a distinct, insulin-sensitive pool of PP1 complexed with the enzymes regulating glycogen metabolism. It is still unknown whether these two pathways are linked. However, exploration of the molecular details underlying the specificity of signal initiation and signal reception in insulin action may elucidate the complex biochemical pathways leading from the binding of insulin at the cell surface to the regulation of intermediary metabolism.

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