

Insulin and insulin antagonists evoke phosphorylation of P20 at serine 157 and serine 16 respectively in rat skeletal muscle

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Abstract We show here that insulin and insulin antagonists differentially modify phosphorylation of three phospho-isoforms of P20 (termed S1, S2 and S3) in rat skeletal muscle. Precise phosphorylation sites of S1 and S2 were mapped to serine 157 and serine 16 respectively. Insulin evoked phosphorylation of P20 at serine 157 through the phosphatidylinositol (PI) 3-kinase pathway. Epinephrine and calcitonin gene-related peptide decreased phosphorylation at serine 157 and increased phosphorylation at serine 16 and other unidentified sites. These results demonstrate that the PI-3-kinase pathway of anabolic insulin and the cAMP pathway of catabolic hormones converge on P20 and suggest a potential role of this protein in regulating energy metabolism of skeletal muscle.

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Key words: Insulin; P20; Epinephrine; Calcitonin gene-related peptide; Phosphorylation; Signal transduction

1. Introduction

In animals, hormones play a central role in controlling intracellular metabolism. Catabolic hormones such as epinephrine, glucagon, calcitonin gene-related peptide (CGRP) and amylin, can evoke the cAMP-mediated signalling cascade in their respective target tissues, thus inhibiting glycogen synthesis and promoting glycogenolysis and lipolysis [1–3]. On the other hand, insulin increases the synthesis and storage of carbohydrate, lipid and protein and also inhibits their degradation. Insulin initiates its biological functions on target tissues by binding with the α -subunits of its receptor on the plasma membrane and activating the intrinsic tyrosine kinase activity of the β -subunit [4]. This is followed by the autophosphorylation of the receptor and the tyrosine phosphorylation of its substrates, IRSs (insulin receptor substrates), which in turn creates binding sites for several signalling molecules/adapters including the phosphatidylinositol 3-kinase (PI-3-kinase) regulatory subunit, Shc-1 and Grb2. Subsequently a variety of serine/threonine protein kinases, such as MAPK (mitogen-activated protein kinase), PI-3-kinase, protein kinase B (PKB) and p70 S6 kinase (p70^{S6k}), are activated to transmit and

amplify the signals of insulin. These multiple protein kinase cascades exist in parallel but functionally interlink to co-ordinately regulate insulin's final metabolic functions.

Anabolic insulin and the catabolic hormones can antagonise each other's metabolic functions by cross-regulating their respective signal transduction cascades [5–7]. For example, the cAMP-mediated pathway inactivates glycogen synthase and activates glycogen phosphorylase by evoking their phosphorylation. On the other hand, insulin acts conversely by promoting dephosphorylation or inhibiting phosphorylation of these two enzymes [8,9]. Although the actions of insulin and its physiological antagonists are believed to be mediated by phosphorylation/dephosphorylation of key intracellular proteins and a great number of protein kinases/phosphatases have been identified, relatively few downstream phosphoproteins have been isolated.

Recently, we have demonstrated that phosphorylation of P20 is responsive to amylin in rat skeletal muscle [10]. P20 is a small heat shock-related protein which is mainly expressed in skeletal muscle [11]. Here, we provide evidence that signalling pathways of insulin and the counter-regulatory hormones epinephrine and CGRP also converge on P20, and that these hormones differentially modify the phosphorylation of the three isoforms of this protein. The phosphorylation sites for each isoform have been characterised and the upstream events responsible for these phosphorylations have been analysed and discussed.

2. Materials and methods

2.1. Preparation of ³²P-labelled muscle strips and two-dimensional gel electrophoresis (2-DE)

Rat extensor digitorum longus (EDL) muscle strips were prepared and radiolabelled with [³²P]orthophosphate (ICN) as previously described [10]. The labelled muscle strips were stimulated with human insulin (Actrapid, Novo Nordisk), epinephrine (David Bull laboratories), rat CGRP-1 (CGRP; Bachem, Torrance, CA) for 1 h at stated final concentrations. The muscle samples were then solubilised and aliquots of lysates with equivalent amounts of radioactivity were separated by 2-DE as before [10]. After electrophoresis the proteins were visualised using autoradiography, phosphorimaging or staining with Coomassie brilliant blue (CBB). In all figures, the gels are displayed with the acid end of the isoelectric focusing dimension to the right and the direction of SDS-PAGE from top to bottom. All the results presented are based on at least three independent experiments.

2.2. Isolation of the tryptic phosphopeptides by reversed-phase high performance liquid chromatography (RP-HPLC)

³²P-labelled proteins separated by CBB-stained preparative 2-DE were excised and gel pieces were subjected to in-gel trypsin digestion as described by Rosenfeld et al. [12]. The digested peptides were fractionated by RP-HPLC on a Jupiter 5 μ C18 column (25 \times 0.2 cm, Phenomenex). The pre-heated column (37°C) was washed for 10 min with 0.1% trifluoroacetic acid (v/v) followed by elution using a 30-min

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Abbreviations: CGRP, calcitonin gene-related peptide; PI-3-kinase, phosphatidylinositol 3-kinase; 2-DE, two-dimensional electrophoresis; EDL, extensor digitorum longus; CBB, Coomassie brilliant blue R250; RP-HPLC, reversed-phase high pressure liquid chromatography

linear gradient from 4% acetonitrile to 56% acetonitrile at a flow rate of 300 μ l/min. Fractions were collected manually and 32 P-labelled phosphopeptides were detected by liquid scintillation counting.

2.3. Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF/MS)

Aliquots of phosphopeptide separated by RP-HPLC were mixed with an equal amount of α -cyano-4-hydroxycinnamic acid. The mixture was loaded on the probe tip and vacuum-crystallised by using the sample preparation accessory (Hewlett-Packard). The MALDI mass spectrum of the peptide was then obtained on a G2025A MALDI-TOF mass spectrometer (Hewlett-Packard). Mass analysis of Glu-C-digested peptide mixtures was performed using a PerSeptive Biosystems Voyager DE mass spectrometer; this instrument was operated in the delayed extraction mode with external calibration against a series of synthetic peptides.

2.4. Two-dimensional phosphopeptide mapping

Trypsin-digested 32 P-radiolabelled peptide mixtures from three phosphoforms of P20 were lyophilised and solubilised in 10 μ l of electrophoresis buffer (1% pyridine/10% acetic acid, pH 3.5). The dissolved sample solution was spotted in the middle of a thin layer chromatography plate (20 cm \times 20 cm; Sigma) 4 cm from the bottom with a trace of basic Fuchsin dye. Electrophoresis was carried out at 350 V, until the basic Fuchsin dye had migrated 2.5 cm towards the cathode. The plates were air-dried and subjected to the perpendicular dimensional chromatography in pyridine/butanol/acetic acid/water (10:15:3:12, v/v) until the solvent front reached the top of the plate. Plates were air-dried and phosphopeptides detected by autoradiography.

2.5. Phosphate release analysis

Peptides were sequenced with a Hewlett-Packard G1000A protein sequencer utilising Routine 3.1 Edman degradation chemistry as recommended by the manufacturer. Phosphopeptides were covalently linked to Sequelon AA filters using the Sequelon AA Reagent Kit (Millipore), and phosphate was extracted at each cycle with three 0.5-ml volumes of 90% methanol, 0.015% phosphoric acid as the solvent in the Routine 3.1 PVDF method. Extracted cycles were diverted via valve number RV6 (line 61), collected directly into fraction collector tubes, and counted on a liquid scintillation counter.

3. Results

3.1. P20 is a common target of insulin and several antagonistic hormones

We have recently found that phosphorylation of three isoforms of P20 is modified by amylin [10]. To further investigate

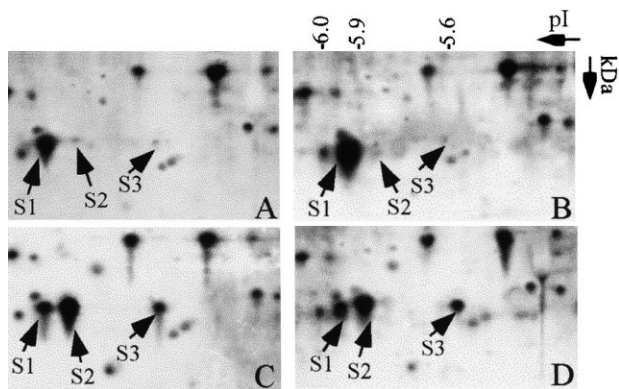


Fig. 1. Phosphorylation of P20 is associated with actions of insulin and two antagonistic hormones. 32 P-radiolabelled EDL muscle strips were incubated for 1 h (A) without or (B) with 100 nM insulin, (C) 100 nM epinephrine, or (D) 100 nM CGRP. Muscle lysates with equivalent amounts of radioactivity were separated by 2-DE and the phosphorylation of the three isoforms of P20 was quantitated by phosphorimaging.

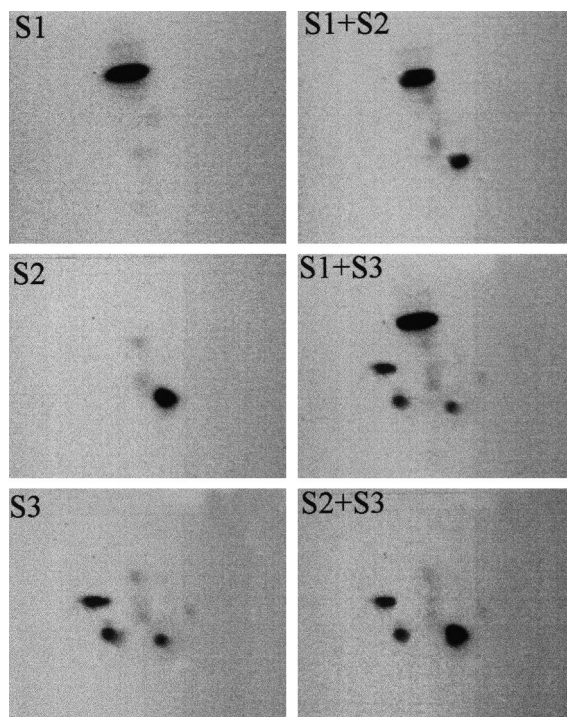


Fig. 2. Comparison of two-dimensional tryptic phosphopeptide maps obtained from the three major phosphorylated species of P20. 32 P-radiolabelled muscle lysates were resolved by 2-DE and the spots corresponding to the three phosphorylated isoforms of P20 (S1, S2 and S3) were excised from the gels and digested by trypsin. The tryptic products from S1, S2 and S3, or their respective mixtures (S1+S2, S1+S3, S2+S3), were then separated by two-dimensional phosphopeptide mapping and analysed by autoradiography as described in Section 2.

the effects of insulin and the antagonistic hormones, epinephrine and CGRP, on phosphorylation of P20, proteins extracted from 32 P-labelled rat EDL muscle strips stimulated by these hormones were separated by analytical 2-DE. Quantitative analysis using phosphorimaging revealed that these metabolic hormones reproducibly alter phosphorylation of the three isoforms of P20 (termed S1 with pI 6.0, S2 with pI 5.9 and S3 with pI 5.6). Insulin increased phosphorylation of S1 by 2.3-fold compared with that of control muscles (Fig. 1A,B). In contrast, epinephrine decreased phosphorylation of S1 by 1.9-fold but increased phosphorylation of S2 and S3 by 8.2-fold and 3.7-fold respectively (Fig. 1C). The effects of CGRP on phosphorylation of S1, S2 and S3 were similar to that of epinephrine (Fig. 1D). Thus we concluded that the signalling cascades of insulin and several of its physiological antagonists converge on P20 and differentially influence the phosphorylation of this protein.

3.2. Two-dimensional phosphopeptide mapping of *in vivo* phosphorylated isoforms of P20

To directly analyse the patterns of radioactive peptides generated from the three phosphorylated isoforms of P20, 32 P-radiolabelled S1, S2 and S3 were individually excised from the preparative 2-DE gels and trypsin-digested as above. The tryptic peptide mixtures were separated by two-dimensional phosphopeptide mapping and detected by autoradiography. The results showed that both S1 and S2 contained only a

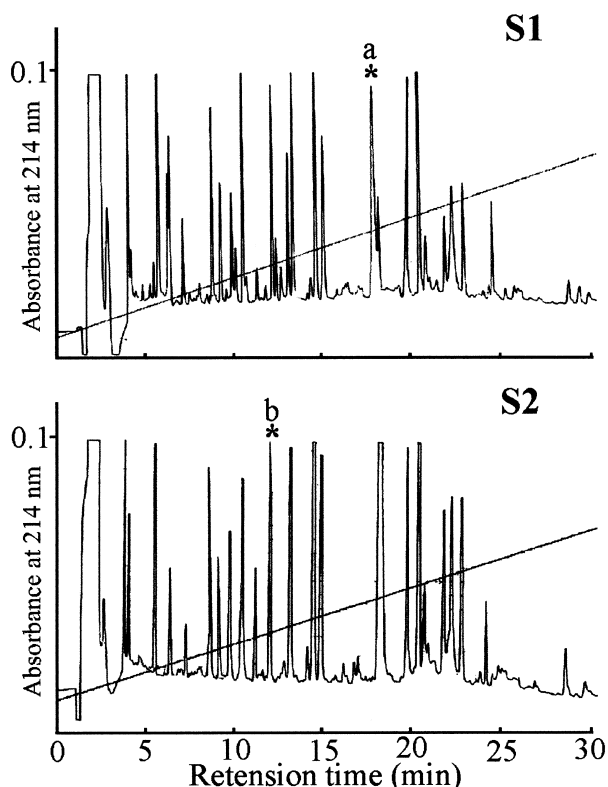


Fig. 3. Purification and identification of ^{32}P -labelled tryptic phosphopeptides of P20. Two ^{32}P -radiolabelled isoforms of P20 (S1, S2) were excised from 2-DE gels, digested by trypsin and separated by RP-HPLC as described in Section 2. The radioactivity of each fraction was determined by liquid scintillation counting and the fractions containing ^{32}P are indicated by a and b.

single prominent ^{32}P -labelled phosphopeptide (Fig. 2). When the tryptic digests from S1 and S2 were mixed and run together, the resulting phosphopeptides migrated as different spots on the plate, suggesting that phosphorylation sites of S1 and

S2 are different. In contrast, three ^{32}P -labelled phosphopeptides were detected from the tryptic peptide mixture of S3. One of these phosphopeptides co-migrated with that of S2. The positions of the other two peptides were distinct from those of S1 and S2. These data indicate that the phosphorylation sites of these three isoforms of P20 are different from each other.

3.3. Isolation and identification of the phosphopeptides from S1 and S2

To precisely determine which tryptic peptides were phosphorylated, the tryptic digests of ^{32}P -labelled S1 or S2 derived from 18 pieces of 2-DE gels were separated by RP-HPLC and aliquots from each fraction were counted for ^{32}P -radioactivity. The result showed that fraction a, from S1 (termed S1-a) and fraction b, from S2 (termed S2-b) contained ^{32}P -radio-labelled phosphopeptides (Fig. 3). Fraction S1-a was eluted at 17.5 min and 35% acetonitrile while fraction S2-b was eluted at 12 min and 24% acetonitrile. Analysis using two-dimensional phosphopeptide mapping revealed that the distribution patterns of S1-a and S2-b on TLC plates were exactly the same with their corresponding tryptic peptide mixtures (data not shown), further confirming that only a single tryptic peptide was phosphorylated in S1 and S2. Subsequent phosphoamino acid analysis showed that both the isolated phosphopeptides are phosphorylated on serine residues (data not shown).

MALDI-TOF/MS analysis was carried out to determine the identity of the two purified phosphopeptides from S1 and S2. This result revealed that fraction S2-b contained a single tryptic peptide with molecular mass of 1494.1 Da (Fig. 4A), which is 81 Da larger than the theoretical mass of the tryptic peptide between residues 14 and 27 of P20, suggesting that this peptide is mono-phosphorylated (Table 1). In fraction S1-a, two peptides with molecular masses of 3846.9 Da and 4166.5 Da respectively were detected by MALDI-TOF/MS analysis (Fig. 4B). The masses of these two peptides are 81 Da larger than the predicted masses of the incomplete trypsin cleavage prod-

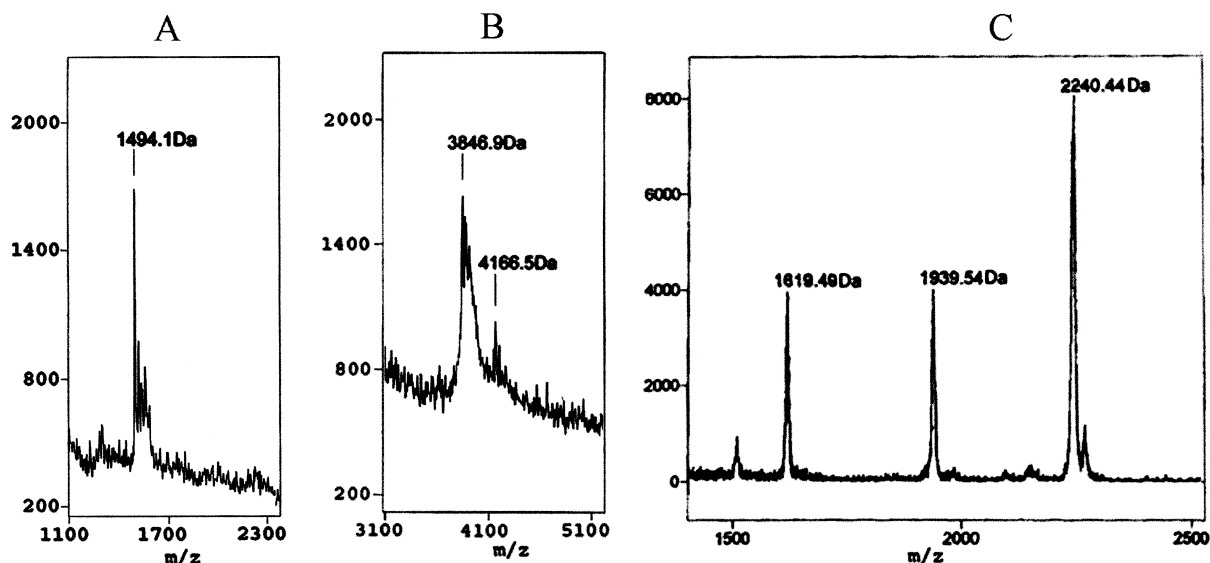


Fig. 4. Mass spectra of the ^{32}P -labelled phosphopeptides purified in Fig. 3. Fraction S2-b (A) and fraction S1-b (B) were analysed by MALDI-TOF/MS as described in the text. The phosphopeptide S1-a was further digested with Glu-C proteinase (0.5 μg in 50 μl) for 16 h at 37°C in 20 mM NH_4HCO_3 , pH 8.0 and analysed by mass spectrometry 4(C).

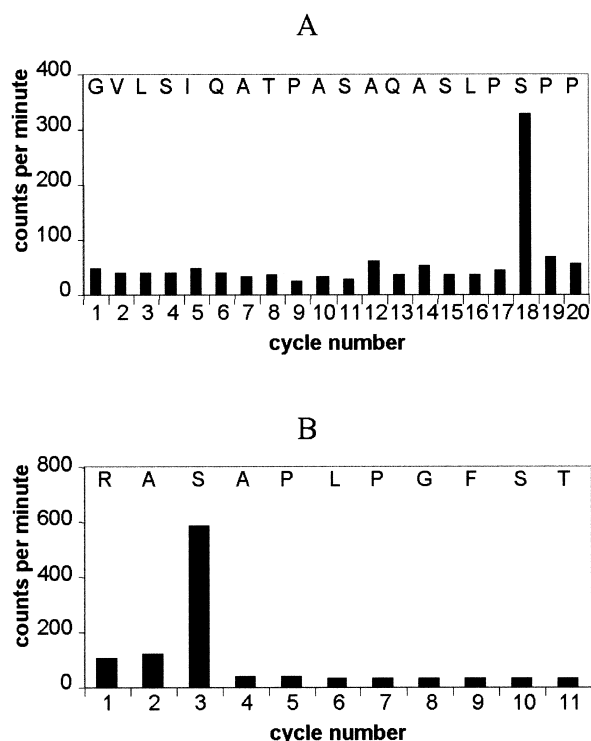


Fig. 5. Phosphate release analysis of the ^{32}P -labelled peptides purified from S1 and S2. Fraction S2-b (A) or Glu-C-digested fraction S1-a (B) were subjected to N-terminal sequencing and ^{32}P released from each cycle was quantitated. The corresponding amino acid residues released from each cycle are shown at the top of each chart.

uct corresponding to residues 121–162 and complete tryptic product from residues 123–162 respectively (Table 1). This result indicated that a single residue between 123 and 162 is phosphorylated. There are six serine residues within this region. To generate shorter ^{32}P -labelled peptides, the original tryptic peptide in fraction S1-a was further digested with endopeptidase Glu-C. MS analysis indicated that the Glu-C protease-digested mixture contained three major peptides (Fig. 4C). Peptides with masses of 1619.49 Da and 1939.54 Da can be assigned to fragments corresponding to residues 123–139 and residues 121–139 respectively (Table 1). The mass of 2240.44 Da matched the mass of the mono-phosphorylated peptide containing residues 140–162, suggesting that the phosphorylated residue is located within this region.

3.4. The precise phosphorylation sites of S1 and S2 are located at serine 157 and serine 16 respectively

The above results revealed that the phosphorylation of

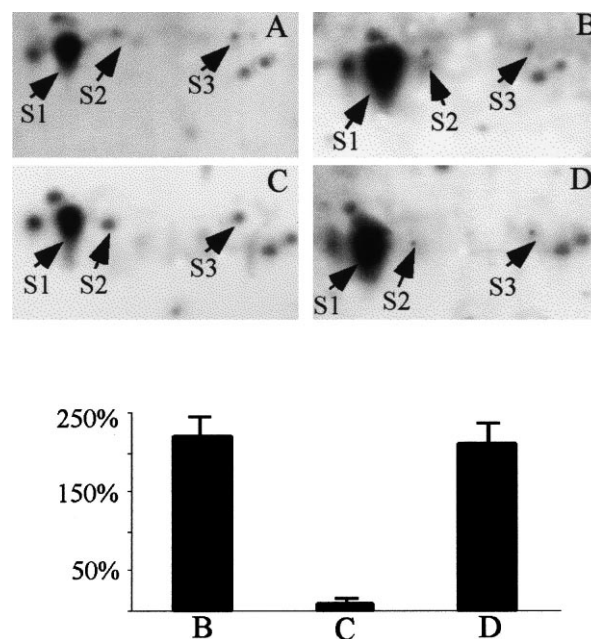


Fig. 6. Effects of wortmannin and rapamycin on insulin-evoked phosphorylation at serine 157. ^{32}P -prelabelled muscle strips were (A) untreated, treated with (B) 100 nM insulin, (C) 100 nM insulin plus 1 μM wortmannin, or (D) 100 nM insulin plus 200 nM rapamycin for 1 h. Wortmannin and rapamycin were added 30 min before the addition of insulin. Muscle lysates were separated by 2-DE and analysed by phosphorimaging. The bar chart represents the increased percentage of radioactivity in P20 isoform S1 relative to that in the untreated muscle strips.

S1 and S2 occurred within residues (GVLSIQATPASAQASLPSP) from 140 to 162 and residues (RASAPLPGFST) from 14 to 27 individually. To determine the precise phosphorylation sites for the two isoforms, the phosphopeptides in S2-b and Glu-C digested S1-a were subjected to automated solid-phase N-terminal sequence analysis and ^{32}P released from each cycle of Edman degradation was counted for radioactivity. The results showed that the majority of ^{32}P was released at cycle 3 for S2-b and cycle 18 for Glu-C digested S1-a (Fig. 5). Thus, we conclude that the phosphorylated residues of S1 and S2 are located at serine 157 (LPSP) and serine 16 (RRAS) respectively. Interestingly, serine 157 is located near the COOH-terminus of P20 while serine-16 is adjacent to the NH_2 -terminus.

3.5. Insulin-evoked phosphorylation of S1 is a downstream event of PI-3-kinase activation

The downstream events of the insulin-evoked PI-3-kinase signalling cascade play a central role in controlling fuel me-

Table 1
Mass determinations of ^{32}P -labelled phosphopeptides from S1 and S2

Mass (observed)	Mass (theoretical)	Mass (theoretical+Pi)	Position	Peptide sequence
1494.1 Da	1413.6 Da	1493.6 Da	14–27	RASAPLPGFSTPGR
3846.9 Da	3765.2 Da	3845.2 Da	123–162	LPFGVDPAAVTSALSPEGVLSIQATPASAQASLPSPPAK
4166.5 Da	4084.6 Da	4164.6 Da	121–162	YRLPPGVDPAAVTSALSPEGVLSIQATPASAQASLPSPPAK
1619.4 Da	1620.8 Da		123–139	LPFGVDPAAVTSALSPE
1939.5 Da	1941.2 Da		121–139	YRLPPGVDPAAVTSALSPE
2240.4 Da	2163.4 Da	2243.4 Da	140–162	GVLSIQATPASAQASLPSPPAK

tabolism. To investigate whether the insulin-evoked phosphorylation of P20 at serine 157 is a downstream target of PI-3-kinase, pre-radiolabelled muscle strips were treated with insulin, or insulin plus the PI-3-kinase selective inhibitor wortmannin, or the p70^{s6k} kinase inhibitor rapamycin, individually. The phosphorylation of P20 was analysed by 2-DE and phosphorimager as above. The results showed that insulin-induced increase in phosphorylation of S1 was completely blocked by wortmannin but not by rapamycin (Fig. 6). This suggests that insulin phosphorylates P20 at serine 157 via the PI-3-kinase pathway but p70^{s6k} kinase is not directly responsible for the phosphorylation.

4. Discussion

Regulation of intracellular metabolism by hormones is often mediated through phosphorylation/dephosphorylation of key phosphoproteins. The results of the present study provide evidence to demonstrate that multiple phosphorylation of P20 is associated with actions of insulin and several of insulin antagonists, in rat hind limb skeletal muscle. Rat P20 is composed of 162 amino acid, including 14 serine, 5 threonine and 4 tyrosine residues [11,13]. 2-DE analysis of ³²P-labelled muscle lysates displayed three phosphorylated isoforms of P20 (S1, S2, S3) with different pI values. Two-dimensional phosphopeptide mapping revealed that phosphorylation of P20 at different sites accounted for the production of these three phosphorylated isoforms. The tryptic phosphopeptides from S1 and S2 were isolated by RP-HPLC and were further shown to be the mono-phosphorylated fragments corresponding to residues 14–27 and 121–162 respectively. The precise phosphorylation sites of S1 and S2 were unequivocally mapped to serine 157 (L-P-S-P-P) and serine 16 (R-R-A-S) by N-terminal sequencing of the purified ³²P-labelled phosphopeptides. We were unable to directly identify the phosphorylation sites of S3 due to its low abundance in CBB-stained gels. However, two pieces of evidence suggested that serine 16 of S3 was also phosphorylated. (I) Two-dimensional phosphopeptide mapping demonstrated that one of the tryptic phosphopeptides of S3 co-migrated with that of S2. (II) Phosphorylation of S2 and S3 simultaneously responded to stimulation by the same hormone.

The sequence surrounding serine 16 (R-R-A-S) exactly matches the recognition motif of protein kinase A [14]. Moreover we found that forskolin also triggers the phosphorylation of P20 at this site in skeletal muscle (data not shown). We therefore conclude that epinephrine, CGRP and amylin evoke phosphorylation of P20 at serine 16 via increasing cAMP-mediated protein kinase A activity. Insulin-evoked phosphorylation of P20 at serine 157 (L-P-P-S-P) is an event downstream of PI-3-kinase, since this action was completely blocked by wortmannin, a selective and irreversible inhibitor of PI-3-kinase [15] (Fig. 6). It is now apparent that activation of PI-3-kinase is indispensable and in some cases sufficient to elicit insulin's actions in controlling fuel metabolism [16]. Several kinases involved in insulin's metabolic functions, such as PDK, PKB/cAkt and p70^{s6k}, act as the downstream effectors of PI-3-kinase [4]. However, LPPSP does not explicitly conform to the substrate recognition motif of these kinases. Further study is required to identify the kinase that directly responsible for insulin-induced phosphorylation of P20 at serine 157.

Several recent studies have suggested that function of P20 might be linked to muscle contractile activity [17–19]. Phosphorylation of P20 was responsive to stimulation by agents that induce either contraction or relaxation of muscle. Interestingly, histamine- and phorbol ester-induced contractions of bovine carotid artery smooth muscle are associated with an increase in phosphorylation of a P20 isoform which is equivalent to insulin-induced S1 in rat skeletal muscle [20]. Both CGRP and amylin have the ability to regulate muscle contractile activity, such as stimulation of vasorelaxation [21] and inhibition of electrically stimulated muscle contraction [22,23]. Notably, the contractile status of muscle is also an important factor in regulating glucose metabolism [24]. Insulin and muscle contraction can enhance each other's sensitivity and synergistically stimulate glucose transport [25,26]. Moreover, both insulin- and contraction-stimulated glucose transport can be blocked by wortmannin [27] and calcium channel blocker [28]. Based on these observations, we suggest that the signalling cascades evoked by stimuli leading to muscle contraction/relaxation stimuli, as well as insulin and catabolic hormones such as epinephrine, CGRP and amylin, might converge on P20, and, by regulating the phosphorylation of this protein, contribute to the co-ordinate regulation of muscle functions such as contractile activity and energy metabolism. Further study is required to test this hypothesis.

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