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PHOSPHORYLATION OF GLYCOGEN SYNTHASE BY A BOVINE THYMUS PROTEIN-TYROSINE KINASE, p40

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SUMMARY: Glycogen synthase from rabbit skeletal muscle was found to be phosphorylated by a protein-tyrosine kinase, p40, purified from bovine thymus. The phosphorylation, to a stoichiometry of 0.4-0.5 mol/mol subunit, was specific for a single tyrosine residue in the sequence EEDGERYDEDEE. This acidic sequence has considerable similarity to the site recognized by p40 in erythrocyte band 3 protein. In the analysis of the phosphorylated peptide, it was noted that the sequence -RY(P)- impeded cleavage by either trypsin or automatic Edman degradation.

Multiple phosphorylation of the enzyme glycogen synthase at serine (1,2) and, in some cases, threonine (3) residues is a well established phenomenon that is believed to be of importance in the regulation of enzyme activity by hormones (1,2). Over ten such phosphorylation sites have been identified *in vitro* in the rabbit skeletal muscle enzyme (1) and many of these are modified *in vivo*. Most of the phosphorylation sites are located in a COOH-terminal CNBr fragment, CB-2, that is 124 residues long. This domain of the protein is characterized by a high proportion of Ser and Pro residues, as well as acidic amino acids. Modification of the sites 3a, 3b and 3c, located between residues 30-38 of CB-2, by the enzyme GSK-3 is the most potent phosphorylation in terms of inactivating the enzyme. Cyclic AMP-dependent protein kinase preferentially recognizes two serines, sites 1a and 1b (residues 87 and 100), in CB-2. Phosphorylation of CB-2 by cyclic AMP-dependent protein kinase has modest or no effect on enzyme activity (1,4). In the present

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report, we describe the modification of a specific Tyr residue, also in the COOH-terminal region of glycogen synthase.

The discovery of tyrosine specific protein phosphorylation, and the possible correlation with the control of cell growth and differentiation, has provoked a significant quest for physiological substrates of protein-tyrosine kinases (5). In addition, it is of importance to understand the structural features of substrates recognized by protein-tyrosine kinases. The protein kinase p40 (6) is one of a limited number of tyrosine specific protein kinases that has been purified directly from mammalian tissue. Erythrocyte band 3 protein is an effective substrate for p40 and one modified tyrosine is located in the sequence MEELQDDYEDDME (7-9). The present work defines a new substrate in vitro for p40 and confirms the requirement for acidic residues in the vicinity of the modified tyrosine residue.

EXPERIMENTAL PROCEDURES

Enzymes. Glycogen synthase was purified from rabbit skeletal muscle following the procedure of Takeda *et al.* (10) as modified by Ahmad *et al.* (11). The protein-tyrosine kinase p40 was purified from bovine thymus as described by Zioncheck *et al.* (6). The p40, 50 µg/ml, was stored in 10 mM HEPES, 100 mM NaCl, 50% glycerol, pH 7.4 at -20 °C.

Protein Phosphorylation. Glycogen synthase phosphorylation followed published methods (12) modified to accommodate the p40 protein kinase for which Mn rather than Mg is the preferred divalent cation. Glycogen synthase, 50 µg, was incubated in 50 mM HEPES, pH 7.4, with 0.11 mM [γ^{32}P]ATP (2,000-6,000 cpm/pmol), and 5 mM $MnCl_2$. The reaction, conducted at 30°C, was initiated by the addition of 5 µl of p40 to give a final volume of 50 µl. Phosphorylation was quantitated by a chromatographic method (assay 2 of Ref. 12). To measure glycogen synthase activity, parallel phosphorylation reactions were run using unlabelled ATP and aliquots were assayed (final dilution 1:300) by the method of Thomas *et al.* (13).

Analysis of Phosphopeptides. Glycogen synthase was phosphorylated as described above and the reaction terminated by the addition of trichloroacetic acid to a concentration of 10 % (w/v). After 2 h at 0°C, the precipitated protein was collected by centrifugation for 5 min in a microfuge. The pellet was washed three times with cold 10% (w/v) trichloroacetic acid and then with cold diethyl ether. After brief drying under vacuum, the protein was suspended in 100 mM ammonium bicarbonate, 50 mM β-mercaptoethanol, pH 8. The protein was incubated for 16h with trypsin (10:1 protein:trypsin) at 30°C. Trpytic peptides were separated by reverse phase HPLC. The first system, used both analytically and preparatively, was an acetonitrile/phosphate gradient with a Synchrom RP C18 column (4.6 X 250 mm) as has been described for separating various glycogen synthase phosphopeptides (14). The second system used for further

purification of the phosphopeptide utilized a Beckman C18 XL-ODS cartridge column (4.1 X 75 mm) with a 25 min linear gradient from 0-100% acetonitrile in the presence of 0.05% trifluoroacetic acid.
Other Methods. Purified peptide was subjected to automatic Edman degradation using an Applied Biosystems model 477A instrument with on-line HPLC analysis of PTH-amino acid derivatives. Phosphoamino acids were identified in protein or peptide partial hydrolysates by standard thin layer electrophoretic separation. Polyacrylamide gel electrophoresis followed previously described methods (15).

RESULTS

The p40 protein-tyrosine kinase was able to phosphorylate rabbit skeletal muscle glycogen synthase (Fig. 1) and, based on phosphoamino acid analysis, the phosphate was associated with phosphotyrosine (not shown). In these studies, the maximum stoichiometry of phosphorylation obtained was in the range 0.4-0.5 mol phosphate/mol subunit. Treatment of phosphorylated glycogen synthase with CNBr followed by polyacrylamide gel electrophoresis indicated that the phosphate was located only in the larger CNBr-fragment, CB-2, which corresponds to the COOH-terminus of the enzyme subunit (not shown). Analysis of a tryptic digest of phosphorylated glycogen synthase by reverse phase HPLC also showed a single dominant tryptic phosphopeptide (Fig. 2). The tryptic phosphopeptide was purified to homogeneity by two reverse phase HPLC steps (see "Experimental Procedures"). Phosphoamino acid analysis of the purified phosphopeptide confirmed that phosphotyrosine, but not phosphoserine or phosphothreonine was present (not shown).

When the peptide was subjected to automatic Edman degradation (Table I), sequence was obtained through nine cycles but at the tenth and at successive cycles no signal for any PTH-amino acid was detected. The sequence obtained, DGLPEEDGE, corresponded to residues 54-62 of the sequence for the CB-2 fragment reported by Cohen and colleagues (1). This result confirmed phosphorylation in the CB-2 fragment of glycogen synthase. However, sequence analysis stopped short of the tyrosine at residue 64 that would be a candidate for phosphorylation by p40. The phosphopeptide was therefore subjected to dephosphorylation by alkaline phosphatase (16,17). The peptide was re-purified by HPLC using a Beckman XL-ODS column from which the ^{32}P eluted in the wash through, and the retention time of the peptide was 6 min greater than that of its phosphorylated parent. The dephosphorylation appeared complete since no phospho-peptide was

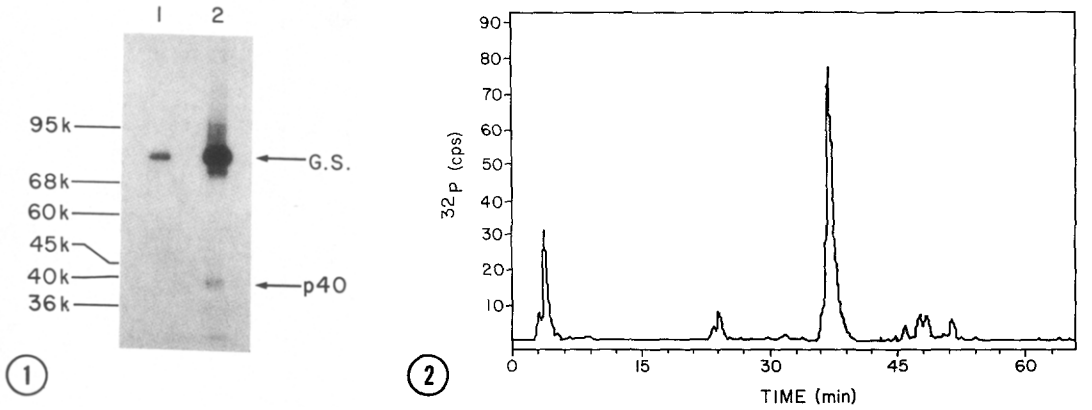

Figure 1. Phosphorylation of Glycogen Synthase by p40. An autoradiogram of polyacrylamide gel electrophoresis of glycogen synthase incubated with p40, as descibed under "Experimental Procedures", is shown. Track 1 corresponds to 2 µg of synthase; Track 2 to 5 µg. Numbers at the side indicate apparent M_r in KDa. Note the autophosphorylation of p40 evidenced by the 40 KDa labelled species.

Figure 2. HPLC Profile of Tryptic ^{32}P-Phosphopeptides of Glycogen Synthase. Glycogen synthase, phosphorylated with p40 and [γ^{32}P]ATP, was digested with trypsin and the mixture chromatographed using reversed-phase HPLC, (see "Experimental Procedures"). Radioactivity was detected using an in-line scintillation monitor (Ramona D).

observed following this treatment. Analysis of the dephosphorylated peptide by automatic Edman degradation led to the clear identification of Arg and Tyr at cycles 10 and 11 respectively.

Table 1: Sequence Analysis of Glycogen Synthase Tryptic Phosphopeptide derived after p40 action[1]

Phosphopeptide:	D G L P E E D G E[2]
Dephosphorylated[3] Peptide:	D G L P E E D G E R Y {D E D E E A A} K
Glycogen synthase[4]:	R D G L P E E D G E R Y D E D E E A A K

[1] PTH-residues of amino acids were identified as described under Experimental Procedures.

[2] After this step in the sequencing run, no identifiable signal was detectable.

[3] Peptide treated with alkaline phosphatase. {} indicates traces of the indicated PTH derivative.

[4] Known sequence of glycogen synthase CB-2 from Arg-53 to Lys-72 (Ref.1).

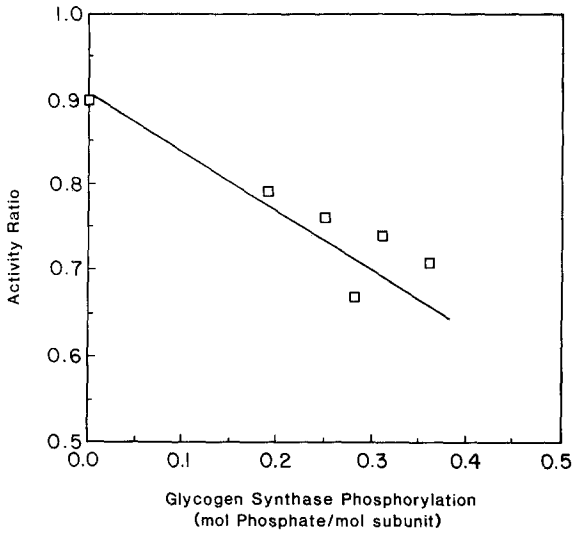

Figure 3. Effects of Phosphorylation of Glycogen Synthase by p40 on enzyme activity. The data are taken from a time course of phosphorylation (0-120 min) in which a parallel incubation without radioactivity was assayed for -/+ glucose-6-P activity ratio.

Signals consistent with the sequence DEDEEAAK were seen to follow thereafter (Table 1). The peptide was thus identifiable as DGLPEEDGERYDEDEEAAK corresponding to residues 54-72 of CB-2 of rabbit muscle glycogen synthase with Tyr-64 the residue modified by p40. The effect of the tyrosine phosphorylation on glycogen synthase activity was monitored (Fig. 3). At a stoichiometry of 0.4 phosphates/subunit, the activity ratio -/+ glucose-6-P decreased from 0.9 to 0.7.

DISCUSSION

The present study revealed two properties of peptides containing phosphotyrosine that we had not anticipated. First, the fact that the phosphopeptide contained an arginine residue in the sequence -Arg-Tyr(P)- suggests that the phosphorylation of the tyrosine blocked trypsin cleavage at the adjacent arginine. There are several known examples where phosphoserine impedes trypsin action at NH_2-terminal basic residues though generally in the sequence Arg/Lys-X-Ser(P) (e.g. 18). The second observation was that the same sequence, Arg-Tyr(P), appeared to have a deleterious effect on the Edman degradation reaction. These two results could be explained if the sequence Arg-Tyr(P) adopts a particularly stable local structure, possibly due to electrostatic interactions, and therefore renders

cleavage by trypsin or Edman degradation less effective. It is unlikely that there is any covalent bond between the two residues since alkaline phosphatase was able to release phosphate and then permit Edman degradation in which arginine and tyrosine were detected normally.

The results show that p40 recognizes a unique tyrosine residue in glycogen synthase. This conclusion is based on the fact that all of the phosphorylation was localized to CB-2 and to a unique tryptic peptide. The latter contained a single tyrosine. From the viewpoint of the control of glycogen synthase, it is of interest that this tyrosine is located in what is the main regulatory domain of glycogen synthase, a region of the molecule that must be exposed and available for interaction with protein kinases. The effect of the tyrosine phosphorylation on glycogen synthase activity is much less than that resulting from phosphorylation of the nearby Ser/Pro rich region containing sites 3, 4, and 5 (15) Nonetheless, in terms of change in activity per mole of phosphate introduced, the inactivation is comparable to that observed for phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. No tyrosine phosphorylation of glycogen synthase *in vivo* has so far been reported although it is also true that the methods used so far were not designed specifically to avoid dephosphorylation at tyrosine residues. Thus, it is necessary to re-examine this question.

Whether or not glycogen synthase is a physiological substrate for p40, the present study is of particular interest with respect to the specificity of protein-tyrosine kinases since we were able to identify the tyrosine residue phosphorylated. The CB-2 region of glycogen synthase alone contains four tyrosines, with 24-32 others predicted from amino acid composition studies of the whole subunit (10,19). The sequence surrounding the modified tyrosine is similar to the p40 site in band 3 protein:

Band 3 protein: M-E-E-L-Q-D-D-Y(P)-E-D-D-M-E-
Glycogen synthase: P-E-E-D-G-E-R-Y(P)-E-D-E-E-A-

In both sequences there are acidic residues both COOH- and NH_2-terminal to the modified tyrosine. In fact, experience with the substrate specificity of p40 to date indicates relatively stringent recognition determinants.

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