INSULIN-DEPENDENT PROTEIN PHOSPHORYLATION IN MEMBRANES

Isolation and characterization of a phosphorylated proteolipid from sarcolemma

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1. Introduction

Insulin can decrease as well as increase phosphorylation of specific plasma membrane proteins in target cells. Direct addition of insulin to adipocyte plasma membranes decreased phosphorylation of a protein M_r 120 000 [1] and increased phosphorylation of a protein M_r 61 000 [2]. In liver plasma membranes insulin decreased phosphorylation of 2 proteins M_r 120 000 and M_r 60 000 [3]. In liver plasma membranes it has also been shown that insulin inhibits the cyclic AMP-dependent phosphorylation of 2 integral proteins M_r 140 000 and M_r 80 000 while insulin in the presence of cyclic AMP increased phosphorylation of 3 peripheral proteins M_r 52 000, 28 000 and 14 000 [4]. Insulin decreases phosphorylation of a protein M_r 90 000 (unpublished) and increases phosphorylation of protein M_r 15 000 [5] in sarcolemma membranes. The effect of insulin on phosphorylation of the latter protein was enhanced by addition of GTP [6]. Control of membrane protein phosphorylation by insulin may be of importance in the initial steps of insulin action. This indicates investigation of the composition of the insulin sensitive phosphorylated membrane proteins. We report here the isolation of the insulin-dependent phosphorylated protein M_r 15 000 from sarcolemma membranes. This protein has now been characterized as a proteolipid consisting of 3 different polypeptide subunits associated with phospholipids. Only one of the polypeptide subunits M_r 3600 is phosphorylated.

2. Experimental

Sarcolemma membranes prepared as in [5] were incubated with [γ-^{32}P]ATP + Mg^{2+}. The [^{32}P]phosphorylated low M_r protein was purified by gel filtration on Sephadex G-100, extracted with acid chloroform/methanol, chromatographed on Sephadex LH-20 according to [7,8] and finally subjected to gel filtration on Sephadex G-25. In a typical experiment sarcolemma membranes (3–4 mg protein) were suspended in 50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and incubated with 100 μM [γ-^{32}P]ATP + 20 mM $MgCl_2$ for 30 s at 30°C in a total volume of 1.0 ml. The reaction was terminated with 10% trichloroacetic acid containing 1 mM ATP + 10 mM K_2HPO_4. After centrifugation the residue was washed 6 times with the trichloroacetic acid–ATP–K_2HPO_4 mixture. After a final wash with 2% trichloroacetic acid the residue was freeze-dried and then dissolved in 50 mM Tris–HCl (pH 7.4) containing 2% SDS. After centrifugation the clear supernatant fraction was subjected to gel filtration on Sephadex G-100. The column was eluted with 50 mM Tris–HCl (pH 7.4) containing 1% SDS. The ^{32}P-labeled fraction identified as the M_r 15 000 protein by SDS–polyacrylamide gel electrophoresis was lyophilized and extracted with chloroform/methanol (2:1) containing 10 mM HCl for 3 h at 20°C and was then applied to a Sephadex LH-20 column equilibrated with the same chloroform/methanol mixture. After elution the ^{32}P-labeled protein fraction was evaporated to dryness under a stream of N_2. The residue was dissolved in 0.1 N acetic acid and subjected to gel filtration on a Sephadex G-25 column equilibrated with 0.05 N acetic acid. The ^{32}P-labeled protein fraction was subjected to amino acid analysis. The fractions were tested for protein as in [9]. SDS–polyacrylamide gel electrophoresis was done as in [10,11]. M_r-values of the ^{32}P-labeled protein fractions were calculated according to appropriate standards as shown on the figures. Samples for

amino acid analysis (140 μg) were hydrolyzed in 6 N HCl for 24 h in vacuo. Analyses were performed with Bio CAL BC 200 amino acid analyzer. Thin-layer chromatography was done according to [12]. ^{32}P-Radioactivity was determined by scintillation counting.

3. Results

As shown in fig.1 ^{32}P-phosphorylation of the membrane protein M_r 15 000 was enhanced by incubation of the membrane in the presence of concanavalin (con) A. The effect of con A was of the same order of magnitude as exerted by insulin + GTP. This effect was not unexpected since con A is known to exert a series of insulin-like effects on membrane and enzymes by triggering the insulin receptor. To obtain information on the composition of the phosphorylated protein M_r 15 000 a purification procedure involving extraction with chloroform/methanol was applied. Initially the ^{32}P-phosphorylated membrane was subjected to gel filtration on Sephadex G-100. As shown in fig.2A two major ^{32}P-labeled fractions were separated by this procedure. Protein fraction II was eluted slightly before cytochrome *c* (standard) and identified as the protein of M_r 15 000. Protein fraction II was freeze dried, extracted with chloroform/methanol and chromatographed on a Sephadex LH-20 column equilibrated with chloroform/methanol. As shown in fig.2B three different protein fractions were separated by this column. The ^{32}P-labeled fraction I was eluted before ATP, P_i and phospholipid standards. After evaporation this fraction was subjected to gel filtration on Sephadex G-25. The ^{32}P-labeled protein was eluted as a single peak slightly before glucagon (standard). Protein fractions II and III eluted from the Sephadex LH-20 column did not contain ^{32}P-radioactivity. The protein content of the fractions I, II and III amounted to 36%, 37% and 26%, respectively, of the protein applied to the

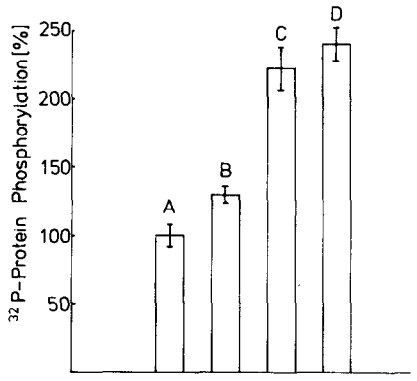
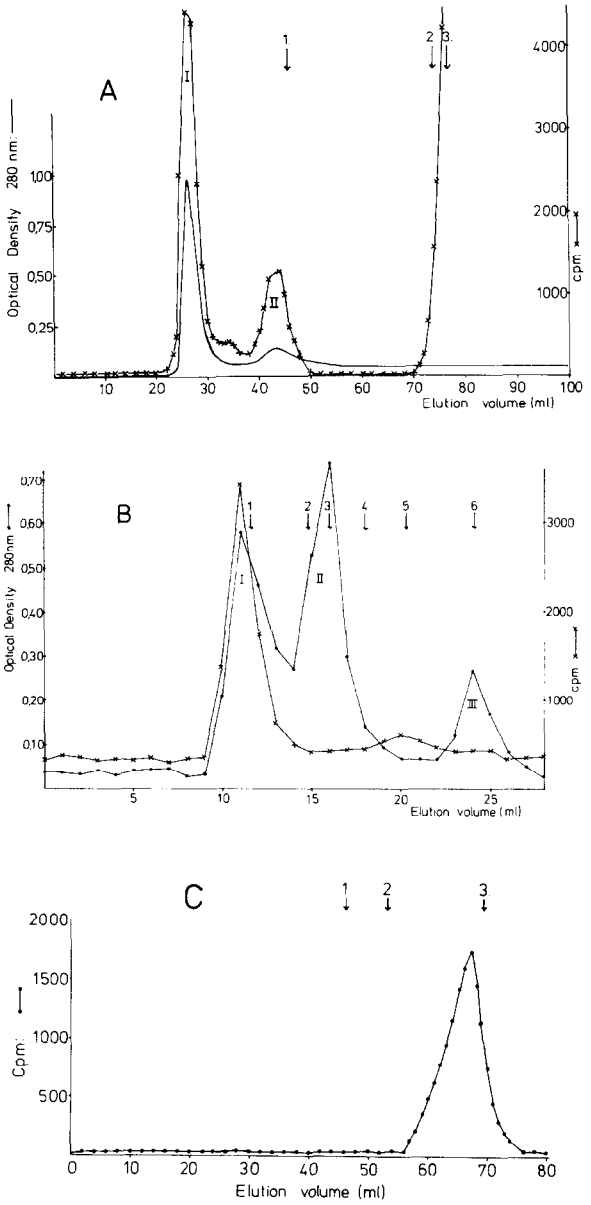


Fig.1. The stimulatory effect of insulin and con A on phosphorylation of sarcolemma membrane protein M_r 15 000. The procedure was as in [5]: (A) control; (B) incubation of the membrane with insulin (1 mU/ml); (C) incubation of the membrane with insulin (1 mU/ml) + 1 μM GTP; (D) incubation of the membrane with 50 μg con A.



column (mean of 3 different expt). Thin-layer chromatography of fraction I failed to reveal the presence of phospholipids. Fraction II contained mainly phosphatidylethanolamine and in addition small amounts of phosphatidylcholine and phosphatidylserine. Fraction III mainly contained phosphatidylserine and in addition phosphatidylcholine. The protein content of the protein fraction soluble in chloroform/methanol amounted to 5% (range 4.6–5.7%) of the total membrane protein. The ^{32}P-labeled subfraction I amounted to 1.8% (range 1.6–2.1%) of the total membrane protein.

M_r-Values of the ^{32}P-phosphorylated fractions were determined by SDS–polyacrylamide gel electrophoresis. Solubilization of the membrane in the presence of SDS followed by electrophoresis revealed a ^{32}P-labeled peak corresponding to M_r 15 500 (mean of 10 expt) (fig.3A). After extraction of ^{32}P-labeled membrane in chloroform/methanol this ^{32}P-labeled peak was greatly decreased in the soluble fraction and almost absent in the residual fraction. However, a large ^{32}P-labeled peak at lower M_r appeared in the soluble fraction (fig.3B). By SDS–urea gel electrophoresis in highly crosslinked polyacrylamide gels [11] the M_r was slightly higher than glucagon (standard). The electrophoretic mobility corresponded exactly with the mobility of fraction I isolated by chromatography on Sephadex LH-20 with an app. M_r 3650 (mean of 5 different expt) as shown in fig.3C.

Fig.2. Isolation of the ^{32}P-phosphorylated proteolipid from sarcolemma membranes. (A) The membranes had been preincubated with [γ-^{32}P]ATP + $MgCl_2$ and treated as in section 2. The solubilized membranes (3 mg protein) were applied on a 1.6 × 45 cm column of Sephadex G-100 and eluted with 50 mM Tris–HCl (pH 7.4) containing 1% SDS. Flow rate 1.0 ml/10 min. Arrows (↓) indicate elution of standards: (1) cytochrome *c*; (2) phosphatidylethanolamine; (3) ATP + P_i; the M_r of fraction II was 15 000 as determined by SDS–polyacrylamide gel electrophoresis (fig.3A). (B) Fraction II (150 μg protein) from fig.2A was extracted with acid chloroform/methanol and chromatographed on a 1.0 × 41 cm column of Sephadex LH-20 equilibrated with acid chloroform/methanol. The flow rate was 1.0 ml/3 min. Arrows (↓) indicate elution of standards: (1) cytochrome *c*; (2) ATP; (3) phosphatidylethanolamine; (4) phosphatidylcholine; (5) P_i; (6) phosphatidylserine. (C) Fraction I (60 μg protein) isolated by chromatography on Sephadex LH-20 (shown in fig.2B) was subjected to gel filtration on 1.6 × 50 cm column of Sephadex G-25 equilibrated with 0.05 N acetic acid. Flow rate 1.0 ml/3 min. Arrows (↓) indicate elution volumes of standards: (1) cytochrome *c*; (2) insulin; (3) glucagon.

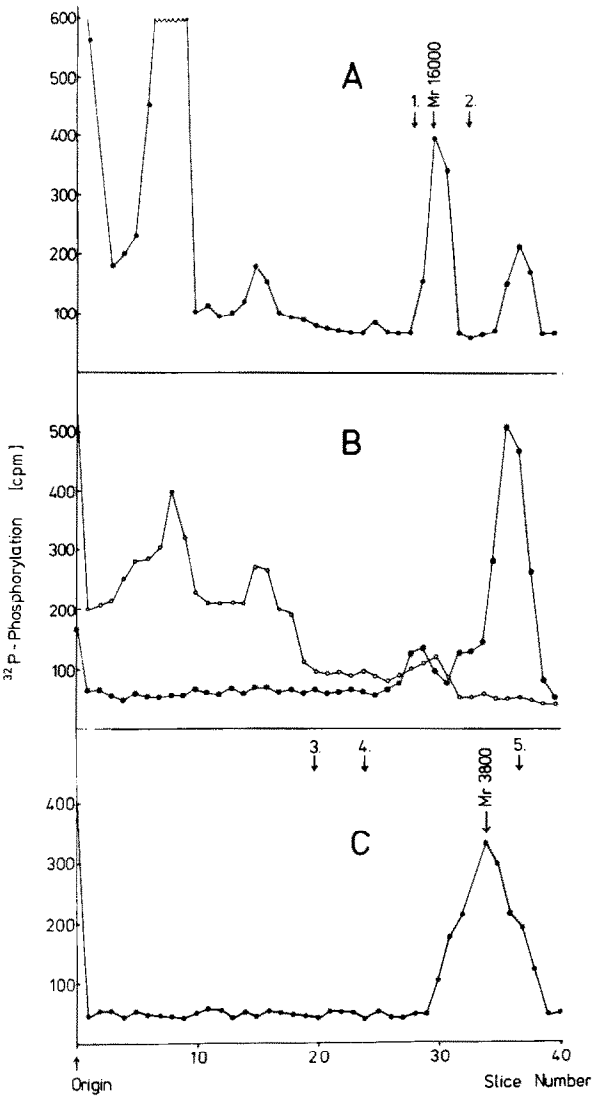


Fig.3. SDS–polyacrylamide gel electrophoresis of ^{32}P-labeled proteolipid from sarcolemma membranes. (A) Membranes (200 μg protein) were incubated with 50 μM [γ-^{32}P]ATP + 10 mM $MgCl_2$ for 10 s at 30°C. The reaction was terminated with SDS–EDTA 'stop solution' as in [5]. (B) Membranes were incubated as described under (A). The reaction was terminated with 10% trichloroacetic acid and the residue extracted with chloroform/methanol as in section 2. (•) Chloroform/methanol soluble fraction; (○) residue after extraction with chloroform/methanol. Electrophoresis in (A) and (B) was performed with 5.6% acrylamide [10]. (C) Fraction I was isolated by chromatography on Sephadex LH-20 (from fig.2B). Electrophoresis was performed with SDS–urea and 12.5% acrylamide [11]. Arrows (↓) indicate electrophoresis of standards: (1) myoglobin; (2) cytochrome *c*; (3) myoglobin; (4) cytochrome *c*; (5) glucagon.

The amino acid composition of the ^{32}P-labeled fractions is shown in table 1. The ^{32}P-labeled fraction II isolated by gel filtration on Sephadex G-100 is characterized by a relative high content of hydrophobic amino acids as is characteristic of proteolipids. Assuming 2 methionine residues an estimated M_r of 16 220 was obtained which correlates well with the value obtained by electrophoresis. The amount of serine residues is somewhat overestimated due to hydrolysis of phophatidylserine associated with the protein. The ^{32}P-labeled fraction I obtained by chromatography on Sephadex LH-20 with chloroform/methanol had a lower content of hydrophobic amino acids. Assuming a total of 34 amino acid residues the M_r was estimated to 3522, in good correspondence with M_r determined by electrophoresis.

Table 1
Amino acid composition of phosphorylated proteolipid isolated from sarcolemma membranes

Amino acid	Phosphorylated proteolipid[a]		Phosphorylated subunit[b]	
	mol/mol Met	Best integral	mol/mol Phe	Best integral
Asp	8.36	8	2.90	3
Thr	3.78	4	2.07	2
Ser	13.12	13	2.74	3
Glu	4.93	5	4.07	4
Gly	9.51	10	4.94	5
Val	4.63	5	1.62	2
Met	1.00	1	0.50	1
Ile	3.06	3	1.31	1
Leu	6.48	6	2.34	2
Tyr	1.33	1	0	0
Phe	3.27	3	1.00	1
His	2.72	3	0	0
Lys	4.72	5	3.71	4
Arg	2.96	3	1.28	1
Ala	5.45	5	2.78	3
Pro	3.0	3	1.9	2
Total residues		78		34
Minimum M_r		8110 (8110 × 2 = 16220)		3522
Total charged		24		12
Total hydrophobic		18		6
SDS electrophoresis M_r		15 500 ± 500 [10]		3 650 ± 200 [5]

[a] Fraction II isolated by gel filtration in Sephadex G-100 (fig.2A)
[b] Fraction I isolated by chromatography on Sephadex LH-20 (fig.2B) followed by gel filtration on Sephadex G-25 (fig.2C)

4. Discussion

Proteolipids are important constituents of cell membranes: three hydrophobic proteolipids of M_r 6000–10 000 have been isolated from mitochondria membranes [13]. Proteolipids have also been identified in sarcoplasmic reticulum as a component of (Ca^{2+})-ATPase [14] and of purified (Na^+,K^+)-ATPase from kidney [15] and cardiac tissue [16]. The amino acid composition of these proteins M_r 12 000 is characterized by a high degree of hydrophobic amino acids. The proteolipid component in (Na^+,K^+)-ATPase is labeled by a photoaffinity derivative of ouabain indicating significance for enzyme activity [17,18].

The proteolipid isolated from sarcolemma membranes here is characterized by phosphorylation in serine residues. Direct addition of insulin or con A to the membrane increases the degree of phosphorylation. The proteolipid has M_r 15 500, contains a relatively high degree of hydrophobic amino acids and is associated with phospholipids. Upon extraction with chloroform/methanol the proteolipid can be dissociated into 3 different protein subunits, one of which is phosphorylated. After delipidation this subunit has been estimated to be $M_r \sim 3600$. Phosphorylation of proteolipids has been reported for (Na^+,K^+)-ATPase [16], erythrocyte membranes [8] and for material isolated from total frog muscle [19].

Proteolipids may represent the functional units responsible for proton channeling in chloroplast and mitochondria membranes [20,13]. Protein-constrained phospholipids have been hypothesised as essential in membrane transport processes in general, the specificity being concealed in the proteins that form the walls of the channels in which phospholipids are contained [21]. The phosphorylated proteolipid characterized here may fill such a transport function. Hormonal control of phosphorylation of serine residues could alter specific properties of the protein controlling entry of solute molecules into the channel and thereby transport capacity. In this connection it is of interest that phosphorylation of an intrinsic protein in turkey erythrocyte membrane correlates well with Na^+,K^+-cotransport under hormonal control [22].

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