Vol. 87, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 13, 1979 Pages 757-763

A GTP-SPECIFIC PROTEIN KINASE IN PLASMA MEMBRANES OF MOUSE FIBROBLASTS

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Received February 17,1979

Summary: Endogenous protein phosphorylation in plasma membranes isolated from SV 40-transformed mouse fibroblasts was studied in the presence of [γ-^{32}P]GTP. The phosphoprotein pattern showed one main component with 100 k which could not be phosphorylated with ATP. Optimal phosphorylation of pp 100 was dependent on protein concentration. Strict substrate specificity of the corresponding endogenous kinase could be demonstrated by comparative electrophoresis with a plasma membrane sample phosphorylated with [γ-^{32}P]ATP and by isotope dilution experiments. GTP-specific phosphorylation of pp 100 was not dependent on cyclic nucleotides but influenced by GDP, GMP and AMP.

The results of numerous studies summarized in recent reviews (1,2) indicate that protein phosphorylation is a common mechanism for the mediation of very different regulatory reactions. Enzymes catalyzing the phosphorylation of proteins are usually divided into two classes (3): the cyclic nucleotide dependent protein kinases which can only utilize ATP as phosphorylating agent and protein kinases not depending on cyclic nucleotides which can utilize ATP or GTP. All preparations of GTP-utilizing protein kinases described so far (4-10) contained also ATP-utilizing activity when tested with purified phosphoproteins such as phosvitin (4,7) or casein (6) or with a complex group of phosphoproteins. On the other hand experimental evidence has been presented that GTP specific phosphorylation might be attributable to formation of labeled ATP from [γ-^{32}P]GTP through isotope exchange and transfer reactions (11). In this communication we give evidence that purified plasma membranes of SV 40-transformed mouse fibroblasts contain a GTP-specific protein kinase which phosphorylates a distinct membrane-bound protein only in the presence of GTP and not in the presence of ATP.

0006-291X/79/070757-07$01.00/0

MATERIALS AND METHODS

Chemicals: [γ-^{32}P]GTP (26.6 Ci/mmol) and [γ-^{32}P]ATP (26.2 Ci/mmol) were purchased from NEN, Boston, USA. Adenosine 3':5'-monophosphate (cAMP), guanosine 3':5'-monophosphate (cGMP), adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) were obtained from Boehringer, Mannheim, Germany; guanosine 5'-triphosphate (GTP), guanosine 5'-diphosphate (GDP) and guanosine 5'-monophosphate (GMP) from Serva, Heidelberg, Germany; $(theophylline)_2$.ethylenediamine (aminophylline) and ethyleneglycol-bis-(ß-amino-ethyl ether) N,N'-tetraacetic acid (EGTA) from Sigma, St.Louis, USA; NCS tissue solubilizer from Amersham/Searle, Arlington, Ill., USA. All other chemicals (analytical grade) were from Merck, Darmstadt, Germany. Kallikrein inactivator was kindly supplied by Bayer, Elberfeld, Germany.

Cells and Isolation of Plasma Membranes: The investigations were performed with STU-51A/232B mouse fibroblasts, a subline derived from SV 40-transformed embryonic cells of STU mice. The cells were grown in suspension culture and harvested in the exponential growth phase. After disruption of the cells the membrane fraction was collected by differential centrifugation and the plasma membranes were purified by repeated sucrose density gradient centrifugation as outlined previously (12,13).

Assay of Kinase Activity: Kinase activity was determined by measuring endogenous phosphorylation at 37°C for 1 min incubation in the presence of [γ-^{32}P]GTP or [γ-^{32}P]ATP. The reactions were carried out in a total volume of 90µl using glycine/phosphate buffer pH 7.8. The final concentrations in the standard reaction mixture were: 25 mM glycine, 25 mM Na-phosphate, 10 mM $MgCl_2$, 1 mM aminophylline, 0.3 mM EGTA and 20 µM [γ-^{32}P]GTP or [γ-^{32}P]ATP. In addition each incubation mixture contained 9 µg kallikrein inactivator per 90 µl to block endogenous proteases. The amount of membrane protein per sample ranged between 15 and 480 µg. The dependence of the reaction on cGMP and cAMP was studied with concentrations between 10^{-4}M and 10^{-9}M. Isotope dilution experiments were performed with concentrations of 1 mM non-labeled GTP or ATP (dilution factor 1:50). The effect of GDP, GMP, ADP and AMP on the phosphorylation reaction was tested at concentrations of 1 mM. Incubation procedures and analysis of the phosphorylated membrane proteins by SDS polyacrylamide gel electrophoresis were performed as described previously (12, 13).

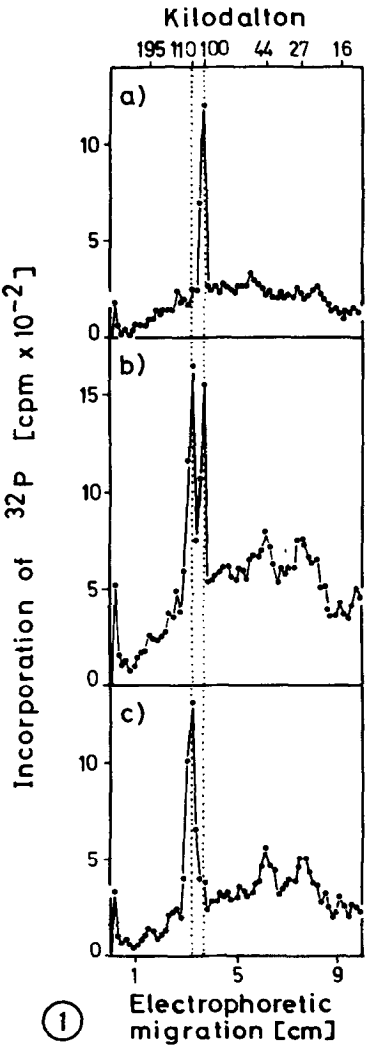
RESULTS

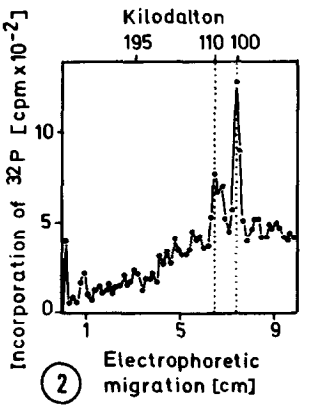
Characterization of the main component of GTP-specific phosphorylation: Endogenous phosphorylation of purified plasma membranes with [γ-^{32}P]GTP yielded

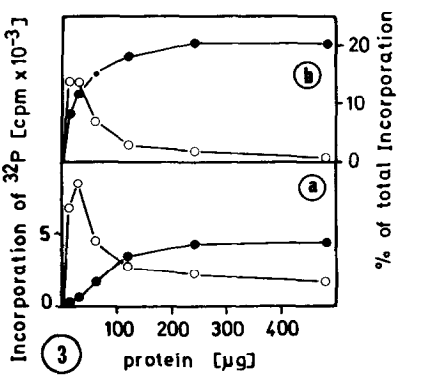
a phosphoprotein pattern with one main component in the range of 100 kilodalton (Fig. 1 a). When [γ-^{32}P]ATP was used a major phosphoprotein with a molecular weight of about 110 kilodalton (Fig. 1 c) could be obtained when phosphorylation was performed with low concentrations of membrane protein (13). Since the reference molecular weight scale used in SDS polyacrylamide gel electrophoresis generally implies an error of at least 5%, one might assume that both phosphoproteins, pp 100 and pp 110, are representing one and the same membrane protein. However, SDS gel electrophoresis of a mixture of two membrane samples, one phosphorylated with GTP and the other with ATP, yielded a clear-cut separation of both phosphoprotein peaks (Fig. 1 b) in 5% gels and after prolonged electrophoresis also in 7.5% gels. Prolonged electrophoresis in 5% gels finally led to splitting of the ATP-specific pp 110 peak into two components whereas the GTP-specific pp 100 peak maintained its original shape (Fig. 2). These findings indicate that GTP-specific pp 100 is different from ATP-specific pp 110.

Dependence on membrane protein concentration: Phosphorylation of ATP-specific pp 110 has been found to be dependent on membrane protein concentration since the phosphoprotein pattern obtained with high protein concentrations did scarcely contain a pp 110 peak (13). Entirely different is the dependence of the GTP-specific phosphorylation of pp 100. In Fig. 3 a the incorporation of ^{32}P into pp 100 and pp 110 is plotted against membrane protein concentration. The absolute incorporation into pp 100 is about linear up to a protein concentration of 120 µg/90 µl and the relative amount of pp 100, given in % of total incorporation (Fig. 3 b) attains a constant level with protein concentrations higher than 200 µg. Since the maximum yields of phosphorylation of pp 100 and pp 110 were attained at different protein concentrations the membrane samples used for co-electrophoresis (Fig. 1 and 2) were matched according to the peak size of pp 100 and pp 110.

Isotope dilution and effects of nucleoside mono- and diphosphates: Triphosphate specificity of phosphorylation of pp 100 and pp 110 was tested in iso-







1. SDS polyacrylamide gel (5%) electrophoresis of plasma membranes after 1 min incubation under standard conditions with (a) [γ-^{32}P]GTP; (c) [γ-^{32}P]ATP; (b) co-electrophoresis of (a) and (c). Time of electrophoresis 7.5 h.

2. SDS polyacrylamide gel (5%) co-electrophoresis as given in Fig. 1 b but after 15 h of electrophoresis.

3. Effect of plasma membrane protein concentration on ^{32}P incorporation into pp 100 after incubation with [γ-^{32}P]GTP (●) and into pp 110 after incubation with [γ-^{32}P]ATP (O) given as cpm (a) and as % of ^{32}P incorporation into total plasma membrane proteins (b).

tope dilution experiments using final triphosphate concentrations 50-fold higher than those of the labeled triphosphates. Under the given conditions ^{32}P incorporation in GTP-specific phosphorylation of pp 100 was fully inhibited by GTP but scarcely influenced by ATP (Table 1). ^{32}P incorporation in ATP specific phosphorylation of pp 110 was not altered by GTP but reduced to 32% of the control by addition of ATP. An explanation for the only partial reduction in the ATP-specific incorporation of ^{32}P into pp 110 can be seen in the possibility that the concentration of [γ-^{32}P]ATP was lower than the K_m for it and hence the isotope dilution effect was partly compensated by the increased incorporation rate at the higher substrate concentration.

GTP-specific phosphorylation of pp 100 was neither influenced by cyclic GMP nor by cyclic AMP in concentrations between 10^{-4} and 10^{-9} M. The effects of cyclic nucleotides on ATP-specific phosphorylation of membrane proteins have been reported recently (12).

The effects of various nucleotides on ^{32}P incorporation appeared to be significantly different when GTP-specific phosphorylation of pp 100 was compared with ATP specific phosphorylation of pp 110. GDP as well as GMP inhibited ^{32}P incorporation into pp 100 almost completely whereas these nucleotides scarcely affected ATP-specific phosphorylation of pp 110 (Table 1). ADP only partly reduced ^{32}P incorporation into both pp 100 and pp 110 but AMP showed an inhibiting effect of about 50% in both cases.

D I S C U S S I O N

Selective phosphorylation of plasma membrane proteins with ATP or GTP might offer a basis for an increased variability in regulation of cellular reactions. To our knowledge all the GTP utilizing protein kinases described so far also exhibited ATP-utilizing activity. This might be due to the fact that the kinase samples were still a mixture of ATP- and GTP-specific enzymes or, alternatively, the samples contained only a single kinase which could utilize both triphosphates. Our findings indicate that in plasma membranes of mouse fibroblasts phosphorylation of a distinct phosphoprotein (pp 1oo) by an endogenous

TABLE 1

Effects of various nucleotides on ^{32}P incorporation into pp 100 and pp 110

additions	GTP-specific phosphorylation pp 100		ATP-specific phosphorylation pp 110	
1 mM	cpm	% of control	cpm	% of control
---	6108	100	4325	100
GTP	207	3	4162	96
GDP	427	7	3296	76
GMP	918	15	4644	107
ATP	5015	82	1382	32
ADP	5211	85	3013	70
AMP	2479	41	2374	55

Highly purified plasma membranes were phosphorylated under standard incubation conditions at concentrations of 20 µM [γ-^{32}P]GTP or [γ-^{32}P]ATP. All nucleotide additions were tested in a final concentration of 1 mM.

kinase is strictly GTP-specific. Triphosphate specificity was clearly demonstrated by isotope dilution experiments.

The effects of various nucleotides on GTP-specific ^{32}P incorporation into pp 100 were partly dependent on time of incubation and most likely result from a number of complex side reactions. Inhibition by GDP possibly derives from reversibility of ^{32}P transfer between pp 100 and GDP as had been reported for ATP-specific phosphorylation and ADP (7,14). The molecular basis of inhibition by GMP can be correlated with the effect of AMP on ATP-specific phosphorylation (15,16) but its mechanism is still unknown.

Acknowledgement: The authors wish to thank Ms.S.Hafenrichter for her excellent technical assistance.

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