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Specific dephosphorylation of phosphoproteins by protein-serine and -tyrosine kinases

(reversibility of protein kinases/phosphatase of protein kinases/epidermal growth factor and insulin receptor kinases/proton ATPase)

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ABSTRACT **Five protein kinases are shown to serve as specific phosphatases in the absence of ADP. Although the rates of hydrolysis are very slow compared to the forward phosphorylation rates under optimal conditions, they are of the same order as the reverse reaction in the presence of ADP. Because cells contain ≈3 mM ATP, neither the reverse reaction nor the phosphatase is likely to play a physiological role. β-casein B phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (protein kinase A) is specifically dephosphorylated by protein kinase A but not by polypeptide-dependent protein kinase (protein kinase P). β-casein B phosphorylated by protein kinase P is specifically dephosphorylated by protein kinase P but not by protein kinase A. Histone H1 phosphorylated by protein kinase C is dephosphorylated by the same enzyme in the absence of ADP. In all cases tested addition of ADP and F_1-ATPase accelerates moderately the rate of dephosphorylation. Native H^+-ATPase from yeast plasma membranes is isolated mainly in the phosphorylated form. It is dephosphorylated and rephosphorylated by protein kinase P but not by protein kinase A. Protein-tyrosine kinase of the epidermal growth factor receptor phosphorylates the random synthetic polypeptide poly($Glu^{80}Tyr^{20}$). The phosphorylated polymer is specifically dephosphorylated in the absence of ADP by epidermal growth factor receptor preparations but not by insulin receptor preparations. The same polymer phosphorylated by insulin receptor is dephosphorylated by insulin receptor but not by epidermal growth factor receptor preparations. By using a cycle of dephosphorylation–rephosphorylation, it is possible to identify proteins that are phosphorylated by these protein kinases *in vivo*. Should this method be applicable to additional protein kinases, it should be possible to estimate the quantitative contribution of each protein kinase to a single phosphoprotein.**

It is now well established that phosphorylation–dephosphorylation reactions represent important mechanisms of regulation of enzyme and transport activities as well as receptor functions (1, 2). The fact that cells contain a large number of protein kinases (3) on the one hand and that a single protein may be phosphorylated by several protein kinases complicates exploration of *in vivo* events. Methods developed by Cohen and his collaborators (for review, see ref. 4) to deal with this problem have focused on the identification of specifically phosphorylated serine or threonine residues located in peptides obtained from native proteins, with parallel experiments assessing the capability of various protein kinases to phosphorylate specifically positioned serine or threonine residues. In general, chemical analyses require relatively large quantities of phosphoproteins.

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It was first shown by Lipmann and his collaborators that protein-serine kinases (5) and protein-tyrosine kinases (6) are reversible. In the course of experiments designed to exploit the reversibility of these enzymes for the purpose of identifying *in vivo* phosphorylation reactions, we discovered that under appropriate conditions the five protein kinases that we tested act as specific phosphatases. They are suitable for the limited identification of the phosphotransfer reactions that take place *in vivo*.

MATERIALS AND METHODS

Hepes, histone H1 (type IIIS), poly($Glu^{80}Tyr^{20}$), poly(Arg^{75}-Ser^{25}), polymyxin B, and tyrosine agarose were obtained from Sigma; Triton X-100 (membrane research grade) was from Boehringer Mannheim; Phosvitin was from Calbiochem; and [γ-^{32}P]ATP was from Amersham. Porcine insulin was donated by Eli Lilly; β-casein B was a gift from E. Bingham (Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia); yeast proton ATPase was supplied by K. Smith (Cornell University, Ithaca, NY). The catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) was a generous gift from E. Fischer (University of Washington, Seattle). Epidermal growth factor (EGF) (7), polypeptide-dependent protein kinase (protein kinase P) (8), and protein kinase C (9) were prepared as described. EGF receptor was purified by wheat germ agglutinin- and tyrosine-agarose chromatography from A431 human epidermoid carcinoma cells as described (10). The cell line NIH 3T3 HIR 3.5, rich in insulin receptor (11), was given to us by J. Whittaker (University of Chicago), and was grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Plasma membranes were prepared as described by Thom *et al.* (12) and solubilized with 1% Triton X-100 as described (13).

Phosphorylation of β-Casein B by Protein Kinase P and Protein Kinase A. A mixture containing, in a final volume of 2.5 ml, 20 mM NaHepes (pH 7.4), 5 mM $MgCl_2$, 10 mM thioglycerol, 500 μg of β-casein B, 500 μg of polymyxin B [which serves as protein kinase P activator (14)], 250 ng of protein kinase P, and 10 μM [γ-^{32}P]ATP (5000 cpm/pmol) was incubated for 1 hr at 22°C. The phosphorylated casein was precipitated by addition of an equal volume of ice-cold 10% (wt/vol) trichloroacetic acid/10 mM sodium pyrophosphate. After 1 hr at 0°C, the mixture was centrifuged at 17,000 × *g* for 10 min. The precipitate was washed three times with the precipitating solution and four times with ethanol/ether, 1:1 (vol/vol). The precipitated casein was suspended in 20 mM NaHepes (pH 7.4) at a final concentration of 1 mg/ml. The same procedure was used for the phosphorylation of β-casein B by protein kinase A (40 μg) but in the absence of polymyxin B.

Abbreviation: EGF, epidermal growth factor.

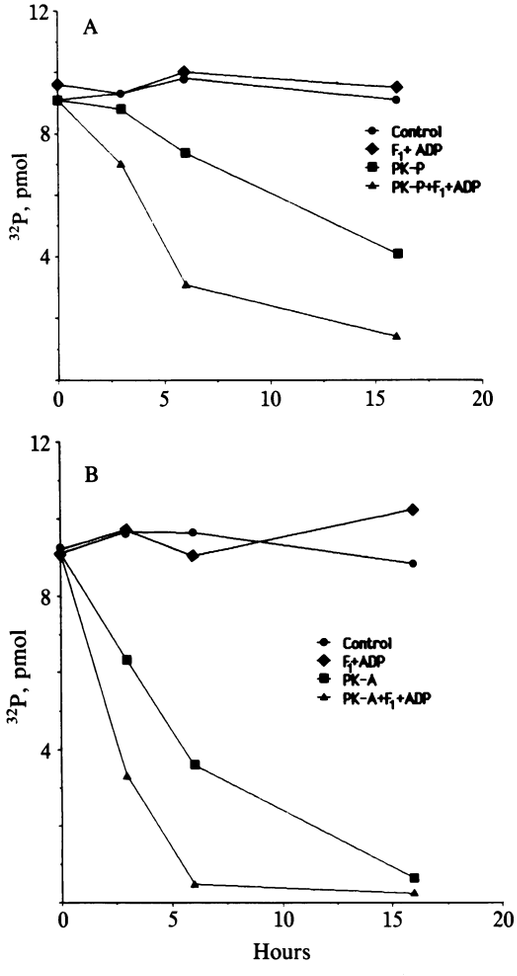

FIG. 1. (*A*) Time course of the dephosphorylation of β-casein B phosphorylated by protein kinase P. The reaction mixture contained, in a final volume of 250 μl, 20 mM NaHepes (pH 7.4), 5 mM $MgCl_2$, 10 mM thioglycerol, 30 μg of casein phosphorylated by protein kinase P, 0.002% sodium azide, with or without 20 μg of F_1-ATPase, and 100 μM ADP. The reaction was started by adding 25 ng of protein kinase P. After incubation at 22°C as indicated, 20-μl samples were applied to a Whatman 3MM filter paper (2 × 2 cm) that was then placed into 10% (wt/vol) trichloroacetic acid containing 10 mM pyrophosphate and shaken for 1 hr gently with four changes of the washing fluid. The filter papers were dried and the radioactivity was measured by Cerenkov counting. Samples (20 μl) were also taken for PAGE analysis and autoradiography. (*B*) Time course of the dephosphorylation of β-casein B phosphorylated by protein kinase A. The reaction was as described under *A*, except that 2 μg of protein kinase A was used instead of protein kinase P.

Phosphorylation of Histone H1 by Protein Kinase C. In a final volume of 0.5 ml, 100 μg of histone H1 was phosphorylated by 2 μg of protein kinase C in a reaction mixture as described (9). After 10 min at room temperature, an equal volume of 10% (wt/vol) trichloroacetic acid, 10 mM sodium pyrophosphate, was added and the phosphoprotein was processed as described above for casein. The redissolved protein was dephosphorylated under the same conditions used for the forward direction except that a final volume of 150 μl was used and no ATP was added. In the experiments indicated, 100 μM ADP and 12 μg of mitochondrial ATPase (F_1-ATPase) were added instead. At the time intervals indicated, 20-μl samples were placed on filter paper and assayed for radioactivity.

Phosphorylation of Poly(Glu^{80}Tyr^{20}) by EGF and Insulin Receptor Kinases. *Phosphorylation of poly(Glu^{80}Tyr^{20}) by EGF receptor.* The reaction mixture, in a final volume of 2.5 ml, containing 20 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.5 mg of poly(Glu^{80}Tyr^{20}), 500 μg of purified EGF receptor, and 250 ng of EGF was incubated for 10 min at 22°C to activate the receptor and then for 10 min in ice. The phosphorylating reaction was initiated by addition of 10 μM [γ-^{32}P]ATP (5000 cpm/pmol). After 30 min at 0°C, the phosphorylated poly(Glu^{80}Tyr^{20}) was precipitated and washed as described above for casein.

Phosphorylation of poly(Glu^{80}Tyr^{20}) by insulin receptor. The reaction mixture, in a final volume of 2.5 ml, containing 20 mM Hepes (pH 7.4), 20 mM $MgCl_2$, 4 mM $MnCl_2$, 500 μg of solubilized insulin receptor, and 200 nM insulin was incubated and precipitated as described above.

Table 1. Specificity of phosphorylation and dephosphorylation of β-casein B by protein kinases A and P

Dephosphorylating enzyme	Time, hr	Phosphocasein, pmol remaining: Protein kinase P	Phosphocasein, pmol remaining: Protein kinase A
None	1	7.5 ± 0.4	6.9 ± 0.6
Protein kinase P		5.9 ± 0.4	7.0 ± 0.6
Protein kinase A		7.5 ± 0.4	5.3 ± 0.2
None	3	7.5 ± 0.4	6.9 ± 0.4
Protein kinase P		4.3 ± 0.3	6.7 ± 0.5
Protein kinase A		7.5 ± 0.3	3.5 ± 0.4
None	6	7.8 ± 0.2	6.7 ± 0.7
Protein kinase P		3.6 ± 0.4	6.0 ± 0.5
Protein kinase A		7.8 ± 0.4	2.3 ± 0.4
None	16	7.9 ± 0.5	6.1 ± 0.8
Protein kinase P		2.4 ± 0.4	6.1 ± 1.0
Protein kinase A		7.6 ± 0.4	1.6 ± 0.3

Reaction mixture was as described in the legend of Fig. 1 but in the absence of F_1-ATPase and ADP and with 150 ng of protein kinase P or 6 μg of protein kinase A. The values are averages ± SD of four experiments.

RESULTS AND DISCUSSION

Specificity of Phosphorylation and Dephosphorylation of β-Casein B by Protein Kinase P and Protein Kinase A. It can be seen from Fig. 1*A* that β-casein B, phosphorylated with [γ-^{32}P]ATP by protein kinase P, was dephosphorylated by protein kinase P; similarly, β-casein B phosphorylated by protein kinase A was dephosphorylated by protein kinase A (Fig. 1*B*). Dephosphorylation was accelerated in the presence of ADP and F_1 but took place in their absence. In other words, in the absence of ATP and ADP, the protein kinases function as phosphatases. In this experiment azide was used (to prevent bacterial growth) in the presence of excess F_1. In the absence of azide (an inhibitor of F_1) the rate of dephos-

Table 2. Phosphorylation–dephosphorylation of histone H1 by protein kinase C in the presence and absence of ADP and F_1-ATPase

Dephosphorylation time, hr	Phosphohistone, pmol remaining in 20 μl: No protein kinase	Protein kinase C	Protein kinase C, ADP, and F_1
0	4.3	—	—
0.5	4.0	3.1	2.0
1	3.6	2.6	1.5
3	3.1	1.3	0.7
4	3.3	1.0	0.6
6	3.2	0.7	0.3

After 3 hr, a second addition of 2 μg of protein kinase C was made.

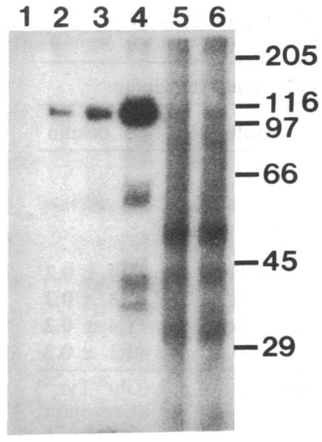

FIG. 2. Specific dephosphorylation and rephosphorylation of the H^+-ATPase of yeast plasma membranes by protein kinase P. For exposure to protein kinase P or protein kinase A, the reaction mixtures contained, in a final volume of 50 μl, 20 mM NaHepes (pH 7.4), 5 mM $MgCl_2$, 10 mM thioglycerol, 0.002% sodium azide, 2.6 μg of yeast H^+-ATPase, and 50 ng of protein kinase P or of 1 μg of protein kinase A. The tubes were incubated at 22°C for 4 hr and then 100 μM sodium vanadate, 5 μg of histone, and 10 μM [γ-^{32}P]ATP (4000 cpm/pmol) were added. As controls native H^+-ATPase was incubated for 4 hr without a kinase, and then protein kinase A or protein kinase P (± histone) were added with [γ-^{32}P]ATP. The reactions were terminated after a 30-min incubation at 22°C by adding $NaDodSO_4$ sample buffer (8). The samples were electrophoresed on a 7.5% $NaDodSO_4$/polyacrylamide gel. The gel was dried and autoradiography was performed with Kodak XAR-5. Lanes: 1, native H^+-ATPase plus protein kinase P; 2, same plus protein kinase P and histone; 3, H^+-ATPase dephosphorylated and rephosphorylated by protein kinase P; 4, same plus histone; 5, native H^+-ATPase plus protein kinase A; 6, same after prior exposure for 4 hr to protein kinase A.

phorylation was about twice as rapid. $NaDodSO_4$/PAGE analysis, performed on samples (20 μl) at all time points, confirmed the data obtained by the filter paper assay and ruled out loss of counts by proteolysis. It was also established by isobutanol/benzene extraction [1:1 (vol/vol)] that P_i was released during dephosphorylation.

The specificity of the dephosphorylation process is documented in Table 1. In this experiment, large amounts of protein kinases in the absence of F_1 were used to allow the dephosphorylation to proceed nearly to completion. It can be seen that 50% of casein phosphorylated by protein kinase was dephosphorylated after 6 hr by protein kinase but not at all by protein kinase A. Protein kinase A phosphorylated casein was dephosphorylated by protein kinase A to ≈70% but not at all by protein kinase P. Although very large amounts of protein kinases were used for dephosphorylation, there was very little or no overlap in the dephosphorylation, even after 16 hr when casein phosphorylated by protein kinase P was exposed to protein kinase A and vice versa. We conclude that these protein kinases acting as phosphatases are highly specific.

It should be noted that >100 times more protein kinase A than protein kinase P was used for the phosphorylation of casein and 40 times more was used for the dephosphorylation. The reason for this is that even β-casein B (15) is a relatively poor substrate for protein kinase A. This is not surprising because acidic proteins are phosphorylated more readily by protein kinase P and basic proteins by protein kinase A. We have shown (14) that a synthetic random polymer of glutamate and tyrosine (4.4:1) served as substrate for protein kinase P but not for protein kinases A and C, cGMP-dependent protein kinase, and calmodulin protein kinase II. On the other hand, a synthetic random polymer, poly($Ser^{25}Arg^{75}$), was readily phosphorylated by protein kinase A and C (but not by protein kinase P). It thus appears that just as protein-tyrosine kinases (16), the protein-serine kinases are not as specific as has been claimed. Ras 2 protein from yeast that contains no "consensus sequence" for protein kinase A is an excellent substrate for this enzyme resulting in functional changes as measured by the stimulation of yeast adenylyl cyclase (17). It seems likely that the surface charges in the substrate and the distances between the serine (threonine) or tyrosine residues and charged amino acids, play at least as important a role as the sequence of intervening amino acids. It is, therefore, not surprising to find that various serine residues within a single protein can be phosphorylated by protein kinase A and C and a variety of other kinases (4).

Histone H1 phosphorylated by protein kinase C in the presence of [γ-^{32}P]ATP was exposed to protein kinase C in the absence and presence of ADP and F_1. It can be seen from Table 2 that >90% of the radioactivity was removed from the protein after a 6-hr incubation at room temperature in the presence of and >80% in the absence of ADP and F_1. There was some instability of the phosphohistone in the absence of protein kinase C (but in the presence of Ca^{2+} and phospholipids) that remains unexplained.

Dephosphorylation and Rephosphorylation of the Native H^+-ATPase of Yeast. We have reported (8) that the H^+-ATPase of the plasma membrane of yeast is isolated in the

Table 3. Specific dephosphorylation by insulin receptor of poly($Glu^{80}Tyr^{20}$) phosphorylated by insulin receptor

Dephosphorylating enzyme	Time, hr	Phosphorylated poly($Glu^{80}Tyr^{20}$), pmol remaining			
		− insulin	+ insulin	− EGF	+ EGF
Insulin receptor	0	2.5 ± 0.2	2.5 ± 0.2		
	1	1.4 ± 0.2	1.2 ± 0.2		
	3	1.1 ± 0.1	0.8 ± 0.2		
	6	1.0 ± 0.1	0.6 ± 0.1		
EGF receptor	0			2.5 ± 0.2	2.5 ± 0.2
	1			2.4 ± 0.1	2.5 ± 0.3
	3			2.3 ± 0.2	2.4 ± 0.2
	6			2.3 ± 0.2	2.4 ± 0.2

Dephosphorylation was performed in a final volume of 100 μl, containing 20 mM Hepes (pH 7.4), either 5 mM $MgCl_2$ for dephosphorylation by EGF receptor or 20 mM $MgCl_2$ and 4 mM $MnCl_2$ for the dephosphorylation by insulin receptor, 20 μg of poly($Glu^{80}Tyr^{20}$) phosphorylated by insulin receptor, and either 20 μg of purified EGF receptor or 20 μg of solubilized insulin receptor in either the absence and presence of 10 ng of EGF or 200 nM insulin. The tubes were incubated at 4°C, 20-μl samples were taken at the indicated times, and the amount of the dephosphorylated protein was determined as described in the legend of Fig. 1. The values are averages ± SD of three experiments.

Table 4. Specific dephosphorylation by EGF receptor of poly(Glu^{80}Tyr^{20}) phosphorylated by EGF receptor

Dephosphorylating enzyme	Time, hr	Phosphorylated poly(Glu^{80}Tyr^{20}), pmol remaining			
		− EGF	+ EGF	− insulin	+ insulin
EGF receptor	0	1.9 ± 0.2	1.8 ± 0.2		
	1	1.1 ± 0.2	1.1 ± 0.2		
	3	0.5 ± 0.2	0.5 ± 0.2		
	6	0.2 ± 0.1	0.2 ± 0.1		
Insulin receptor	0			1.8 ± 0.2	1.8 ± 0.2
	1			1.8 ± 0.2	1.8 ± 0.3
	3			1.5 ± 0.2	1.4 ± 0.2
	6			1.4 ± 0.2	1.1 ± 0.3

Reaction mixture was as described in the legend of Table 3, except that 20 μg of poly(Glu^{80}Tyr^{20}) phosphorylated by EGF receptor, instead of the poly(Glu^{80}Tyr^{20}) phosphorylated by insulin receptor, was used.

phosphorylated form. After treatment with potato acid phosphatase, the protein was rephosphorylated by protein kinase P with a stoichiometry of ≈1 mol per mol of protein. Since the enzyme catalyzes the hydrolysis of ATP, vanadate was added to inhibit the ATPase activity in all phosphorylation experiments with protein kinases. Neither protein kinase A nor protein kinase C phosphorylated the dephosphorylated enzyme (data not shown).

It can be seen from Fig. 2 that the native ATPase preparation, after exposure to protein kinase P or protein kinase A in the absence of vanadate, was rephosphorylated by [γ-^{32}P]ATP only in the presence of protein kinase P (lanes 3 and 4) but not of protein kinase A (lanes 5 and 6). Prior to dephosphorylation little phosphorylation was observed (lanes 1 and 2). It is of interest to note that there are some impurities in the ATPase preparation that are phosphorylated by protein kinase A, some by protein kinase P, but the positions of these bands are clearly different. We suggest that the H^+-ATPase of yeast is phosphorylated *in vivo* by protein kinase P (or a kinase with similar specificity).

Specific Dephosphorylation Catalyzed by EGF and Insulin Receptors. It was shown (6) that protein-tyrosine kinases generate ATP when operating in reverse. It was, therefore, of interest to explore whether specific phosphatase activity can be demonstrated with this group of enzymes as well. It was shown (16, 18) that a random synthetic polymer, poly(Glu^{80}Tyr^{20}), was phosphorylated by every protein-tyrosine kinase thus far tested. We, therefore, chose phosphorylated poly(Glu^{80}Tyr^{20}) as the substrate and two receptor-protein kinase preparations to explore the usefulness of enzyme reversal to establish the specificity of dephosphorylation. A431 cells, which are known to contain several million EGF receptors per cell, have been widely used for studies of its protein-tyrosine kinase activity (19). From plasma membranes of these cells EGF receptor was purified (10). A cell line was developed that contains similar amounts of insulin receptor (11). Solubilized plasma membranes of this cell line were used as a source of insulin-receptor-tyrosine kinases. As shown in Table 3, a preparation of poly(Glu^{80}Tyr^{20}) phosphorylated by the insulin receptor was dephosphorylated by insulin receptor but not by EGF receptor. There was a slight stimulation of dephosphorylation in the presence of insulin. Table 4 shows that poly(Glu^{80}Tyr^{20}) phosphorylated by purified EGF receptor was dephosphorylated by EGF receptor, but the presence of EGF appeared to have no effect. Vanadate at 100 μM, which inhibits a phosphatase that hydrolyzes phosphate from tyrosine residues (20), had no effect on the rate of dephosphorylation by the purified EGF receptor. When a crude extract of A431 membranes was used, vanadate inhibited the dephosphorylation by ≈20%. Thus, it is important to use highly purified protein kinases and to check for the presence of nonspecific phosphatases in experiments on the specific dephosphorylation of native phosphoproteins. It, therefore, is not surprising that some dephosphorylation was observed in the presence of crude insulin receptor, probably due to some phosphatase activity in the membrane extract.

It has been recorded that kinase and phosphatase activities may reside in a single protein. La Porte and Koshland (21) have shown that isocitrate dehydrogenase kinase and phosphatase activities are catalyzed by the same protein. A similar association has been described for 6-phosphofructo-2 kinase and fructose-2,6-bisphosphatase. Evidence favors the concept that the two activities are catalyzed by the protein but at two different sites (22). The remarkable specificity of the phosphatase activation of the serine- and tyrosine-specific protein kinases described in this paper should allow for an analysis of events that take place *in vivo*.

We dedicate this paper to Dr. A. Kornberg on the occasion of his 70th birthday. We thank Dr. E. Bingham for gifts of β-casein B and Dr. E. H. Fischer for generous supplies of protein kinase A (catalytic subunit), for suggesting the experiments with vanadate, and for critical comments on the manuscript. This investigation was supported by Public Health Service Grant CA-08964, from the National Cancer Institute and by a Fellowship from the Cystic Fibrosis Foundation to M.A.-G.

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