REVERSAL OF HEPARIN INHIBITION OF NUCLEAR PROTEIN KINASE NII BY POLYAMINES AND HISTONES

Toshiro HARA, Keikichi TAKAHASHI and Hideya ENDO

Cancer Research Institute, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1. Introduction

Evidence is accumulating to suggest that the phosphorylation of histone and non-histone chromosomal proteins plays a key role in the regulation of the eukaryotic gene expression. Many of the non-histone chromosomal proteins are phosphoproteins and their phosphorylation is catalysed by protein kinases including both cyclic AMP-dependent and -independent protein kinases [1–3]. Two cyclic AMP-independent nuclear protein kinases, NI and NII [4], have been purified from rat liver nuclei and characterized [5,6]. These two kinases, involved mainly in the phosphorylation of non-histone chromosomal proteins, are not regulated by direct acting effectors [7]. Cytosol cyclic AMP-independent protein kinases from rabbit reticulocytes and bovine adrenal cortex were found to be inhibited by glycosaminoglycan [8,9]. We now report that nuclear protein kinase NII is also selectively inhibited by heparin, a glycosaminoglycan, when either casein or phosvitin is used as the substrate, while no inhibition occurs with histone. Histone is rather effective in the recovery of the enzyme activity inhibited by heparin. A similar release of inhibiton by heparin was seen with spermine and spermidine, but not with putrescine. Analysis with heparin–Sepharose affinity chromatography suggested that polyamines exert the effect without influencing the binding between heparin and the enzyme.

2. Materials and methods

[γ-^{32}P]ATP was prepared as in [10]. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, casein, histone, phosvitin

and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. Heparin was from Nakarai Chemicals, Kyoto. DEAE–Sephadex A-25, activated CH–Sepharose 4B, Sephacryl S-300 superfine and heparin–Sepharose CL-6B were from Pharmacia. Hydroxyapatite was from Bio-Rad. Phosphocellulose was from Whatman. Spermidine–Sepharose was prepared as in [11].

2.1. Extraction and purification of nuclear protein kinases

Nuclei were isolated from rat liver (130 g) as in [12] with slight modification. Details of the procedure will be reported elsewhere [13]. The purified nuclei were extracted twice with 75 mM NaCl, 25 mM EDTA (pH 8.0), 0.5 mM PMSF and successively 6 times with 10 mM Tris–HCl (pH 8.0), 0.5 mM PMSF. Combined extracts were used for purification of protein kinases. Extracted proteins (700 mg) were dialysed against 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol and 0.5 mM PMSF (buffer A) containing 0.3 M NaCl and applied to a phosphocellulose column (2.5 × 10 cm) equilibrated with the same buffer. Cyclic AMP-independent protein kinase activities were eluted with a linear gradient from 0.3–1.0 M NaCl in buffer A. Active fractions were directly applied to a hydroxyapatite column (1.6 × 6 cm) equilibrated with buffer A containing 1.0 M NaCl. With this step, protein kinases were separated into NI and NII [13]. The flow-through fraction was used as a source for protein kinase NI. NII was eluted at 140 mM phosphate with a linear gradient from 10–500 mM potassium phosphate buffer (pH 7.4) containing the same additives. NII kinase was precipitated with ammonium sulfate at 80% saturation, dissolved in 3 ml buffer A containing 1.0 M NaCl and

subjected to a Sephacryl S-300 column (2.5 × 88 cm) equilibrated with buffer A containing 1.0 M NaCl. The active fractions were dialysed against buffer A containing 0.1 M NaCl and applied to a DEAE–Sephadex A-25 column (1.6 × 3.0 cm) equilibrated with the same buffer. NII kinase was recovered at 280 mM NaCl with a linear gradient elution from 0.1–0.5 M NaCl. The protein kinase was dialysed against buffer A containing 0.1 M NaCl and applied to a spermidine-column (1.5 × 2.0 cm) equilibrated with buffer A containing 0.1 M NaCl. NII kinase was eluted at 330 mM NaCl with a linear gradient elution from 0.1–1.0 M NaCl in buffer A and used as NII preparation. NII enzyme was purified ~2000-fold from nuclear extracts.

2.2. Assay of protein kinase activity

The standard reaction mixture (100 μl) contained 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 mg substrate/ml, 0.1 mM [γ-^{32}P]ATP (100 000 cpm/nmol) and enzyme. After the incubation at 30°C for 10 min, the reaction was terminated by transferring 100 μl of aliquots onto Whatman 3 MM filters (1.5 × 3.0 cm) which were then processed as in [4].

3. Results

3.1. Effect of heparin on nuclear cyclic AMP-independent protein kinases

As shown in fig.1, protein kinase NII was strongly inhibited by heparin when either casein or phosvitin was used as the substrate. Inhibition of 50% was seen

with 100 ng/ml and 30 ng/ml of heparin for casein and phosvitin, respectively. In contrast, heparin at ≤100 μg/ml showed no inhibition when histone was used as a substrate, although histone is a poor phosphate acceptor of protein kinase NII [14]. There was no inhibitory effect of heparin (≤10 μg/ml) on nuclear protein kinase NI, with any substrate used (not shown). To determine the kinetics of heparin inhibition, the rate of phosphorylation by NII was studied at various concentrations of casein or ATP using 3 different concentrations of heparin. A double reciprocal plot of the data revealed that heparin is a competitive inhibitor of protein kinase NII with respect to casein (fig.2), but is non-competitive toward ATP (not shown). These results are consistent with those of casein kinases from rabbit reticulocytes and bovine adrenal cortex cytosol [8,9].

3.2. Effect of polyamines and histone on the inhibition of protein kinase NII by heparin

We have found that protein kinase NII is stimulated 7.5-fold with 2 mM spermine, 6.5-fold with 6 mM spermidine and 1.4-fold with 10 mM putrescine when casein was used as a substrate [13]. These findings prompted further investigation of the effect of polyamines on the heparin inhibition of protein kinase NII. The inhibitory effect of heparin was released with spermine and spermidine, but not with putrescine (≤10 mM), as shown in fig.3. Spermidine and spermine reversed the heparin inhibition even if added after the enzyme was incubated with heparin at 4°C for 1 h (not shown).
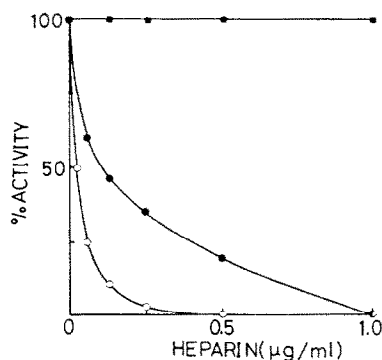

Fig.1. Effect of heparin on nuclear protein kinase NII. Protein kinase activity was assayed in the standard reaction mixture containing 10 ng enzyme, 2 mg histone/ml (■), casein (●) or phosvitin (○), and the indicated amount of heparin. The values assayed without heparin were taken as 100%.
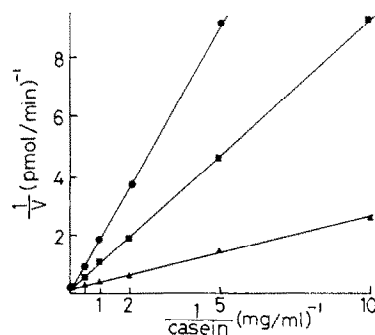

Fig.2. Lineweaver-Burk plots for the inhibition of protein kinase NII by heparin. Assays were carried out in the standard reaction mixture containing 10 ng enzyme, various concentrations of casein from 0.1–10.0 mg/ml in the absence (▲) and presence of heparin. Heparin was 0.1 μg/ml (■) and 0.5 μg/ml (●).
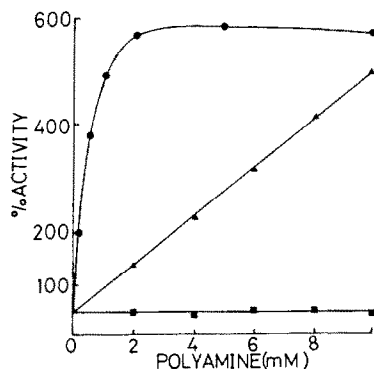
Fig.3. Effect of polyamines on the inhibition of protein kinase NII by heparin. Assays were performed in the standard reaction mixture containing 10 ng enzyme, 0.25 μg heparin/ml, 2 mg cascin/ml and the indicated concentrations of spermine (●), spermidine (▲) and putrescine (■). 100% activity corresponds to the value assayed in the absence of heparin and polyamine.

As heparin solubilizes chromatin through the interaction with histones [15], we examined the effect of histone on the inhibition of protein kinase NII by heparin. Histone stimulated the protein kinase NII activity 2.3-fold at 0.4 mg/ml and the inhibition by heparin disappeared with increasing doses of histone (fig.4).

3.3. *The relationship among polyamines, heparin and protein kinase NII*

To determine the mechanism of reversal of the heparin inhibition by polyamines, the interaction among polyamines, heparin and enzyme was assessed using heparin—Sepharose affinity chromatography. Protein
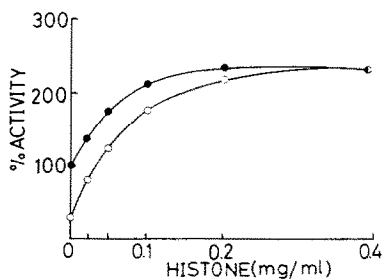

Fig.4. Effect of histone on the haparin inhibition of NII. Protein kinase activity was measured in the standard reaction mixture containing 10 ng enzyme, 2 mg casein/ml and the indicated amount of histone in the absence (●) and presence (○) of heparin (0.2 μg/ml). The value assayed in the absence of heparin and histone was taken as 100%.
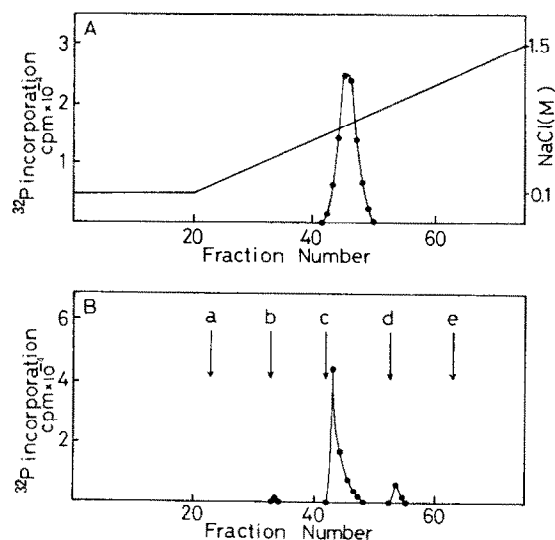

Fig.5. Heparin—Sepharose affinity chromatography. (A) Purified protein kinase NII in buffer A containing 0.1 M NaCl was applied to a heparin—Sepharose column (1.0 × 1.5 cm) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with buffer A containing 0.1 M NaCl and eluted with a 50 ml linear gradient from 0.1—1.5 M NaCl in buffer A. (B) Purified protein kinase NII in buffer A containing 10 mM spermidine was applied to a heparin column (1.0 × 1.5 cm) equilibrated with buffer A containing 10 mM spermidine. After wash with buffer A containing 10 mM spermidine, stepwise elution was performed with buffer A containing 50 mM (a), 100 mM (b), 150 mM (c), and 200 mM (d) spermidine and finally 1.5 M NaCl (e). Fractions of 1 ml were collected at 20 ml/h. Protein kinase activity (●) was assayed in the standard reaction mixture containing 2 mg casein/ml, 0.15 M NaCl and enzyme.

kinase NII bound to the column and eluted at 0.65—0.84 M NaCl in buffer A as depicted in fig.5A or 5 mg/ml of heparin in buffer A. It is conceivable that, if polyamine affects the binding between heparin and enzyme, a low concentration of polyamine should not only prevent the adherence of enzyme to a heparin-column but should also release the enzyme from the column. Contrary to our expectations, the enzyme in buffer A containing 10 mM spermidine adhered to the heparin column equilibrated with buffer A containing 10 mM spermidine. Once the enzyme had bound to the heparin-column, it eluted only with buffer A containing 150 mM spermidine, which is almost the same ionic strength as that of NaCl used for the elution (fig.5B). These results suggest that spermidine does not alter the binding between heparin and the enzyme.

4. Discussion

Heparin is a highly sulfated glycosaminoglycan with anticoagulant activity and selectively interacts with different enzymes to bring about significant changes in their activities. Although heparin is a constituent of various tissues, particularly liver and lung, little is known of precise intracellular distributions of glycosaminoglycan [15]. Several workers have shown that glycosaminoglycan might be associated with cell nuclei, in significant amounts [16,17]. Close interaction of glycosaminoglycan with nuclear components is also suggested by the facts that heparin induces changes in nuclei by loosening the chromatin structure and alters the activities of enzymes involved in nucleic acid metabolism, such as DNA polymerase, RNA polymerase and ribonuclease [15]. This study has shown that heparin selectively inhibits nuclear protein kinase NII, which may play an important role in the regulation of gene activity by phosphorylating the high mobility group (HMG) and other non-histone chromosomal proteins [18,19]. It is generally considered that the inhibition of enzyme activity by heparin is not mediated by simple ionic interaction but presumably by enzyme-inhibitor interaction [20,21]. This also seems pertinent to protein kinase NII because NII enzyme, despite being an acidic protein, binds to a polyanionic heparin-column and is selectively eluted by heparin.

Heparin—Sepharose affinity chromatography appears to be feasible for assessing the interaction among polyamines, heparin and enzyme as well as for purification of protein kinases inhibited by heparin. The fact that polyamines affect neither the adherence nor the release of the enzyme from the column suggests that polyamines reverse the inhibitory effect of heparin without affecting the binding between protein kinase NII and heparin. In [22] polyamines dissociated and endogenous inhibitor from the casein kinase of adrenal cortex cytosol. Therefore, the interaction among nuclear protein kinase NII, heparin, and polyamines might be different from that of the casein kinase with an endogenous inhibitor and spermine. Nuclear protein kinase NII is not regulated by cyclic AMP, cyclic GMP or calcium [14]. Heparin could be a physiological regulator, interacting with polyamines and histones, of nuclear protein kinase NII.

References

[1] Dabauvalle, M. C., Meggio, F., Creuzet, C. and Loeb, J. E. (1979) FEBS Lett. 107, 193–196.
[2] Ohtsuki, K., Yamada, E., Nakamura, M. and Ishida, N. (1980) J. Biochem. 87, 35–45.
[3] Stahl, H. and Knippers, R. (1980) Biochim. Biophys. Acta 614, 71–80.
[4] Desjardins, P. R., Lue, P. F., Liew, C. C. and Gornall, A. G. (1972) Can. J. Biochem. 50, 1249–1258.
[5] Thornburg, W., O'Malley, A. F. and Lindell, T. J. (1978) J. Biol. Chem. 253, 4638–4641.
[6] Thornburg, W. and Lindell, T. J. (1977) J. Biol. Chem. 252, 6660–6665.
[7] Krebs, E. G. and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923–959.
[8] Hathaway, G. M., Lubben, T. H. and Traugh, J. A. (1980) J. Biol. Chem. 255, 8038–8041.
[9] Feige, J. J., Pirollet, F., Cochet, C. and Chambaz, E. M. (1980) FEBS Lett. 121, 139–142.
[10] Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149.
[11] Bartos, F. and Bartos, D. (1979) Res. Commun. Chem. Pathol. Pharmacol. 23, 547–559.
[12] Chauveau, J., Moule, Y. and Rouiller, C. (1956) Exp. Cell Res. 11, 317–321.
[13] Hara, T. et al. (1981) in preparation.
[14] Thornburg, W., Gamo, S., O'Malley, A. F. and Lindell, T. J. (1979) Biochim. Biophys. Acta 571, 35–44.
[15] Lindahl, U. and Höök, M. (1978) Annu. Rev. Biochem. 47, 385–417.
[16] Bhavanandan, V. P. and Davidson, E. A. (1975) Proc. Natl. Acad. Sci. USA 72, 2032–2036.
[17] Fromme, H. G., Buddecke, E., von Figura, K. and Kresse, H. (1976) Exp. Cell Res. 102, 445–449.
[18] Inoue, A., Tei, Y., Hasuma, T., Yukioka, M. and Morisawa, S. (1980) FEBS Lett. 117, 68–72.
[19] Hasuma, T., Yukioka, M., Nakajima, S., Morisawa, S. and Inoue, A. (1980) Eur. J. Biochem. 109, 349–357.
[20] Farooqui, A. A. (1980) J. Chromatog. 184, 335–345.
[21] Srivastava, P. N. and Farooqui, A. A. (1979) Biochem. J. 183, 531–537.
[22] Job, D., Pirollet, F., Cochet, C. and Chambaz, E. M. (1979) FEBS Lett. 108, 508–512.