Effect of heparin on phosphorylation site specificity of neuronal Cdc2-like kinase

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Abstract Neuronal Cdc2-like kinase (Nclk) can be stimulated by heparin in a substrate-dependent manner. While phosphorylation of tau is markedly enhanced by heparin, phosphorylation of histone H1 by Nclk is essentially unaffected. A histone H1 peptide, HS(9–18): PKTPKKAKKL, and its substitution analogues were used to examine the basis of the differential heparin effect. In the presence of heparin, the phosphorylation site specificity of Nclk is altered and only proline immediately following the phosphorylation site is still an essential substrate determinant. This change in the site specificity may adequately account for the differential heparin effect on histone H1 and tau phosphorylation.

Key words: Neuronal Cdc2-like kinase; Tau; Heparin; Alzheimer's disease

1. Introduction

Neuronal Cdc2-like kinase (Nclk) is a proline-directed Ser/Thr kinase composed of Cdk5 and p25/p35 [1–3]. Its primary phosphorylation sequence has been characterized as S/T-P-X-H/R/K (single amino acid symbols, X for any amino acid) by independent studies using systematically substituted peptide substrates and an oriented degenerate peptide library [4,5]. In addition to the requirement of a Pro at the position +1 and a basic residue (Lys, Arg or His) at the position +3 relative to the phosphorylation site, a string of positively charged residues carboxyl terminal to the minimal phosphorylation sequence markedly enhances the phosphorylation efficiency of the substrate peptides [4].

Several lines of evidence have suggested that the microtubule associated protein tau is a physiological substrate of Nclk [6,7]. Tau is hyperphosphorylated in brains of Alzheimer's disease patients and the phosphorylation is essentially found on proline-directed Ser/Thr residues [8]. However, most of the tau phosphorylation sites fall into the S/T-P-X_1-X_2 (X_2 is not a basic amino acid) motif, which is a poor substrate sequence of Nclk. It has been shown most recently that the Nclk-catalyzed phosphorylation of tau is dramatically enhanced by heparin [9]. Tau was observed to co-localize with sulfated glycosaminoglycans such as heparan sulfate in Alzheimer's brains [10,11]. Moreover, the binding of heparin or heparan sulfate to tau prevents tau from binding to microtubules and induces microtubule disassembly, suggesting that heparin or heparan sulfate plays important roles in neurofibrillary pathology [10]. In the present study, we observed that the effect of heparin on Nclk activity depended strongly on protein substrates. While tau phosphorylation by Nclk was dramatically enhanced by heparin, the activity of Nclk towards histone H1 was essentially unaffected. Using systematic analogues of the Nclk substrate peptide from histone H1, the substrate-dependent stimulation of Nclk by heparin was analyzed in detail. The result shows that heparin caused a change of the Nclk phosphorylation site motif from S/T-P-X-K/R to S/T-P.

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; MOPS, 4-morpholinepropane sulfonic acid; PBS, phosphate buffered saline

2. Materials and methods

2.1. Phosphorylation reactions

Nclk applied in this study was a reconstituted enzyme from bacterially expressed Cdk5 and p25 [12]. Purified recombinant tau was a 441-amino acid isoform of human tau (hT40) [13]. Heparin was purchased from Sigma. Phosphorylation of a histone H1 peptide HS(9–18) (PKTPKKAKKL) and its analogues was performed as described previously [4]. Kinetic constants were determined from Lineweaver-Burk plots. Tau phosphorylation was carried out following the procedure of the peptide phosphorylation.

2.2. Binding of Nclk to heparin-Sepharose

170 μg GST-Cdk5 was mixed with 200 μg GST-p25 and 3.7 units of thrombin (Sigma) in 0.2 ml PBS. The sample was allowed to sit at room temperature for 2 h for reconstitution of Nclk and cleavage of GST. The mixture was diluted to 1 ml with a column buffer (20 mM MOPS, pH 7.4, 1 mM DTT, and 1 mM EDTA) before loading onto a heparin-Sepharose CL-6B (Pharmacia) column (0.3 ml of bed volume), which was pre-equilibrated with the column buffer. The column was then sequentially washed with 10 bed volumes of the column buffer containing NaCl at the following conditions: 0, 0.15 M, 0.3 M, 0.5 M, and 1.0 M. Aliquots from each collected fraction were measured for kinase activity by phosphorylation of the histone H1 peptide HS(9–18). Immunoblots were performed with monoclonal and polyclonal antibodies recognizing Cdk5 and p25, respectively. The blots were visualized by enhanced chemiluminescence (Amersham Life Science, Inc.).

3. Results and discussion

3.1. Differential effects of heparin on tau and histone H1 phosphorylation

Among in vitro protein substrates identified for Nclk, histone H1 is phosphorylated with the highest efficiency. Tau has been suggested as a physiological substrate of Nclk, but it is phosphorylated in vitro at a slow rate [7]. Very recently, Hasegawa et al. have shown that Nclk-catalyzed phosphorylation of tau can be stimulated by heparin [9]. As shown in Fig. 1,

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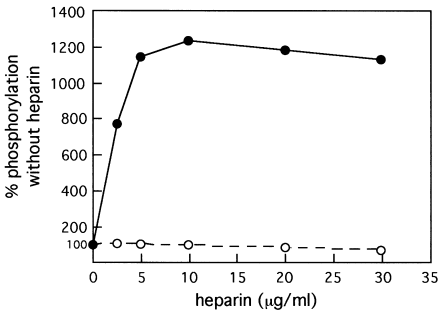

Fig. 1. Effect of heparin on phosphorylation of tau and histone H1 by Nclk. 0.3 μg of tau or histone H1 protein was phosphorylated by Nclk in the presence of different amounts of heparin. The phosphorylation rates in the absence of heparin are 0.13 pmol PO_4/min of tau and 15.72 pmol PO_4/min of histone H1. The phosphorylation rates in the presence of heparin are expressed as percentages of the respective rates in the absence of heparin. The dashed line and open circles represent phosphorylation of histone H1. The solid line and circles are the reactions of tau.

heparin stimulates the phosphorylating activity of Nclk towards a recombinant human tau protein in a dose-dependent manner with the maximal stimulation achieved at about 10 μg/ml heparin. Under this condition, the tau phosphorylation rate was increased by approximately 12-fold. In contrast, Nclk-catalyzed histone H1 phosphorylation was essentially unaffected (Fig. 1), indicating that the heparin effect on the Nclk activity depends on the protein substrate applied in the phosphorylation reaction. Previous studies have shown that Nclk catalyzes tau phosphorylation to a stoichiometry of 3.8 mol of phosphate/mol of tau at seven sites: Ser-195, Ser-202, Thr-205, Thr-231, Ser-235, Ser-396, and Ser-404 [7]. In the presence of heparin, tau becomes more heavily phosphorylated by Nclk to about 12 mol of phosphate/mol of tau, indicating that additional sites in tau are phosphorylated [9]. In fact, phosphorylation of some other sites besides these seven residues was observed in the presence of heparin [9].

3.2. Effect of heparin on synthetic peptide phosphorylation

Synthetic peptides are useful tools for studies of phosphorylation site specificity. We have previously shown that a synthetic peptide derived from the Nclk phosphorylation site in

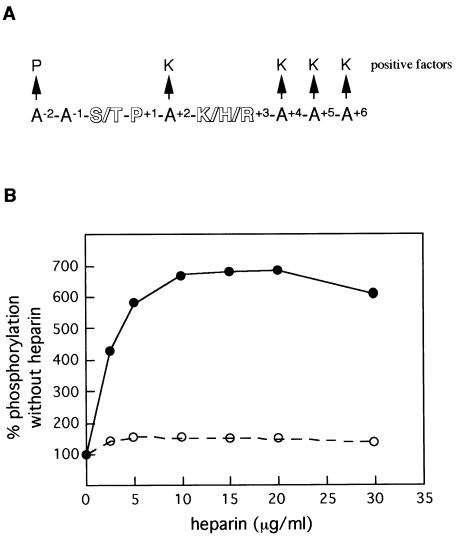

Fig. 2. Effect of heparin on phosphorylation of the S/T-P-X-K and S/T-P-X_1-X_2 peptides by Nclk. A: The consensus sequence motif of Nclk substrates. Outlined letters in the sequence are residues crucial for the substrate activity. B: Phosphorylation of HS(9–18) (PKTPKKAKKL) and its analogue HS(9–18)A14 (PKTPKAAKKL). HS(9–18) and HS(9–18)A14 contain the S/T-P-X-K and S/T-P-X_1-X_2 motifs, respectively. 5 μM of the peptides and different amounts of heparin as indicated were applied in the reactions. The phosphorylation rates are expressed as percentages of the respective rates without heparin, which are 2.91 pmol PO_4/min of HS(9–18) and 0.21 pmol PO_4/min of HS(9–18)A14. Dashed line and open circles represent the reactions of HS(9–18). Solid line and circles are the reactions of HS(9–18)A14.

histone H1, HS(9–18): PKTPKKAKKL, has essentially the same substrate activity for Nclk as histone H1 protein [4]. Using a set of substitution analogues of the HS(9–18) peptide, a number of amino acids in the peptide have been found as determinants of Nclk substrate activity [4]. These include Pro(+1) and Lys(+3) as strong determinants whose substitutions result in elimination of the substrate activity, and Pro(−2), Lys(+5) and Lys(+6) as weak determinants (Fig. 2A). As shown in Fig. 2B, heparin displays little activating activity towards Nclk when the peptide HS(9–18) was used as a substrate, but it greatly stimulates the Nclk activity towards

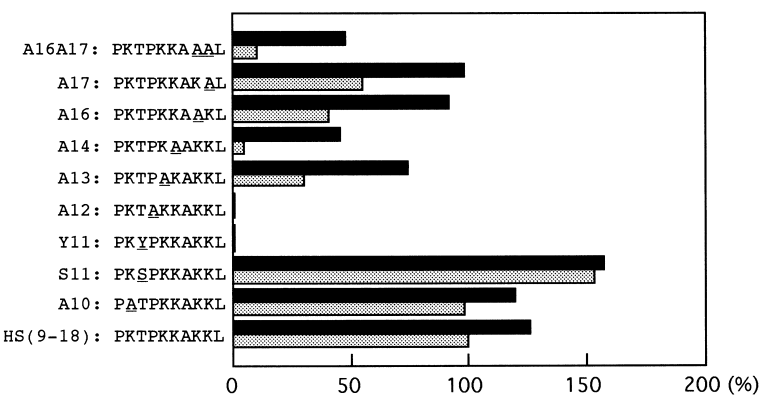

Fig. 3. Relative phosphorylation rates of the peptide analogues of HS(9–18) with and without heparin. Phosphorylation of the synthetic peptides was performed with reconstituted Nclk and 5 μM of HS(9–18) or its analogues. The phosphorylation rates are expressed as percentages of phosphorylation of the parent peptide HS(9–18) in the absence of heparin. Shaded bars represent the phosphorylation in the absence of heparin, and solid bars are the reactions in the presence of 15 μg/ml heparin. Underlined residues are substitutions from the parent peptide.

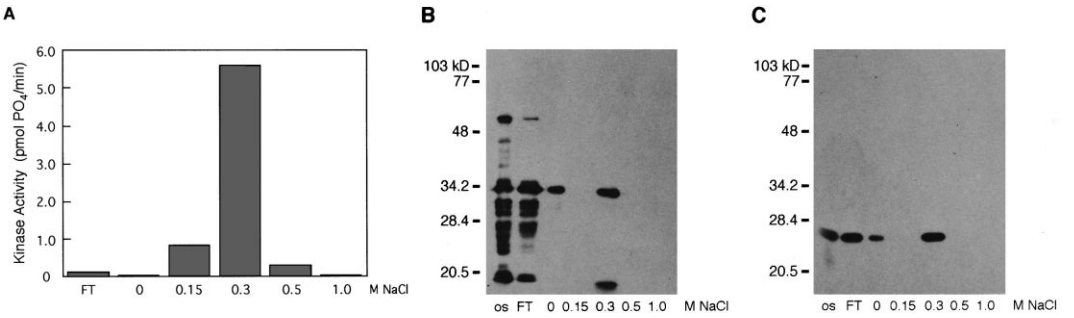

Fig. 4. Binding of Nclk to heparin-Sepharose. A: Kinase activity of the column fractions. The column was sequentially washed at different salt conditions as indicated. 15 μl aliquots from each fraction were subjected to a kinase assay using the substrate HS(9–18). B and C are immunoblots with antibodies recognizing Cdk5 and p25, respectively. *os* is the sample loaded on the column. *FT* is the flow-through.

the Lys(+3) substituted peptide HS(9–18)A14, indicating that the phosphorylation site specificity was modified by heparin.

A set of histone H1 peptide analogues was used to further characterize the heparin effect on Nclk phosphorylation site specificity. Fig. 3 shows that, in general, phosphorylation of the better Nclk substrates are not or only slightly affected by heparin, whereas heparin significantly stimulates phosphorylation of many of the poor substrates. Kinetic characterization of the peptide phosphorylation has revealed that the stimulation effect of heparin is due to pronounced decrease in K_m values (Table 1). In fact, heparin caused V_{max} values to decrease somewhat rather than to increase. An important consequence of this substrate sequence-dependent heparin effect is that the consensus motif of the Nclk phosphorylation sequence is altered from S/T-P-X-K/R/H to S/T-P by heparin. It eliminates the strong dependence of a basic residue (Lys/Arg/His) at the position +3 in its phosphorylation sequence, and effects of most of the secondary substrate determinants on the Nclk activity. On the other hand, some basic features of its substrate specificity which include the specificity for Ser or Thr but not Tyr, and the dependence on Pro(+1) are independent of heparin.

3.3. Direct interaction between Nclk and heparin

An effector of a protein kinase reaction may exert its action by binding either to the kinase or to the protein substrate, termed enzyme-directed or substrate-directed effect respectively. Synthetic peptide substrates can be applied to distinguish the two mechanisms. It is reasonable to assume that peptide substrates that contain minimal structures required for substrate recognition are unlikely to bind to the effector. The observation that heparin can stimulate the Nclk activity towards the peptide substrates suggests that heparin stimulates Nclk in an enzyme-directed mechanism. To provide direct demonstration of the physical interaction between Nclk and heparin, a heparin-Sepharose column was used to isolate Nclk from a crude sample that was reconstituted by incubating a mixture of bacterially expressed GST-Cdk5 and GST-p25 with thrombin. The sample was applied to the column in a buffer condition similar to that of the Nclk kinase reactions. The column was then washed with the same column buffer, and eluted step-wise with the buffered solutions containing 0.15 M, 0.3 M, 0.5 M and 1.0 M NaCl. The eluents were analyzed for the kinase activity of Nclk. Fig. 4A shows that essentially all of the kinase activity was bound to the affinity column and most of the bound activity was eluted at 0.3 M NaCl. As reported previously, bacterially expressed GST-Cdk5 and GST-p25 contained large amounts of partially proteolyzed and incorrectly folded proteins which were inactive [12]. Most of the inactive species of the bacterially expressed Nclk subunits appear not to bind to heparin-Sepharose. As shown in Fig. 4B and C, most of the bacterially expressed Cdk5 and p25 detected by the respective antibodies was found in the flow-through fraction which displayed little kinase activity. The apparently specific interaction between heparin and active Nclk suggests that a heparin-Sepharose column may be used to purify active Nclk from the crude sample reconstituted from its subunits.

In summary, we show in this study that heparin displays different effects on phosphorylation of tau and histone H1. Using synthetic peptides as model protein substrates, the differential effect of heparin on the Nclk activity is shown to be due to the difference in phosphorylation site structures of the two proteins. The substrate motif of Nclk is altered from S/T-P-X-K/R/H to S/T-P by heparin. This heparin effect is at least partially attributed to its direct binding to Nclk. On the other hand, the direct interaction between tau and heparin has also been demonstrated and heparin stimulation of tau phospho-

Table 1
Influence of heparin on kinetic parameters of Nclk

	K_m (μM)		V_{max} (% of control)		V_{max}/K_m (% of control)	
	−Heparin	+Heparin	−Heparin	+Heparin	−Heparin	+Heparin
HS(9–18)	14	6	100	97	100	226
HS(9–18)A13	61	11	124	84	28	107
HS(9–18)A14	339	15	106	40	4	37
HS(9–18)A16	29	3	95	60	46	280
HS(9–18)A17	24	3	100	63	58	294
HS(9–18)A16A17	163	21	78	59	7	39

Kinetic parameters were determined with reconstituted Nclk in the absence or presence of 15 μg/ml heparin. V_{max} and V_{max}/K_m were expressed as relative rates (%) to those of the parent peptide HS(9–18) in the absence of heparin.

rylation by a few other protein kinases has been reported, thus suggesting that contribution of the heparin-tau interaction to the kinase activation could not be completely ruled out [14–17]. Therefore, heparin may facilitate the Nclk-catalyzed tau phosphorylation by affecting both the enzyme and the substrate. Tau and heparan sulfate have been shown to coexist in neurofibrillary tangles. Our findings indicate that an increased level of sulfated glycosaminoglycans in neurons may account for the aberrant Nclk activity and hyperphosphorylation of tau in the development of Alzheimer's disease.

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References

[1] Ishiguro, K., Kobayashi, S., Omori, A., Takamatsu, M., Yonekura, S., Anzai, K., Imahori, K. and Uchida, T. (1994) FEBS Lett. 342, 203–208.
[2] Lew, J., Huang, Q.Q., Qi, Z., Winkfein, R.J., Aebersold, R., Hunt, T. and Wang, J.H. (1994) Nature 371, 423–426.
[3] Tsai, L.H., Delalle, I., Caviness, V.S.J., Chae, T. and Harlow, E. (1994) Nature 371, 419–423.
[4] Beaudette, K.N., Lew, J. and Wang, J.H. (1993) J. Biol. Chem. 268, 20825–20830.
[5] Songyang, Z., Lu, K.P., Kwon, Y.T., Tsai, L.H., Filhol, O., Cochet, C., Brickey, D.A., Soderling, T.R., Bartleson, C., Graves, D.J., DeMaggio, A.J., Hoekstra, M.F., Blenis, J., Hunter, T. and Cantley, L.C. (1996) Mol. Cell Biol. 16, 6486–6493.
[6] Hisanaga, S., Ishiguro, K., Uchida, T., Okumura, E., Okano, T. and Kishimoto, T. (1993) J. Biol. Chem. 268, 15056–15060.
[7] Paudel, H.K., Lew, J., Ali, Z. and Wang, J.H. (1993) J. Biol. Chem. 268, 23512–23518.
[8] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Watanabe, A., Titani, K. and Ihara, Y. (1995) Neurobiol. Aging 16, 365–371.
[9] Hasegawa, M., Crowther, R.A., Jakes, R. and Goedert, M. (1997) J. Biol. Chem. 272, 33118–33124.
[10] Goedert, M., Jakes, R., Spillantini, M.G., Hasegawa, M., Smith, M.J. and Crowther, R.A. (1996) Nature 383, 550–553.
[11] Snow, A.D., Lara, S., Nochlin, D. and Wight, T.N. (1989) Acta Neuropathol. 78, 113–123.
[12] Qi, Z., Huang, Q.Q., Lee, K.Y., Lew, J. and Wang, J.H. (1995) J. Biol. Chem. 270, 10847–10854.
[13] Goedert, M., Cohen, E.S., Jakes, R. and Cohen, P. (1992) FEBS Lett. 312, 95–99.
[14] Brandt, R., Lee, G., Teplow, D.B., Shalloway, D. and Abdel-Ghany, M. (1994) J. Biol. Chem. 269, 11776–11782.
[15] Mawal-Dewan, M., Sen, P.C., Abdel-Ghany, M., Shalloway, D. and Racker, E. (1992) J. Biol. Chem. 267, 19705–19709.
[16] Moreno, F.J., Munoz-Montano, J.R. and Avila, J. (1996) Mol. Cell Biochem. 165, 47–54.
[17] Yang, S.D., Yu, J.S., Shiah, S.G. and Huang, J.J. (1994) J. Neurochem. 63, 1416–1425.