

HETEROGENEITY OF PROTEIN KINASE NII  
FROM RAT LIVER NUCLEI

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Received July 24, 1984

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Summary: Protein kinase NII from rat liver nuclei was resolved into two fractions, NIIa and NIIb, by DEAE-Sephadex column chromatography. NIIa was eluted at 151 mM  $(\text{NH}_4)_2\text{SO}_4$  and NIIb at 175 mM. They had an identical molecular size (125,000 daltons, 7.0S) and subunit composition ( $\alpha\alpha'\beta_2$ ). However, they showed significantly different  $K_m$  values and turnover numbers for casein substrate. Furthermore, NIIa was found predominantly as a form bound to the chromatin, while NIIb was in the nucleoplasmic-soluble fraction in addition to the chromatin-bound fraction. These observations suggest that NII consists of a heterogeneous population of at least two molecular species, differing in the activity and functional states in the cell nucleus.

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Protein kinase NII has been found in the nuclei of many eukaryotic cells (eg. 1-3), and the same type of enzymes have been identified in the cytoplasmic or whole cell extracts from various species. The enzymes have a  $\alpha_2\beta_2$  or  $\alpha\alpha'\beta_2$  subunit structure (4-6), and the  $\alpha$  subunit has recently been shown to possess an ATP-binding site (7) or a catalytic site (8,9). Kinase NII is not influenced by cyclic nucleotides, preferentially phosphorylates acidic proteins such as casein and phosvitin, and modifies serine and threonine residues of substrate proteins. NII also phosphorylates nonhistone proteins (10), among which are HMG 14 and 17 (11,12) that confer DNase I-sensitivity of the transcriptionally active chromatin (13). This enzyme also phosphorylates and activates RNA polymerases I and II in vitro (14,15).

During the preparation of protein kinase NII from rat liver nuclei, we observed an enzyme activity eluting from DEAE-Sephadex slightly ahead of the major NII activity. We report herein that protein kinase NII consists of a heterogeneous population.

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Abbreviations used: PMSF: phenylmethylsulfonyl fluoride;  
TG(25)MEMP: 50 mM Tris/HCl, 25% (v/v) glycerol, 5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.2 mM PMSF, pH 8.0, the number in parentheses indicates the concentration of glycerol at v/v; TG(25)EMP: TG(25)MEMP minus 5 mM  $\text{MgCl}_2$ ;  
turnover number: moles of phosphate transferred/min/mole of enzyme at 30°C, pH 7.2.

### Materials and Methods

Extraction of nuclear protein kinases. Livers (145 g) from Sprague-Dawley rats were homogenized and centrifuged in 2.3 M sucrose, 3 mM  $MgCl_2$ , 0.2 mM PMSF as described (10), and the pelleted nuclei were suspended in 32 ml of 0.3 M sucrose, 3 mM  $MgCl_2$ , 0.2 mM PMSF, 10 mM Tris/HCl (pH 7.6). The suspension was mixed with 2 vol Buffer A (50 mM NaCl, 10% (v/v) glycerol, 5 mM mercaptoethanol, 20 mM Tris/HCl, pH 7.2), and centrifuged at 2,000 g to yield the soluble nucleoplasmic fraction. The nuclear pellet was washed again with 100 ml of Buffer A and suspended in 75 ml of 0.4 M NaCl, 1 mM  $MgCl_2$ , 5 mM 2-mercaptoethanol, 20 mM Tris/HCl (pH 7.85) in a loose glass-teflon homogenizer, and centrifuged at 15,000 g. The 0.4 M NaCl extract, dialyzed against 40 mM  $(NH_4)_2SO_4$  in TG(25)MEMP, was centrifuged at 15,000 g and used as the chromatin-bound fraction.

Purification of NIIa and NIIb, and rechromatography of the purified enzymes on parallel columns. Chromatography of protein kinase extracts on DEAE-Sephadex was performed as described (11). NIIa and NIIb eluting from DEAE-Sephadex at 151 mM and 175 mM  $(NH_4)_2SO_4$  were concentrated to 7 ml each, dialyzed against 50 mM NaCl in TG(25)MEMP and applied on 2 parallel columns (see below) of phosphitin-Sepharose (1.0 x 8.2 cm, 1.5 ml/15 min per tube). After elution with a 0.05 to 1.2 M NaCl linear gradient in TG(10)MEMP (80 ml for 2 columns), the enzyme fractions were dialyzed against 0.2 M NaCl in TG(25)EMP and chromatographed on phospho-cellulose (Brown Corp., Type P, 1.0 x 3.8 cm, 1 ml/10 min per tube). The phospho-cellulose enzymes, eluted with a 40 ml (for 2 columns) 0.2 to 1.0 M NaCl linear gradient in TG(10)EMP, were dialyzed against 0.1 M NaCl/TG(25)MEMP, and purified finally on heparin-Sepharose (0.8 x 3.0 cm, 0.75 ml/7.5 min per tube), employing a 45 ml 0.2 to 0.8 M NaCl linear gradient in TG(25)MEMP. They were purified about 700 to 1,000-fold starting from 0.4 M NaCl extracts, or about 50-70 times from DEAE-Sephadex fractions.

To 100  $\mu$ l each of purified NIIa and NIIb, bovine serum albumin (0.2 mg/ml) was added. The samples in 40 mM  $(NH_4)_2SO_4$ /TG(25)MEMP were chromatographed on parallel columns of DEAE-Sephadex (0.86 x 4.4 cm, 0.5 ml/10 min per tube), employing a linear 40 to 300 mM  $(NH_4)_2SO_4$  gradient in TG(25)MEMP (40 ml for two columns). In this chromatography, the gradient was made using a single gradient maker and introduced onto 2 parallel columns by way of a small three way connector and thin tubings (1 mm diameter). Effluents from the columns were collected simultaneously with a single fraction collector. Other conditions were also equally adjusted.

Preparative and analytical methods. Assay for protein kinase activity, preparation of 0.25 M HCl soluble nonhistone proteins from calf thymus, and SDS-polyacrylamide gel electrophoresis were performed as previously described in (10), (11) and (16). Gels were silver-stained with a kit from Bio-Rad Lab. Protein concentration was determined with Bio-Rad dye reagent, using bovine serum albumin as a standard.  $(NH_4)_2SO_4$  concentration of the column fractions was determined by conductimetry. Phosvitin-Sepharose and heparin-Sepharose were prepared as described in (5) and (17).

### Results

#### Heterogeneity of protein kinase NII.

Rat liver nuclei contain two major casein kinases, NI and NII. They are eluted from DEAE-Sephadex at the flow-through and at 170 mM  $(NH_4)_2SO_4$ , respectively (10). As shown in Fig. 1, when the 0.4 M NaCl nuclear extract was chromatographed on a long column of DEAE-Sephadex, two peaks of protein kinase activity, in addition to a large flow-through peak of NI enzyme, were

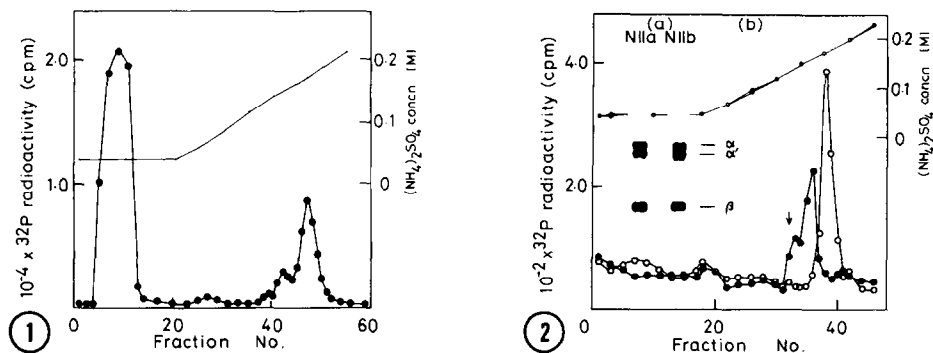


Fig. 1. Chromatography of nuclear protein kinases on DEAE-Sephadex. The 0.4 M NaCl extract prepared from 145 g rat liver was applied on DEAE-Sephadex (1.6 x 52 cm) at 6.7 ml/10 min per tube. After the column was washed, adsorbed proteins were eluted with a linear 40 to 300 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> gradient in TG(25)-MEMP (460 ml). Protein kinase activity was assayed for 20  $\mu$ l of each fraction with casein substrate. The flow-through enzyme is protein kinase NI, another major nuclear casein kinase.

Fig. 2. Purified NIIa and NIIb (a) (insert) SDS-polyacrylamide gel electrophoresis. NIIa and NIIb from DEAE-Sephadex were purified by 3 successive chromatographies, as described in Methods. The active fractions (2.25 ml) from heparin-Sepharose were processed as described (16) for electrophoresis on a 13% (w/v) polyacrylamide gel. A half to one-third of the samples in 0.1% SDS, 14% glycerol, 4 mM dithiothreitol, 10 mM Na phosphate were applied in the slots. (b) Rechromatography on DEAE-Sephadex. Purified NIIa (●) and NIIb (○) were chromatographed on 2 parallel columns of DEAE-Sephadex as described in Methods. Arrow indicates a minor protein kinase which is assumed to be another form of NII enzyme.

observed in the region of NII enzyme; one eluting at 150 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the other at 175 mM (Fig. 1). We refer here to these kinases as NIIa and NIIb, respectively.

To examine the possibility that one kinase was a degradation product of the other, samples at various stages of the enzyme preparation were incubated at 22°C for 30 min to enhance possible proteolytic degradation. The incubation was performed at 3 stages; (1) immediately after isolation of the nuclei, (2) upon enzyme extraction in 0.4 M NaCl, and (3) before application on a DEAE-Sephadex column. However, no difference in the relative abundance of NIIa and NIIb was observed between these samples and the control (prepared in a usual manner at 0-4°C throughout), suggesting that the occurrence of NIIa and NIIb was not artificially generated during preparation of the enzymes.

After fractionation by DEAE-Sephadex, NIIa and NIIb were purified by successive column chromatographies on phosphitin-Sepharose, phospho-cellulose and heparin-Sepharose. SDS-polyacrylamide gel electrophoresis revealed that they were reasonably pure (Fig. 2). Using the purified enzymes, rechromatography on 2 parallel DEAE-Sephadex columns was performed, which unambiguously confirmed that NIIa eluted at 151 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NIIb at 175 mM (Fig. 2). Fig. 2 further demonstrates that they had identical subunit

compositions of  $\alpha, \alpha'$  and  $\beta$ , the molecular weights of which were 42,000, 38,500 and 24,500, respectively. From staining stoichiometry and the molecular sizes (125,000-130,000, see below), these polypeptides are estimated in a ratio close to 1.2:0.8:1.7-2.0, constituting  $\alpha\alpha'\beta_2$  or a mixture of  $\alpha_2\beta_2$ ,  $\alpha'_2\beta_2$  and/or  $\alpha\alpha'\beta_2$  structure, for both NIIa and NIIb.

#### Some properties of purified NIIa and NIIb.

NIIa and NIIb were of similar molecular size. In gel-filtration on Bio-Gel A-1.5m, they eluted exactly at the same elution position. With proteins of known molecular weights, their mass was estimated to be 125,000-135,000 daltons. On sucrose density centrifugation in 0.4 M NaCl, both of the enzymes sedimented at the identical position, as 7S particles. The enzyme activities at various NaCl concentrations showed very similar profiles with maximum activities at 0.10 M NaCl.

NIIa and NIIb preferred acidic protein casein as the substrate and basic nonhistone proteins that were extracted in 0.25 M HCl (10) were poorer substrates. However, the relative phosphorylation rates toward the substrate proteins were not the same. NIIa phosphorylated 0.25 M HCl soluble nuclear proteins and casein at relative rates of 1:4.5, whereas the ratio for NIIb was 1:14. Lineweaver-Burk plots revealed that NIIa and NIIb had identical  $K_m$  values for ATP (5.8  $\mu$ M) or for 0.25 M HCl soluble proteins (0.87 mg/ml). However, for casein substrate, they showed different kinetics: NIIb exhibited a 2.3-fold larger  $K_m$  value (0.65 vs 0.30 mg/ml) (Fig. 3), and a 5-fold greater turnover number (120 vs 24  $\text{min}^{-1}$ , estimated from  $V_{\text{max}}$  values and  $M_r = 125,000$ ). These different turnover numbers contrasted to the results obtained with 0.25 M HCl soluble proteins (NIIa, 7.9  $\text{min}^{-1}$ ; NIIb, 7.6  $\text{min}^{-1}$ ).

Fig. 4 shows the elution profiles on DEAE-Sephadex of protein kinases extracted in the nuclear soluble and chromatin-bound fractions. It is evident

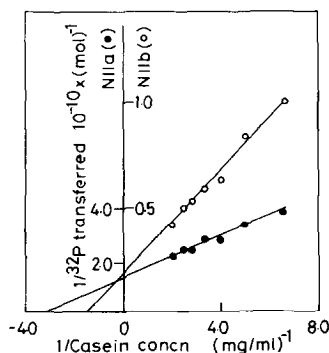


Fig. 3. Casein phosphorylation by NIIa and NIIb. Casein (0~500  $\mu$ g/ml) was phosphorylated by purified NIIa (●) or NIIb (○) (5  $\mu$ l each) under the standard assay condition (10), and the obtained velocity vs substrate concn curves were transformed to Lineweaver-Burk double reciprocal plots.

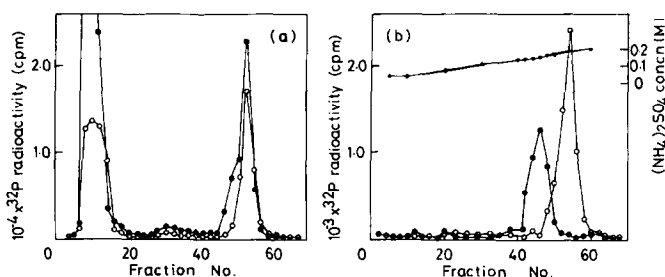


Fig. 4. (a) Protein kinase NII of nucleoplasmic-soluble and chromatin-bound fractions. The nucleoplasmic-soluble (○) and chromatin-bound (●) fractions prepared from 54 g liver were chromatographed on a column of DEAE-Sephadex (2.6 x 34 cm). Elution of a linear 40 to 400 mM  $(\text{NH}_4)_2\text{SO}_4$  gradient in TG(25)MEMF (700 ml, 10 ml/15 min per tube) was started at the 10th tube. (b) Recchromatography. Nucleoplasmic NII (○), fractions 50-55 in (a), and NIIa of the chromatin-bound fraction (●), fractions 45-48 in (a), were collected with  $(\text{NH}_4)_2\text{SO}_4$  at 62% saturation. Aliquots (one-fifth) of the enzymes in 40 mM  $(\text{NH}_4)_2\text{SO}_4$ /TG(25)MEMF were analyzed on two parallel columns (1.6 x 5.0 cm) of DEAE-Sephadex employing a linear 40 to 300 mM  $(\text{NH}_4)_2\text{SO}_4$  gradient (260 ml for 2 columns, 1.5 ml/9 min per tube).

that the nucleoplasmic-soluble fraction contained only NIIb, while the chromatin-bound fraction contained both NIIa and NIIb (Fig. 4a).

Rechromatography on two parallel columns demonstrated that the nucleoplasmic protein kinase and NIIa of the chromatin-bound kinases indeed differed in the elution positions (Fig. 4b). The former eluted at 175 mM  $(\text{NH}_4)_2\text{SO}_4$ , and the latter at 151 mM.

### Discussion

DEAE-Sephadex column chromatography of the nuclear extract revealed two forms of protein kinase NII, NIIa and NIIb. Using purified enzymes, elution positions were determined at 151 mM  $(\text{NH}_4)_2\text{SO}_4$  for NIIa and 175 mM for NIIb (Fig. 2). In addition, a small protein kinase activity eluting as a shoulder of NIIa was detected at 144 mM  $(\text{NH}_4)_2\text{SO}_4$  as seen in Fig. 2. This minor activity is also seen in Fig. 1. Therefore, there seem to be at least 3 forms of NII that can be resolved by DEAE-Sephadex chromatography: NIIa, NIIb and the minor one eluting slightly ahead of NIIa.

We showed previously that NII phosphorylates a set of basic non-histone proteins in the 0.25 M HCl soluble fraction (10). In the present study, NIIa and NIIb exhibited very similar kinetics for these substrate proteins. In fact, the phosphorylated protein species, and their extents of phosphorylation were indistinguishable between NIIa and NIIb as evidenced by SDS-gel electrophoresis and autoradiography (not shown). On the other hand, for acidic protein casein, they exhibited the kinetics with  $K_m$  values (Fig. 3) and turnover numbers significantly different from each other. Furthermore, NIIa was found predominantly as a form bound to chromatin, while NIIb was in both

the nucleoplasmic-soluble and chromatin-bound fractions (Fig. 4). Although the physiological significance of these observations is not known, they indicate that NII consists of a heterogeneous population, with respect to both activity and functional states in the cell nucleus.

#### Acknowledgements

We thank M. Uda for preparation of the polyacrylamide gels and secretarial services, and M. Ohara for reading the manuscript.

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