TWO PROTEIN KINASES FROM NUCLEI OF CULTURED TOBACCO CELLS WITH PROPERTIES SIMILAR TO THE CYCLIC NUCLEOTIDE-INDEPENDENT ENZYMES (NI AND NII) FROM ANIMAL TISSUE

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1. Introduction

In the nucleus of the mammalian cell protein kinases were detected which are cyclic nucleotide-independent and whose phosphorylation site is directed towards acidic peptide segments. Such enzymes have been purified to homogeneity from the nuclei or chromatin of rat liver [1], porcine liver [2,3], calf thymus [4], Morris hepatoma [5] and murine cells [6,7]. The properties of these enzymes clearly indicate 2 distinct types. Type I protein kinases, termed NI [8,9], are small monomeric proteins (M_r 25 000–35 000), bind to CM-cellulose at pH 7, are strictly ATP-specific and are not inhibited by low concentration of heparin. Type II protein kinases, NII enzymes, are bound to DEAE-cellulose at pH 7, are larger proteins (M_r 120 000–200 000) with a complicated quaternary structure [10], can utilize both ATP and GTP [11] and are inhibited by low concentrations of heparin [5,12,13].

The few protein kinases from the plant kingdom described hitherto are all cyclic nucleotide-independent. Some of these are located in the nucleus and also phosphorylate acidic peptide segments. Such plant nuclear protein kinases which have been purified to homogeneity were reported from cauliflower (M_r 39 000) [14] and soybean hypocotyl (M_r 55 000) [15]. This work describes the partial purification of 2 protein kinases from the nuclei of tissue-cultured tobacco cells. Their properties are shown to be very similar to those of the nuclear enzymes from the mammalian cell.

2. Experimental

2.1. *Preparation of nuclei*

A culture of *Nicotiana tabacum* var. White Burley was used which had been transformed by the *Agrobacterium tumefaciens* strain A6 and was originally obtained from Dr R. Schilperoort, Leiden. The cells were grown at 25°C in roller bottles each containing 1.5 litres. Linsmaier and Skoog medium [16] without phytohormones and subculturing was performed every week. Cells were harvested 4–5 days after subculturing. Usually 6 roller bottles gave a yield of 1.6–2.0 kg cells (fresh wt) and ~1.2×10^9 nuclei. The preparation of the nuclei was performed as in [17]; the method comprises a combination of enzymatic and mechanical disruption of the cell wall and centrifugation in Percoll density gradients. The preparation lasts ~3.5 h and yields highly purified nuclei with high intrinsic enzyme activities [17]. The nuclei were stored by freezing at –20°C.

2.2. *Purification of the protein kinases*

All the buffers contained 5 mM $MgCl_2$, 2 mM EDTA, 10 mM mercaptoethanol, 2 mM DTT and 0.02% NaN_3. Buffer A used for extraction contained in addition 0.35 M NaCl, 2 μM ATP, 0.2 mM pepstatin and 20 mM Tris–HCl (pH 8). Buffer B, the basic buffer for column chromatography, contained in addition 0.2 mM PMSF and 20 mM MOPS (pH 7). All operations were performed at 0–4°C. The nuclei (the yield from 6 roller bottles) were suspended in 2 ml buffer A, homogenized in a motor-driven Potter-Elvehjem apparatus (1500 rev./min) for 1 min, incubated for a further 15 min by stirring and centrifuged at 5000 × *g* for 10 min. This extraction procedure was repeated 3 times with the pellet. The combined supernatants were diluted with buffer B, to give 50 mM final [NaCl] and applied to a DEAE-Sephacel column (15 ml). The column equilibrated with 50 mM NaCl in buffer B was washed with 30 ml of this solu-

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tion and eluted with a NaCl gradient (0.05–2.0 M) in buffer B (60 ml). Protein kinase activity was eluted as a homogeneous peak at 0.5 M NaCl. The pooled active fractions were again diluted to 50 mM NaCl with buffer B and applied to a blue Sepharose 6B column (6 ml). After washing with 15 ml 0.1 M NaCl in buffer B, the protein kinase was eluted (reversed flow) with a double gradient of NaCl (0.1–2.0 M) and ATP (0–4 mM) in buffer B. The active fractions appeared as a homogeneous peak at 0.5 M NaCl and 0.4 mM ATP. These were pooled, concentrated by dialysis with 300 ml 20% poly(ethylene) glycol and 0.25 M NaCl in buffer B, and taken up in 0.5 ml 0.25 M NaCl in buffer B. Gel permeation chromatography was done as in fig.1.

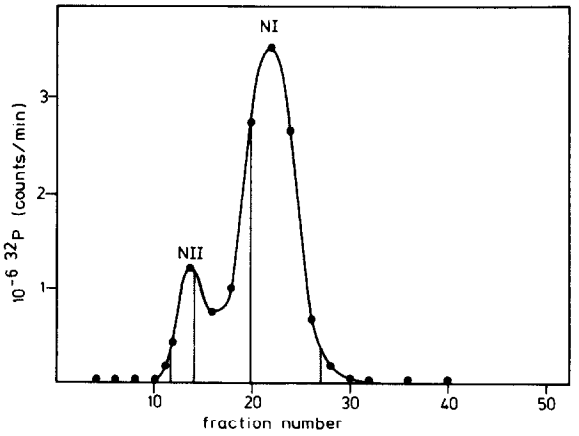

Fig.1. Gel permeation chromatography on Sephadex G-100. The active fractions obtained from the blue Sepharose step were concentrated as in section 2 and applied (0.5 ml) to a 1 × 40 cm Sephadex G-100 column. The column was equilibrated and run with 0.25 M NaCl in buffer B (2 ml/h; 1 ml fractions). Fractions 12–14 (NII) and 20–27 (NI) were pooled. Activity was assayed as in section 2 with an incubation time of 10 min at 30°C.

2.3. *Enzyme assays*

Protein kinase was assayed at 30°C and pH 8 with purified casein (Difco Labs) [3]. The standard incubation mixture of 100 μl contained 50 mM Hepes (pH 8), 10 mM $MgCl_2$, 0.15 M NaCl, 2.5 mM DTT, 8 mg casein/ml and 50 μM [γ-^{32}P]ATP (50 cpm/pmol). The incubation time was 5 min and the filter technique [18] was used for collection and counting the phosphorylated protein. The K_m- and K_i-values were determined as described in [11] and in the legends of table 2 and fig.2. Protein was determined according to [19].

3. Results and discussion

3.1. *Protein kinase purification*

The purification procedure summarized in table 1 had to be adjusted to handle small amounts of proteins and small volumes, and to avoid the time-consuming and deleterious exchange of buffer by dialysis. Gel permeation chromatography (fig.1) separates the total activity into 2 fractions, the large peak with the lower M_r was termed protein kinase NI and the small peak with the higher M_r NII (cf. nomenclature for the rat liver nuclear enzymes [8]). The activity ratio of NI/NII (fig.1), i.e., ~85% NI and only 15% NII, is different from that found in the mammalian cell. In the nucleus of porcine liver [3] 40% of the total protein kinase activity was assigned to NI and 60% to NII. The degree of purification obtained for

Table 1
Purification of the tobacco nuclear protein kinases

Purification steps	Total protein (mg)	Total activity (nmol/min)	Yield (%)	Specific activity (nmol . min^{-1} . mg^{-1})	Purification
Extract of nuclei	16.5	41	100	2.5	1
DEAE–Sephacel	5.8	37	90	5.9	2.5
Blue Sepharose	1.8	27	66	15.6	6.3
Sephadex G-100					
Fraction NI	0.16	11.8	29	74	30
Fraction NII	0.64	0.4	1	0.6	

The protein kinase activities were determined with casein and [γ-^{32}P]ATP as substrates. The fractions NI and NII were pooled as indicated in fig.1

fraction NI of only 30 is actually higher, for one has to take into account the purification of the nuclei not included in this figure. However, the present procedure did not result in homogeneous proteins, as could be seen from the SDS gel electrophoresis assay. This is partly due to the small amount of nuclei available ($\sim 10^9$). For the purification of the porcine liver protein kinases $\sim 3 \times 10^{11}$ nuclei were available as starting material [2].

3.2. *Properties of the two nuclear protein kinases*

The native M_r-value was estimated by gel permeation chromatography (cf. [10]); the value obtained for protein kinase NI (23 000) is very similar to that of NI from porcine liver (25 000) [3]. However, the mammalian nuclear protein kinases NII (M_r 130 000–200 000 [10]) are larger proteins than the present plant nuclear enzyme NII which exhibited M_r 85 000. The pH optima of the plant enzymes determined with casein are 8.5 (NI) and 8.0 (NII), which is slightly higher than those of the liver nuclear enzymes [20]. The optimal ionic requirement are 0.15 M NaCl and 15 mM Mg^{2+}, which is very similar to the properties of the mammalian nuclear enzymes NI and NII [1,2].

Mammalian nuclear protein kinases NI and NII exhibit distinct differences in their substrate sites [1,10,21]. Table 2 lists kinetic parameters for the present plant protein kinases, and allows a rough estimation of their specificity to be made. The plant NII enzyme is similar to the respective enzyme from porcine liver nuclei [10] having a lower K_m for phosvitin relative to casein and phosphorylating a histone mixture with about the same velocity (30% relative to casein), whereas the NI enzymes from both liver and tobacco cells phosphorylate histones with much lower efficiency.

However, the mammalian nuclear protein kinases NI and NII have very distinct ATP sites [2,3,11]; only

Table 2
Kinetic parameters of the tobacco nuclear protein kinases for ATP, GTP and different protein substrates

	K_m (μM)		Relative V_{max} (%)	
	NI	NII	NI	NII
Casein	24	30	100	100
Phosvitin	26	8.5	94	86
Mixed histone	24	18	2	29
ATP	19.3	14.3	100	100
GTP	120	11.4	9	100

The data were determined from plots made according to Lineweaver-Burk. For the different protein substrates labelled ATP was used. Casein, [γ-^{32}P]ATP or [γ-^{32}P]GTP was used for the determination of the kinetic parameters for ATP or GTP. The relative V_{max} were also extracted from the Lineweaver-Burk plots and set to 100% for ATP and casein, respectively. The assays were performed as in section 2 using 20 μl pooled fractions, NI and NII indicated in fig.1 and table 1

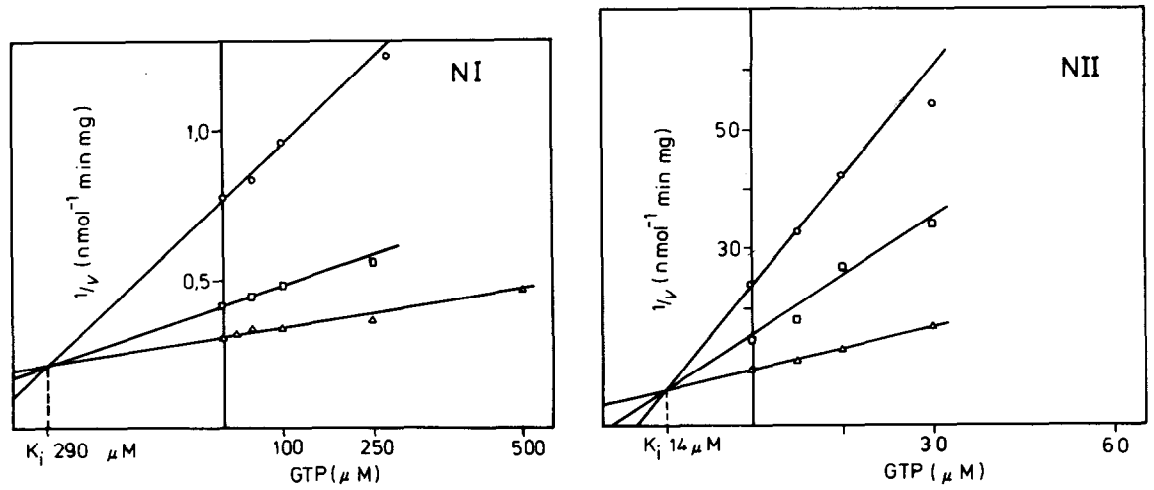

Fig.2. Determination of the K_i-values for GTP. The competition of unlabelled GTP with [γ-^{32}P]ATP in the phosphorylation of casein was measured. The data are plotted according to Dixon [22]. The assays were performed as in section 2 using 20 μl of the pooled protein kinase fractions (cf. fig.1 and table 1). The labelled ATP concentration was: (○) 1 μM; (□) 2 μM; (△) 4 μM.

Table 3
K_i-Values (μM) for the tobacco nuclear protein kinases

	NI	NII
GTP	290	14
ATP	16	12.5
ADP	14	–
AMP	235	–
Ado	10	–
Adenine	120	–

The values were determined with casein and [γ-^{32}P]ATP as indicated for GTP in fig.2

type II enzymes can utilize GTP as well. With the limited amount of enzyme activity available the kinetic parameters for ATP and GTP (table 2) and the inhibitor constants (competitive inhibition) of GTP and a few ATP-derived compounds were determined (fig.2, table 3). The K_m- and K_i-values for ATP and GTP clearly indicate a similar distinction to that found with the mammalian enzymes. NII has a significantly higher affinity for GTP than NI; the K_m- and K_i-values are of the same magnitude as those for ATP, whereas those for NI and GTP are much higher than for ATP. This difference, however, is less pronounced than with the respective mammalian enzymes, for example, the K_i-values for GTP of the nuclear enzymes from porcine liver are 3000 μM (NI) and 9 μM (NII).

The K_i-values of ATP, ADP and AMP (NI) confirm the findings for 3 mammalian protein kinases [3] that the β-phosphoryl contributes significantly to bindings, as its removal drastically enhances the K_i-value. An interesting result for the present plant nuclear enzyme is the low K_i-value for adenosine which was not observed with the mammalian protein kinases. Its implications remain to be elucidated.

The mammalian protein kinase NII is very sensitive to inhibition by heparin [5,13], with $\leqslant$1 μg/ml being effective, whereas NI is insensitive. A similar test showed that the present enzymes only respond at much higher concentrations. For 50% inhibition 120 μg/ml are required for NII and 500 μg/ml for NI. This indicates at least a similar trend in their behavior with heparin to that found with the mammalian protein kinase, although at much higher concentrations.

In conclusion, the nucleus of *Nicotiana tabacum* contains 2 different cyclic nucleotide-independent protein kinases specific for acidic peptides which strongly resemble a pair of protein kinases isolated from the nucleus of different mammalian cells (NI and NII). Although little is known at present about the physiological role of these enzymes, their ubiquitous existence, now established in the plant kingdom, might indicate very basic celllular functions.

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