Oligomeric Structure and Autophosphorylation of Nucleoside Diphosphate Kinase from Rat Mucosal Mast Cells†

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ABSTRACT: Nucleoside diphosphate (NDP) kinases have been found to be involved in a wide range of fundamental biological processes ranging from developmental control to signal transduction and metastasis. We have recently cloned and sequenced a cDNA encoding an NDP-kinase of the rat mucosal mast cell line RBL-2H3 [Hemmerich, S., Yarden, Y., & Pecht, I. (1992) *Biochemistry* (preceding paper in this issue)]. The enzyme itself has been isolated by means of its affinity to the bischromone cromoglycate. Here we report several of its biochemical characteristics: A structural model for the native protein is proposed in which two disulfide-linked pairs of similar 18-kDa subunits (p18) associate to form a 72-kDa tetramer (p72). This is based on the migration properties of the purified enzyme on gel filtration columns, sodium dodecylsulfate gel electrophoresis, and two-dimensional electrophoresis, together with peptide mapping data. In the absence of NDP, both intact p72 and the dissociated 18-kDa subunits (p18) were shown to undergo Mg^{2+}-dependent stoichiometric autophosphorylation utilizing adenosine and guanosine triphosphate or γ-thiotriphosphate as phosphate donor. This autophosphorylation activity was found to be retained by the 18-kDa subunits even following fractionation by SDS–PAGE and electrophoretic transfer to nitrocellulose. The Michaelis constant of this autophosphorylation reaction with either ATP, ATPγS, GTP, or GTPγS was determined to be 6.5 ± 1 μM, and maximally 2 mol of phosphate were found to be incorporated per p72 molecule, thus indicating that phosphorylation occurs at a single site on only two of the four 18-kDa subunits of the holoenzyme. This covalently bound phosphate is labile to hydroxylamine and alkaline treatment but is acid stable, suggesting the formation of an activated aspartyl or glutamyl phosphate as the reaction intermediate that is characteristic for the ping-pong reaction mechanism of NDP-kinases. At a much lower stoichiometry (<0.01 mol of phosphate per mole of p72), serine residues were also found phosphorylated. Moreover, at micromolar enzyme concentrations, p72 was found to phosphorylate serine residues in casein and histone 2b. Using antibodies raised specifically to this NDP kinase, p72 was shown in the rat to be present in most organs. In mice and humans, immunologically cross-reactive autophosphorylating proteins were also identified.

Nucleoside diphosphate kinase (NDP[1] kinase, EC 2.7.4.6) is a ubiquitous enzyme which catalyzes the transfer of the terminal phosphate from 5′-triphosphate [and also 5′-(γ-thio)triphosphates] nucleotides to nucleoside 5′-diphosphates by a ping-pong mechanism involving a high-energy phosphorylated enzyme intermediate (Parks & Agarwal, 1973). Recently, genes encoding NDP kinases were implicated in diverse biological processes such as in tumor metastasis (the human *NM23* gene) (Rosengard et al., 1989) as well as in developmental control (the highly homologous *Drosophila melanogaster* developmental *awd* gene) (Biggs et al., 1988).

We have shown recently that introduction of the antiallergic drug 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-hydroxypropane (cromoglycate, CG) as its lipid-soluble bisacetoxymethyl ester into rat mucosal mast cells of the line RBL-2H3 (Barsumian et al., 1981) uncouples $^{45}Ca^{2+}$ influx and exocytosis from type I Fc_ϵ receptor ($Fc_\epsilon RI$) aggregation in these cells (Hemmerich et al., 1991). During experiments aimed at resolving cellular components that may be involved in $Fc_\epsilon RI$-mediated exocytosis of RBL cells, we have used CG as a ligand in affinity chromatography. In the preceding paper, we reported the isolation and molecular cloning of a cromoglycate-binding cytoplasmic NDP-kinase that exhibits an apparent molecular mass of 18 kDa on reducing sodium dodecylsulfate–polyacrylamide electrophoresis (SDS–PAGE) gels (p18). Its deduced amino acid sequence was found identical to that of a rat NDP-kinase reported very recently by Kimura et al. (1990) and very similar to the products of the human *NM23* (Rosengard et al., 1989) and *Drosophila awd* genes (Biggs et al., 1988) as well as to the recently reported NDP-kinases from *Myxococcus xanthus* II (Muñoz-Dorado et al., 1990a,b) and *Dictyostelium discoideum* (Lacombe et al., 1990). In the present study, we investigated several biochemical properties of the CG-binding rat NDP-kinase, especially its oligomeric structure and autophosphorylation stoichiometry. Our results should provide further insights into structure and function of these important enzymes.

Materials and Methods

Materials

Ampholyte pH 3–10 (Isodalt, no. 42951) was from Serva, Heidelberg, F.R.G. Tris-dodecylsulfate (TDS) required for acidic SDS–PAGE was prepared by passing an aqueous so-

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[1] Abbreviations: NDP, nucleoside diphosphate; CG, cromoglycate; DSCG, disodium cromoglycate; $Fc_\epsilon RI$, type I cell surface receptor for the F_c domain of class E immunoglobulin; p18, the 18-kDa subunit of CG-binding rat NDP-kinase; p72, the native CG-binding rat NDP-kinase tetramer composed of four p18 subunits; RBL, rat basophilic leukemia cells of the line 2H3; SDS–PAGE, sodium dodecylsulfate–polyacrylamide electrophoresis.

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lution of sodium dodecylsulfate (SDS) through an acidic cation exchanger column (Dowex SOW-X2). The resulting aqueous solution of dodecylsulfuric acid was then neutralized with Tris-base. Permafluor III scintillation liquid was from Packard, Downers Grove, IL. Nitrocellulose membranes (type BA85) were from Schleicher & Schüll, Dassel, F.R.G. For all other reagents cf. Hemmerich et al. (1992).

Buffers. PBS, 10 mM phosphate, 135 mM NaCl, pH 7.4 (NaOH); BBS, 200 mM borate, 160 mM NaCl, pH 7.4 (NaOH); BBS–EDTA, BBS with 1 mM EDTA; BBS–Ca^{2+}, BBS with 2 mM $CaCl_2$; HTE, 20 mM HEPES, 1 mM EDTA, pH 7.4 (Tris); TM, 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0; TX, 50 mM Tris-HCl, 0.6 M NaCl, 0.5% Triton X-100, pH 8.0; tyrode, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4; TBST, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20.

Analytical Procedures

Sodium Dodecylsulfate–Polyacrylamide Electrophoresis (SDS–PAGE). SDS–PAGE at basic pH was done with the discontinuous buffer system described by Laemmli (1970). Prior to electrophoresis, protein samples were dissolved in either nonreducing or reducing sample buffer containing 3% SDS, 0.625 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.6 mg of bromophenol blue, and 5% (v/v) of either ethylene glycol or β–mercaptoethanol, respectively (final concentrations). These samples were fractionated in 10–15% polyacrylamide gradient gels with the following MW standards: phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Proteolytic digests (cf. below) were also resolved by SDS–PAGE using a 15% polycrylamide separation gel that contained a 2-fold concentration of Tris (0.75 M instead of 0.375 M), with myoglobin (17.2 kDa), myoglobin fragments I + II (14.6 kDa), I (8.24 kDa), II (6.38 kDa), and III (2.56 kDa) as molecular weight markers. In this case, electrophoresis was interrupted for 1 h following arrival of the dye front above the interphase between stacking and separation gel, then continued. Proteins or peptides were visualized by either Coomassie Brilliant Blue or silver staining (Goldmann et al., 1981). The R_f values of the markers were plotted versus the logarithm of their molecular mass, thus yielding a calibration curve relating the position of any observed protein band to its apparent molecular mass. For detection of radiolabeled polypeptides, the stained gels were later dried in vacuo and exposed on Agfa Curix or Kodak XAR-5 film at −70 °C.

SDS–PAGE at acidic pH (2.6) was done essentially as described by Lichtner and Wolf (1979). Separation and stacking gel buffer contained 93.8 mM citrate, 12.4 mM phosphate–Tris, pH 2.6, 10% glycerol, and 1% Tris dodecylsulfate (TDS). The polyacrylamide concentrations in the stacking and separation gel were 3% and 10%, respectively. Prior to electrophoresis, samples were treated with 0.5 volumes of 3-fold concentrated acidic gel buffer supplemented with 2 mg/mL Pyronin Y (Eastman, Rochester, NY). During electrophoresis, the gel was thermostated at 4 °C. When the dye had reached the anodic end, the gel was dried immediately in vacuo without further processing and exposed to X-ray film.

Two-Dimensional Electrophoresis and Peptide Mapping. Thirty-eight micrograms of [^{125}I]p72 (1.65×10^9 cpm/μg) and in parallel 16 μg of [^{32}P]pp72 (5×10^5 cpm/μg, 2.18 mol of phosphate per mole of p72) were resolved by two-dimensional electrophoresis essentially as described (O'Farrell, 1975). Prior to first dimension isoelectric focusing, protein samples were transferred into 40 μL of IEF sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1% ampholytes, pH range 3–10) by repeated dilution and reconcentration on Centricon ultramembranes (Amicon, Lexington, MA, 10-kDa cutoff). Then these samples were loaded onto 4% polyacrylamide gels in 9 M urea, 0.5% Triton X-100, and 2% ampholytes, pH 3–10, that were prepared within glass rods (130 mm × 2.5 mm i.d.). These gels were run to equilibrium at 500 V between 0.02 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). Thereafter, the focused gel rods were equilibrated for 20 min in reducing SDS sample buffer and layered onto 10–15% SDS gradient gels. SDS–PAGE was carried out as described above. Following electrophoresis, the gels were exposed directly to X-ray film. Gel pieces containing the [^{125}I]p18 or [^{32}P]pp18 isozymes were excised and equilibrated in either 100 or 50 μL, respectively, of 125 mM Tris-HCl, pH 6.8, and 0.1% SDS overnight at 4 °C. Aliquots of the supernatant (20 μL) of the [^{125}I]p18 samples were treated with 2 μg of either V8 protease, papain, trypsin, or pepsin, and 10 μL of reducing and 3-fold concentrated SDS sample buffer, while the entire [^{32}P]pp18 samples (gel piece plus supernatant) were digested with 2 μg of V8 protease in 25 μL of the latter buffer. Then these samples were subjected to electrophoresis as described above.

Western Blots. The protein samples were subjected to electrophoresis, and then the gel content was transferred electrophoretically (200 mA, 4 h) onto nitrocellulose (transfer buffer: 120 mM glycine, 15 mM Tris). The blot was quenched in 10% BSA in PBS containing 0.1% NaN_3 and was incubated with the relevant probe. Nucleotides were employed at a concentration of 1 μM and specific activity of 1000 Ci/mol in 50 mM HEPES, 40 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.2 M NaCl, and 0.1% Tween 20, pH 8.0. Rabbit IgG specific for p72 was used at a specific titer of 10 μg/mL in 1% BSA, 0.1% NaN_3, and 0.05% Tween-20 in PBS. Nucleotide blots were then washed three times in buffer TM, dried, and exposed to X-ray film. Antibody blots were washed three times in buffer TBST and then incubated with 2×10^5 cpm/mL [^{125}I]p72 in 1% BSA, 0.1% NaN_3, and 0.05% Tween-20 in PBS. Following three washes in TBST, the blot was dried and exposed to X-ray film.

Immunoprecipitations. The tissue or cell pellets were homogenized in a >5-fold volume of cold HTE buffer supplemented with leupeptin (5 μg/mL), pepstatin (5 μg/mL), aprotinin (0.22 TIU/mL), 1 mM PMSF, DNAse (25 μg/mL), and RNAse (20 μg/mL). The homogenates were sonicated 1 min on ice, and then the insoluble manner was sedimented, and total protein concentration in the supernatants was determined by the Lowry assay (Lowry et al., 1951). Aliquots of these samples were either subjected to SDS–PAGE with subsequent electrophoretic transfer onto nitrocellulose (cf. above) or diluted to 2 mL with PBS and added to 50 mL of a 50% slurry of protein-A Sepharose saturated with rabbit IgG specific for p72. These suspensions were shaken head over 4 h at 4 °C, and then beads were sedimented and washed with three 10-mL portions of buffer TX and once with 10-mL of 2-fold concentrated buffer TM. [γ-^{32}P]ATP (25 μL) (2 μM, 250 μCi/mL) was added to the washed beads followed by 30 min of gentle shaking at 25 °C. Then the beads were washed with 3 × 10 mL of TM and boiled in 50 mL of reducing 1.5-fold concentrated SDS sample buffer. The sample buffer extracts were then analyzed by SDS–PAGE and autoradiography.

Protein Chemical Procedures

The preparation of the cromoglycate-Sepharose affinity matrix and the purification of p18 from RBL-2H3 cells have

been described earlier (Hemmerich et al., 1992).

Protein Determination. Concentrations of p72/p18 were usually determined by the Lowry assay (Lowry et al., 1951) with BSA as the standard. In order to obtain correct values for the concentration of p72 or p18 by this assay, 5 μL of a p72 solution, in which the concentration of p72 relative to that of the BSA standard was determined to be 10 mg/mL, was subjected to amino acid analysis in a Biotronic LC 6001 amino acid analyzer. Integration of the molar amounts of each amino acid yielded 45.5 μg of protein in the sample. Therefore all [p72/p18] values obtained with the Lowry assay were corrected by a factor of 0.91.

Reduction and Alkylation of p72. Samples of p72 (4 mg/mL) in 0.2 M Tris-HCl (pH 8.0) were reacted with 2.5 mM dithiothreitol (DTT) for 1 h at 25 °C. Following addition of 10 mM iodoacetamide and a further 30-min incubation at 25 °C in the dark, the reduced and alkylated protein was isolated by gel filtration through Sephadex G-25 (8 mm i.d.; bed volume, 25 mL) equilibrated in PBS. The protein-containing fractions were pooled and concentrated on a Centricon ultramembrane (10000 dalton cutoff) yielding a 2.7 mg/mL stock solution, which was aliquoted into 40-μL portions, quickly frozen in liquid N_2, and stored at −70 °C.

Radioiodinations. Protein (100 μg) in 0.1 mL of PBS was reacted with 1 mCi of either [^{125}I]NaI or [^{131}I]NaI and 10 μg of chloramine T for 7 min on ice. Then the reaction was quenched by addition of 10 μL of saturated aqueous tyrosine, and the radioiodinated protein was isolated by gel filtration through Sephadex G-25 (column i.d., 8 mm; bed volume, 10 mL) equilibrated in PBS. A specific activity of 10 μCi per microgram of protein was usually achieved.

M_r Determination by Gel Filtration. Ten micrograms of intact [^{125}I]p72 and 10 μg of [^{131}I]p18 (reduced and alkylated p72) together with 128 μg of phosphorylase B (93 kDa), 166 μg of BSA (67 kDa), 194 μg of ovalbumin (43 kDa), 166 μg of carbonic anhydrase (30 kDa), 160 μg of soy bean trypsin inhibitor (20.1 kDa), and 242 μg of α-lactalbumin (14.4 kDa) in 500 μL (PBS) were loaded onto a 500 mm × 8 mm i.d. column of Sephadex G-75 equilibrated in BBS. The column was eluted with BBS, and 1-mL fractions were collected. Ten microliters of each fraction were counted in a Kontron GAMMAmatic γ-radiation counter using both a high-energy (320–400 keV, cpm_a) and a low-energy (15–90 keV, cpm_b) window. The measured counts were converted into micrograms of [^{131}I]p18 (m_{18}) and μg ^{125}I-p72 (m_{72}) using the equations $m_{18}\cdot a_{11} + m_{72}\cdot a_{12} = cpm_a$ and $m_{18}\cdot a_{21} + m_{72}\cdot a_{22} = cpm_b$. The coefficients of this matrix were determined experimentally by counting samples of [^{131}I]p18 and [^{125}I]p72 separately both in channel a and b: $a_{11} = 2.28 \times 10^6$ cpm_a per microgram of [^{131}I]p18; $a_{12} = 2050$ cpm_a per microgram of [^{125}I]p72; $a_{21} = 925\,500$ cpm_b per microgram of [^{131}I]p18; $a_{22} = 6.53 \times 10^6$ cpm_b per microgram of [^{125}I]p72. In addition, 50 μL of each fraction was analyzed by SDS–PAGE and autoradiography.

Isolation of [^{32}P]pp72. 12.5 μM p72 (0.9 mg/mL) was reacted with 0.25 mM [γ-^{32}P]ATP (specific activity, 5000 Ci/mol) in 100 μL of buffer TM. Then the phosphorylated protein [^{32}P]pp72 was isolated by gel filtration through Sephadex-G25 equilibrated in PBS (column i.d., 8 mm; bed volume, 10 mL). Five microliters of each fraction was mixed with 2 mL of scintillation liquid and counted in a Kontron BETAmatic β-radiation counter. The amount of protein-bound phosphate was calculated from the ratio of the integral of the area under the first radioactivity peak to the sum of the integrals under both first and second peak. 2.1 ± 1 mol of [^{32}P]phosphate was found incorporated per mole of p72 under these conditions.

Stability of the Phosphate Bound to pp72. Aliquots (10 μL) of a solution of freshly prepared [^{32}P]pp72 in PBS (1.1×10^6 cpm [^{32}P]phosphate/mL) were added in parallel to 50 μL of (1) 0.8 M NaCl, 0.1 M NaOAc, pH 5.4; (2) 0.8 M NH_4Cl, 0.1 M NaOAc, pH 5.4; (3) 0.8 M $HONH_3Cl$, 0.1 M NaOAc, pH 5.4. Following a 30-min incubation at room temperature, these samples were diluted with 1 mL of TM and filtered rapidly through 25-mm diameter nitrocellulose filters. The filters were washed four times with 2-mL portions of TM and dried, and their β-radiation was determined. Each data point was done in triplicate.

Phosphoamino Acid Analysis of pp72. p72 (35 μM) was reacted with 35 μM [γ-^{32}P]ATP (specific activity, 16700 Ci/mol) in 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0, for 30 min at 25 °C. Then 5-μL aliquots were subjected to SDS–PAGE through a 10–15% polyacrylamide gradient gel under reducing conditions. The gel was stained (Comassie Brilliant Blue), dried, and exposed to X-ray film. The radioactive 18-kDa band was excised from the dry gel and treated overnight at 37 °C with trypsin (0.2 mg/mL) in 20 mM NH_4HCO_3; then this solution was cleared and lyophilized. The residue was hydrolyzed in 0.3 mL of 6 N HCl at constant volume and 110 °C overnight. This hydrolysate was evaporated and redissolved in 3 mL of H_2O; then the acid-free residue was taken up in an aqueous solution of phosphoserine, phosphothreonine, and phosphotyrosine (each 1 mg/mL). From this a 5-μL sample was applied at the bottom of a thin layer of cellulose on glass. Following electrophoresis at 1500 V in pyridine/AcOH/H_2O, 1:10:189 (v/v), the plate was stained with ninhydrin and exposed to X-ray film.

Phosphorylation Assays

Autophosphorylation. For each assay 1 μM p72 (= 4 μM p18) was incubated with the indicated concentrations of [γ-^{32}P]nucleoside triphosphate (NTP) or nucleoside [γ-^{35}S]-thiotriphosphate (NTP-γS) with a specific activity of 1550 Ci/mol in buffer TM for 30 min at 25 °C. Then each sample (40 μL) was either subjected to acidic SDS–PAGE or, following addition of 1 mL of TM, filtered rapidly through 25 mm diameter nitrocellulose filters (Schleicher & Schüll BA85). The filters were washed four times with 2-mL portions of TM, dried, and then placed into 10 mL of Permafluor II/xylene (1:9 v/v) and counted. Each data point is the average of triplicate. The nonspecific absorption of either of the nucleotides to the filters was always found negligible (<1% of the protein-bound counts). In each experiment, a calibration relating the measured counts per minute to the concentration of phosphate or thiophosphate was done for each nucleotide substrate. With the proportionality coefficient obtained, the average of the radioactivity measured on the filters of each triplicate was translated into $[phosphate]_{bound}$ or $[thiophosphate]_{bound}$.

Data Analysis. The data obtained for the dose response of autophosphorylation to [nucleotide substrate] were replotted as log ($[(thio)phosphate]_{bound}$ over $(R - [(thio)phosphate]_{bound})$ as function of log $[NTP(\gamma S)]_{free}$ with R being the concentration of phosphorylation sites (set to 2 μM). The straight line through these data was constructed with a linear least-squares algorithm, and then, using a locally developed computer program, this line was fitted iteratively for a minimal deviation from the experimental data by ±10% variation of R. From the best fit, the Hill coefficient n ($= \Delta y/\Delta x$) and the Michaelis constant [$K_m = 1$ μM × exp(y-intercept)] were calculated. With these parameters, the theoretical saturation

curve $[(\text{thio})\text{phosphate}]_{\text{bound}} = f[[\text{NTP}(\gamma\text{S})]_{\text{total}}]$ was constructed using analytical forms of the Hill equation $[(\text{thio})\text{phosphate}]_{\text{bound}}/[\text{NTP}(\gamma\text{S})]_{\text{free}}^{n} = K_m \times [R - [(\text{thio})\text{phosphate}]_{\text{bound}}]$.

Phosphorylation of Exogenous Substrates by p72. The reaction mixture contained the protein substrate [either 2.5 mg/mL histone 1 (Sigma no. H5055), 2.5 mg/mL histone 2b (Sigma no. H4255), or 12.5 mg/mL casein (Sigma no. C4765)] and 0.625 mM [γ-^{32}P]ATP (specific activity, 2 Ci/mmol) in 10 mM HEPES, pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT (optional, cf. Results). Following addition of p72 to final concentrations ranging from 2.5 μg/mL to 2.5 mg/mL, 20 μL of the mixture was incubated for 30 min at 37 °C, and then 1 mL of ice-cold 15% trichloroacetic acid was added. The precipitate was pelleted, washed three times in cold 15% trichloroacetic acid, and redissolved by boiling in 30 μL of 3-fold concentrated reducing SDS sample buffer. Then the sample was subjected to SDS–PAGE and autoradiography. The radioactive substrate bands were then excised and processed for phosphoamino acid analysis as described above.

Generation of Polyclonal IgG Specific for p72

New Zealand White rabbits were immunized with p72 by intradermal injection at multiple sites [0.5 mg per animal in complete Freund's adjuvant (CFA) emulsion]. Boost immunizations were given twice, one week and two weeks following the primary immunization, in the same fashion using incomplete Freund's adjuvant (IFA) instead of CFA. The animals were bled thereafter weekly until the antibody titer (detected qualitatively by precipitin formation) dropped considerably. Immune serum (100 mL) was cleared by centrifugation (1 h at 12 500 rpm), and then solid $(NH_4)_2SO_4$ was added to yield 50% saturation. The precipitate was collected, washed once with 50% saturated aqueous $(NH_4)_2SO_4$, and redissolved in a minimal volume of 10 mM phosphate buffer, pH 8.0. This solution was dialyzed against several changes of 10 mM sodium phosphate, pH 8.0. The precipitated protein was centrifuged off, and supernatant was loaded onto a DE-52 (Whatman, Maidstone, U.K.) anion-exchanger column (30 mm i.d.; 700-mL bed volume) equilibrated in 10 mM sodium phosphate, pH 8.0. The column was washed with the same buffer, and flow-through was collected until $A_{280\text{nm}}$ had dropped beyond 0.25 OD. The very pure (>90%) IgG in this fraction was concentrated by $(NH_4)_2SO_4$ precipitation and redissolved in PBS to 24 mg of IgG/mL. The titer of specific antibodies in this solution was assayed by quantitative immunoprecipitation of p72 to 0.8 mg of p72-specific IgG/mL (3.3% of total IgG). Additional less pure IgG was obtained by further elution of the column with 75 mM NaCl in 10 mM sodium phosphate, pH 8.0. Following concentration, this fraction was found to contain 0.57 mg of p72-specific IgG/mL and 30 mg/mL total protein.

Results

Structural Characterization of Rat Nucleoside Diphosphate Kinase. Rat NDP-kinase was isolated from RBL-2H3 cells by affinity chromatography on cromoglycate-Sepharose as described earlier (Hemmerich et al., 1992). We have shown that the respective mRNA encodes a 17 286-dalton polypeptide, which is in agreement with the migration properties of the enzyme on reducing SDS gels. However, on gel filtration columns, the intact protein exhibited a molecular mass of 70–75 kDa (Figure 1). Following reduction with dithiothreitol and subsequent alkylation, a single polypeptide migrated on the same column at a slower rate equivalent to ≈18 kDa (Figure 1). On nonreducing SDS gels, the intact protein

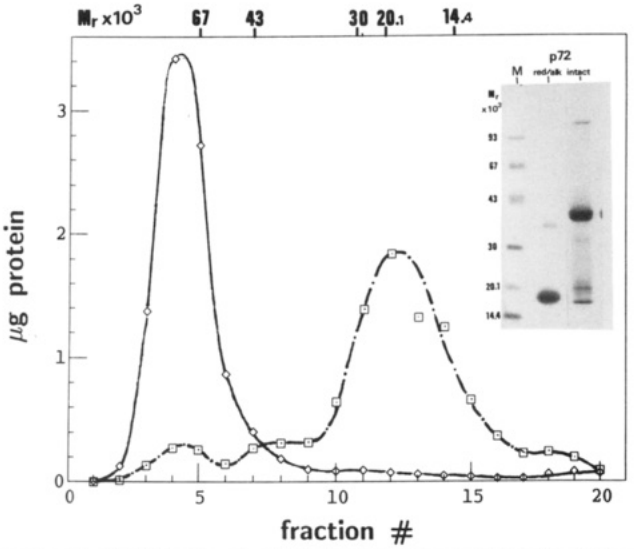

FIGURE 1: Gel filtration of radioiodinated p72 and p18 on Sephadex G-75. 10 μg of native [^{125}I]p72 and 10 μg of [^{131}I]p18 (reduced and alkylated p72) together with 100–200 μg of each of the following proteins: phosphorylase B (93 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soy bean trypsin inhibitor (20.1 kDa), and o-lactalbumin (14.4 kDa) in 500 μL (PBS) were loaded onto a 500 mm × 8 mm i.d. column of Sephadex G-75 equilibrated in BBS. The column was eluted with BBS, and 1-mL fractions were collected and analyzed by counting their radioactivity and SDS–PAGE autoradiography. Labeled protein contents of the fractions, computed from the high (^{131}I) and low (^{125}I) energy γ-radiation, are presented. (Insert) Intact and reduced/alkylated enzyme on nonreducing SDS gels. 10 mg of intact p72 and 10 mg of reduced and alkylated p72 (p18) were subjected to SDS–PAGE under nonreducing conditions. While the nonreduced p72 migrates as a closely spaced doublet at 36 kDa, reduced and alkylated protein exhibits a single band at 18 kDa.

migrated as a closely spaced doublet centered at a position equivalent to 36 kDa, while the reduced and alkylated polypeptide exhibited a single band at 18 kDa on these gels (Figure 1, insert). Thus the intact protein with an apparent molecular mass of 72 kDa appears to be composed of four pairwise disulfide-linked 18-kDa subunits. Therefore the holoenzyme was termed p72, while its 18-kDa subunits are referred to as p18. Two-dimensional electrophoresis combining isoelectric focusing with reducing SDS–PAGE (Figure 2) resolved p18 into two dominant isozymic forms with pIs of 7.0 and 6.5 at a 1:1 ratio. In addition, three minor more acidic species amounting together to less than 5% of the total protein were also observed. Peptide maps (Cleveland et al., 1977) of all these isozymes using different proteases were found to be practically identical to each other (Figure 3).

Characterization of the Autophosphorylation Activity of p72. Both the intact (p72) and the reduced and alkylated (p18) protein were shown to incorporate [^{32}P]phosphate or [^{35}S]thiophosphate from either [γ-^{32}P]ATP, [γ-^{32}P]GTP, [^{35}S]ATPγS or [^{35}S]GTPγS, requiring no cofactors but Mg^{2+} ions. On gel filtration columns, this radioactive phosphate or thiophosphate coeluted with the respective protein. This capacity of p72 to incorporate phosphate from nucleoside triphosphate substrates was found retained even following SDS gel electrophoresis under reducing conditions and transfer onto nitrocellulose (Figure 4). Since [α-^{32}P]nucleoside triphosphates yielded none or only faint signals on these blots, autophosphorylation rather than NTP binding has to account for high phosphate incorporation of p72 from [γ-^{32}P]NTP or [^{35}S]NTPγS.

This autophosphorylation activity was quantitated by reacting p72 with the nucleoside triphosphate substrate in

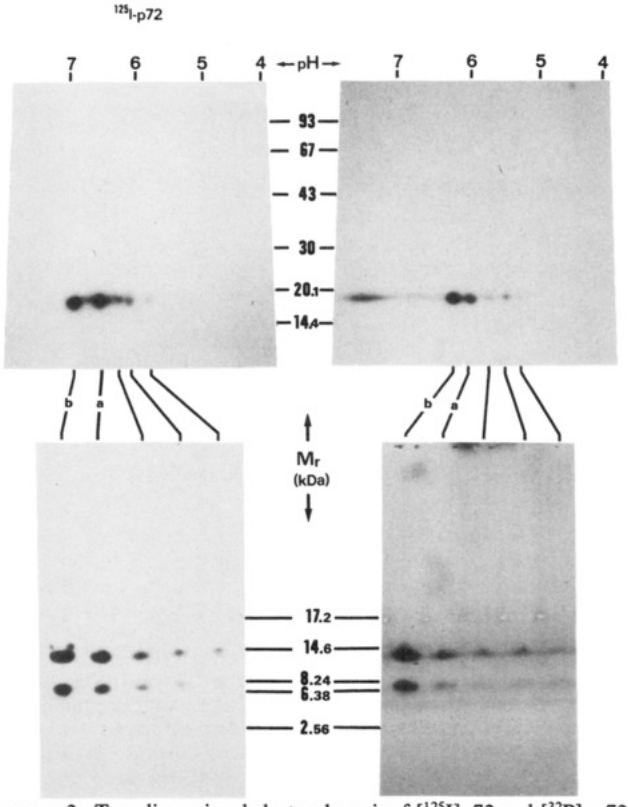

FIGURE 2: Two-dimensional electrophoresis of [^{125}I]p72 and [^{32}P]pp72 with subsequent V8 protease peptide mapping. 38 μg of [^{125}I]p72 (left) and in parallel 16 μg of [^{32}P]pp72 (2.18 mol of phosphate per mole of p72, right) were resolved by two-dimensional electrophoresis (O'Farrell, 1975). Autoradiograms of these gels are depicted in the upper panel. The dominant 18-kDa isozymes at pH 7.0 and 6.6 (phosphorylated forms at pH 6.4 and 6.0) are indicated with b (basic) and a (acidic), respectively. The lower panel depicts autoradiograms of V8 protease peptide maps (Cleveland et al., 1977) of the respective [^{125}I]p18 or [^{32}P]pp18 isozymes.

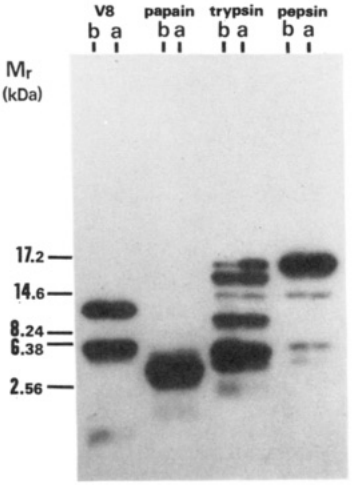

FIGURE 3: Peptide mapping of [^{125}I]p18 isozymes by different proteases. Samples of the dominant pI 7.0 (b) and pI 6.6 (a) [^{125}I]p18 isozymes isolated by two-dimensional electrophoresis (cf. Figure 2) were digested by the indicated proteolytic enzymes. The figure depicts autoradiograms of the respective Cleveland peptide maps.

Mg^{2+}-containing buffer and adsorbing the protein on nitrocellulose filters. Thus, the extent of autophosphorylation measured as function of NTP concentration was found always saturable at 2 × [p72], irrespective of the nature of the phosphate donor (Figure 5). This stoichiometry was also determined independently in phosphorylated p72 that had been obtained by reaction of p72 with a 20-fold molar excess of ATP

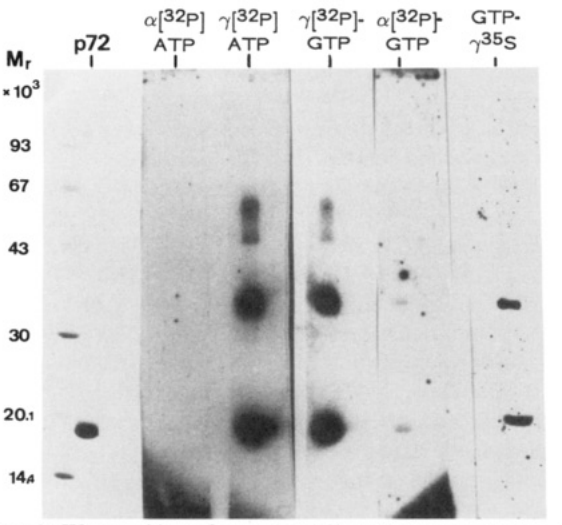

FIGURE 4: Western blots of p72 with different NTP substrates. p72 was subjected to reducing SDS–PAGE and transferred electrophoretically onto nitrocellulose. The blots were then probed with the indicated radiolabeled nucleoside triphosphates. While [α-^{32}P]nucleosides labeled the blots only poorly, [γ-^{32}P]nucleoside triphosphates and [γ-^{35}S]thiotriphosphates labeled the blots strongly at positions equivalent to molecular masses of 18 and 36 kDa. The latter signal appears to be due to traces of p18 dimer present even on reducing gels. Controls made with equivalent amounts of bovine serum albumin and γ-globulin did not produce any labeled product under the above conditions.

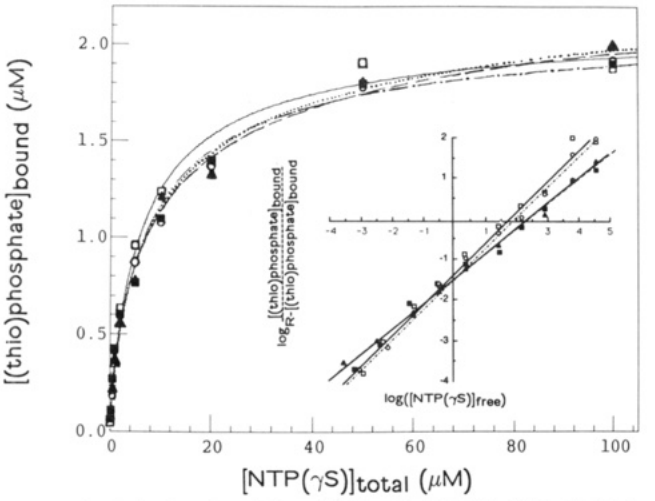

FIGURE 5: Autophosphorylation of p72 with ATPγS, ATP, GTPγS or GTP. Quantitation of p72 autophosphorylation with nucleotide substrates using a filter assay and analysis of the data are described under Materials and Methods. [p72] was always 1 μM. (□) ATPγS; (○) ATP; (■) GTPγS; (▲) GTP. The depicted data are averages of triplicates. (Main panel) Self-phosphorylation as function of total substrate concentration. Experimental data are shown along with theoretical binding isotherms. Maximally, 2 mol of phosphate were found to be incorporated into pp72 at high substrate concentrations. (Insert) Hill plot of the data sets along with their respective best fitting straight lines. R is the concentration of phosphorylation sites. It was set to 2 μM ± 10%, with the error margin allowing for best fit of the straight lines to the experimental data.

and separated from unreacted NTP by gel filtration. Reduction and alkylation of p72, shown above to cause dissociation of its 18-kDa subunits, did not alter the phosphorylation stoichiometry of maximally 0.5 mol of phosphate per mole of p18. The shapes of the $[(\text{thio})\text{phosphate}]_{\text{incorporated}}$ versus $[\text{nucleotide}(\gamma\text{-thio})\text{triphosphate}]_{\text{total}}$ isotherms were very similar for all four different nucleotides examined (Figure 5). In Eadie–Hofstie plots, the data did not fit straight lines as expected for a Michaelis–Menten-type mechanism, but rather

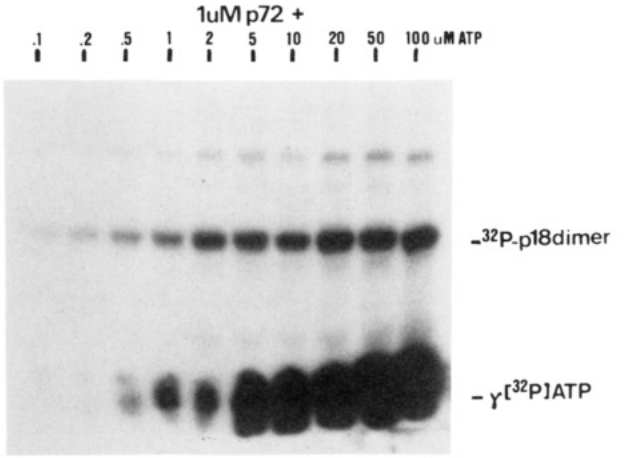

FIGURE 6: Acidic SDS gel of [^{32}P]p72. 1 μM p72 was reacted with the indicated concentrations of [γ-^{32}P]ATP, and then 40-μL samples were subjected to nonreducing acidic (pH 2.6) SDS–PAGE at 4 °C (Lichtner & Wolf, 1979). On the depicted autoradiograph (exposure time, 5 min) the ratio of [^{32}P]phosphate bound to the p18 dimer (upper bands) to unreacted substrate (lower bands) as function of ATP concentration agrees well with the results of the filter assays (Figure 5).

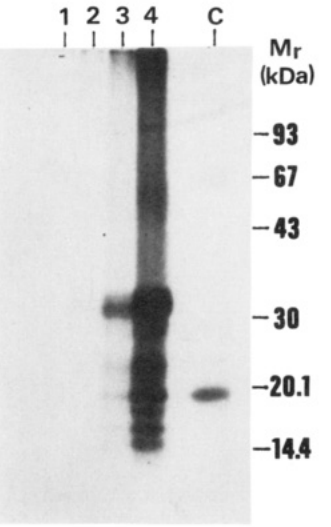

FIGURE 7: Phosphorylation of casein by p72. p72 (lane 1, 2.5 μg/mL; lane 2, 25 μg/mL; lane 3, 0.25 mg/mL; lane 4, 2.5 mg/mL) was incubated (30 min, 37 °C) with 12.5 mg/mL casein (Sigma no. C4765) and 0.625 mM [γ-^{32}P]ATP (specific activity, 2 Ci/mmol) in 10 mM HEPES, pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT. Then the protein was precipitated with ice-cold 15% trichloroacetic, and the pellet was washed three times, dissolved in reducing SDS sample buffer, and subjected to SDS–PAGE. The figure presents the autoradiograph of this gel. As a control, bovine serum albumin (12.5 mg/mL) was reacted with 2.5 mg/mL p72 (C). In this case only autophosphorylation of p72 was observed.

to hyperbolas (data not shown). However, the data could be fitted well to straight lines in Hill plots (Figure 5, insert), yielding apparent Michaelis constants and Hill coefficients which are, respectively, very similar for the different nucleotides: 3.75 μM and 0.75 for ATPγS as substrate, 4.24 μM and 0.74 for ATP, 4.47 mM and 0.6 for GTPγS, and 4.33 μM and 0.61 for GTP. Theoretical curves that were computed with these parameters as described under Materials and Methods fitted the experimental data well (Figure 5, main panel). Equivalent concentrations of reduced and alkylated enzyme yielded the same dose–response curve as the intact protein (data not shown).

The rate of autophosphorylation was very fast, and no dependence on temperature and substrate concentration could be resolved. Already, in less than 1 min after addition of the phosphate donor, the final equilibrium was reached. Laemmli SDS–PAGE or treatment of the phosphorylated protein [^{32}P]pp72 with 0.8 M hydroxylamine (but not NaCl or NH_4Cl) at pH 5.4 (30 min, 25 °C) caused the loss of over 80% of the bound phosphate. However, [^{32}P]pp72 was found stable on acidic (pH 2.4) SDS gels run at 4 °C (Figure 6).

A residual rather small amount of the protein-bound phosphate stable toward hydroxylamine and bases was found associated with the respective protein bands on basic (pH 8.8) Laemmli SDS gels. On nonreducing gels of [^{32}P]-phosphorylated p72, the radioactivity was found to comigrate with both bands of the 36-kDa doublet, while on reducing gels it was associated with the 18 kDa subunit. This protein-bound phosphate determined in the respective bands never exceeded 0.01 mol per mole of p72. The radioactive 18-kDa band was excised from the reducing SDS gel, completely hydrolyzed, and subjected to phosphoamino acid analysis. The only labeled amino acid found was phosphoserine (data not shown). Two-dimensional electrophoresis of this alkali-stable phosphorylated p72 has shown serine-bound phosphate being present in all pp18 isozymes, which relative to the non-phosphorylated species are ~0.6 pH-units more acidic (Figure 2).

Exogenous Substrates. The capability of p72 to phosphorylate proteins others than itself was examined using histones, ribosomal subunit S4, casein, and calmodulin. Solutions of these proteins and p72 were reacted with [γ-^{32}P]ATP. Rather high concentrations of the p72 (>25 μg/mL) had to be used and only casein (Figure 7) and histone 2b were found to undergo phosphorylation (on serine residues only).

Species and Tissue Distribution of p72. Polyclonal antibodies specific for p72 were raised in rabbits. The IgG fraction was purified from the total serum to a p72-specific titer of 0.8 mg/mL as described under Materials and Methods. These antibodies were found to immunoprecipitate p72 from Triton X-100 lysates of RBL cells that had been biosynthetically labeled with [^{35}S]methionine. However, no inhibition of the autophosphorylation of p72 with ATP could be induced by these antibodies. Thus, p72 that had been immunoprecipitated with these antibodies either from aqueous solution or from RBL cytosol was found to retain its enzymatic activity even in the immune complex (Figure 9).

A series of rat, murine, and human tissues were examined for the presence of p72 either by Western blot analysis or by immunoprecipitation using the described antibodies. In the Western blot shown in Figure 8 bound antibody was visualized by incubation with radioiodinated p72 that bound to the second antigen combining site of the p72-specific antibodies, while in the immunoprecipitations (Figure 9) the antigen was visualized by in situ autophosphorylation. p72 was found present in all the rat tissues tested: brain, kidney, spleen, liver, heart, lung, thymus, and RBL cells. Its apparent relative amount, however, varied significantly among these tissues and cells types, being most abundant in RBL cells at levels between 2- and 3-fold higher than in the normal rat tissues (Figure 8). Yet, this finding may reflect different reactivities of the enzyme from different tissues with the antibody raised against the RBL enzyme. Of the human tissues examined, namely adenoids, mucosal and adipous tissue, muscle, omentum, leukocytes, and erythrocytes, only the two former were found to contain a 20-kDa polypeptide that is recognized by the antibodies on blots (Figure 8). In immune complexes with these antibodies, this 20-kDa polypeptide was also found to undergo autophosphorylation with ATP as substrate. Analysis of the au-

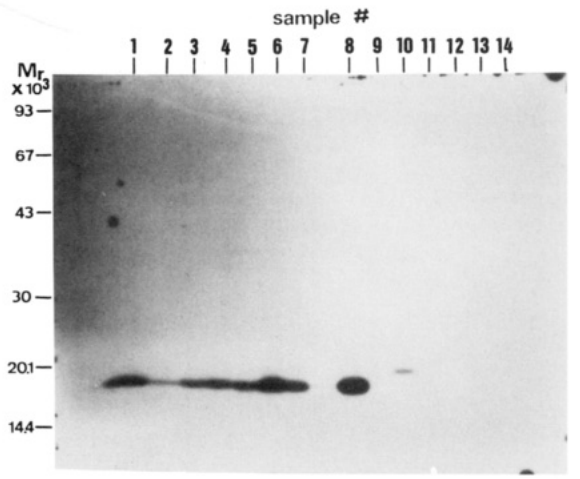

FIGURE 8: Screening of various tissues for presence of p72 by Western blot analysis. Cytosol of the following tissues were fractionated by SDS electrophoresis through a 10–15% polyacrylamide gradient gel and transferred electrophoretically to nitrocellulose: rat heart (1), rat lung (2), rat testis (3), rat liver (4), rat thymus (5), rat kidney (6), rat brain (7), RBL (8), *Xenopus laevis* oocyte (9) (1–9, 250 μg of total protein), human mucosal membrane (10), human adipous tissue (11), human muscle (12), human omentum (13), and human lymphocytes (14) (10–14, 600 μg of total protein). The blot was probed with rabbit IgG specific for p72, and bound antibody was detected with radioiodinated p72. The human p72 analogue exhibited on the blot a single band at 20 kDa.

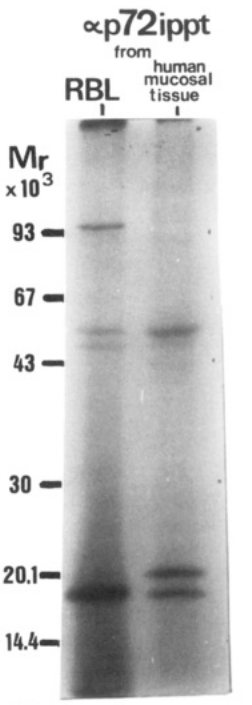

FIGURE 9: Immunoprecipitation of rat p72 and its human analogue. Immunoprecipitation with rabbit IgG specific for p72 was performed in parallel on a sample of RBL cytosol (2.75 mg of total protein) and cytosol of human mucosal tissue (9.8 mg of total protein). The isolated immunoprecipitates were reacted with [γ-^{32}P]ATP, and the products were analyzed by SDS–PAGE and autoradiography.

tophosphorylated human antigen by SDS–PAGE revealed a further phosphorylated 18-kDa polypeptide associated with the 20-kDa band seen on blots (Figure 9).

Discussion

The present study dealt with quaternary structure and autophosphorylation activity of a rat nucleoside diphosphate kinase that had been isolated from the mucosal mast cell line RBL-2H3 by a procedure based on its affinity to cromoglycate. cDNAs with identical open reading frames encoding this protein have been cloned and sequenced by us (Hemmerich et al., 1992) and independently by Kimura et al. (1990), who isolated the kinase using conventional protein chemical methodologies. NDP-kinases have emerged recently as key enzymes in fundamental cellular processes such as signal transduction and growth control.

All NDP-kinases isolated and characterized to date were shown to form oligomeric structures composed of subunits with molecular masses between 16 and 19 kDa. However, the stoichiometry and structural relatedness of these subunits have remained unclear (Nickerson & Wells, 1984; Kimura et al., 1990). Thus rat NDP-kinase was suggested to be a pentamer or hexamer (Kimura & Shimada, 1988), *Drosophila* awd protein was shown to be a hexamer or higher multimer (Biggs et al., 1988), while the *M. xanthus* II NDP-kinase was reported to be a trimer (Muñoz-Dorado et al., 1990). Though the phosphorylated intermediate involved in the ping-pong reaction mechanism of the enzyme has been well established, the stoichiometry of this autophosphorylation has remained poorly characterized.

Since our CG-ligand affinity chromatography fast and reliably yielded milligram amounts of RBL NDP-kinase in high purity, we investigated this enzyme further in order to define its oligomeric structure and autophosphorylating activity: The results of gel filtration and two-dimensional (O'Farrell) SDS–PAGE and peptide mapping (Figures 1, 2, and 3) suggested the holoenzyme to be a 72-kDa pairwise disulfide-linked tetramer (p72) with an $\alpha_2\alpha^*_2$ subunit composition, where α and α^* are structurally almost identical 18-kDa polypeptide chains (p18) with different isoelectric points (pI). Disulfide bonds seem to be essential for the tetrameric structure, since treatment with disulfide-reducing agents caused dissociation of p72 into its 18-kDa subunit even without denaturants. It cannot be inferred from our results, whether the disulfide bonds link between identical subunits $(\alpha\text{–}\alpha)(\alpha^*\text{–}\alpha^*)$ or different subunits $(\alpha\text{–}\alpha^*)_2$. Our model ignores three additional more acidic 18-kDa chains also resolved by isoelectric focusing, since their relative amount compared to the main two subunits is less than 5% (Figure 2). V8 protease peptide maps of these minor, more acidic subunits and the two major p18 chains are identical (Figure 2); therefore, the former may have risen from the latter by posttranslational modification.

In the preceding report, we have shown that purified p72 effectively catalyzes the transfer of the terminal phosphate group from nucleoside 5′-triphosphates to nucleoside 5′-diphosphates. This catalysis is known to function by the following ping-pong mechanism involving a high-energy phosphorylated protein intermediate (Mourad & Parks, 1965; Parks & Agarwal, 1973; Robinson et al., 1981):

$$N_1TP + E \rightleftharpoons E\text{–}P + N_1DP$$

$$N_2DP + E\text{–}P \rightleftharpoons E + N_2TP$$

In the absence of a NDP phosphate acceptor, the phosphorylated enzyme intermediate has been isolated in many cases (Parks & Agarwal, 1973, Lacombe et al., 1990; Muñoz-Dorado et al., 1990). Indeed, upon reacting both the native tetrameric p72 as well as the (reduced and alkylated) p18-monomer with [γ-^{32}P]ATP, a phosphorylated intramediate could be isolated by gel filtration. Autophosphorylation of p72/p18 occurred also on Western blots of SDS gels (Figure 4) and even two-dimensional gels (first dimension, nondenaturing isoelectric focussing; data not shown) that were incubated with [γ-^{32}P]ATP or GTP. These observations clearly suggested that p72/p18 rather than a contaminating kinase is responsible for this activity. The acid stability as compared

with the alkali and hydroxylamine lability of most of the protein-bound phosphate in phosphorylated protein (pp72) indicates that a high-energy acyl phosphate bond is formed, i.e., phosphorylation takes place probably on aspartic or glutamic acid residues (Bitte & Kabat, 1974). Since phosphohistidine is acid-labile and alkali-stable, at least in pp72 there appears to be no histidine phosphorylation as it has been reported for a large number of NDP-kinases described so far (Parks & Agarwal, 1973; Lacombe et al., 1990). However, acyl-phosphate intermediates have been observed before in NDP-kinases (Hossler & Rendi, 1971; Parks & Agarwal, 1973). This notion has also been raised by the amino acid sequence of p18 (Hemmerich et al., 1992) that contains a nine-residue stretch with 80% homology to one of the phosphorylation sites in casein. However, the SerSerSer in the latter is substituted by SerAspSer in p18. Therefore it is conceivable that this aspartate might be the site of acyl-phosphate formation during the catalytic cycle of the enzyme.

The dose-response of acyl-phosphate formation as function of substrate concentration is very similar for the different nucleotides examined (Figure 5). It can be modeled well using the Hill formalism. However, since practically the same dose-response curve was also observed using equivalent amounts of the (reduced and alkylated) free p18 monomer instead of intact tetrameric p72, the measured Hill coefficients ($n < 1$) apparently do not reflect negative cooperativity as a consequence of half-site reactivity. Rather we assume that either the α or the α^* subunit only undergo phosphorylation involving a non-Michaelis-Menten-type reaction mechanism. This assumption is further supported by the autophosphorylation stoichiometry (maximal 2 mol of incorporated phosphate or thiophosphate per mole of p72 or 0.5 mol per mole of p18, irrespective of the substrate) that was found identical both for the intact p72 and for the dissociated p18 subunits.

Our initial biochemical experiments probed the possibility that p72 is a novel protein kinase, and, indeed, rather low nonstoichiometric (0.01 mol of phosphate/mol of p72) serine phosphorylation was found to occur on all four p18 subunits at least on two sites (Figure 2) and also on exogenous substrates that are typical for protein serine kinases, such as casein (Figure 7) or histone 2b. However, the enzyme concentrations required to phosphorylate casein were rather high (>25 μg/mL = 0.36 mM p72), clearly indicating that it is certainly a nonphysiological substrate. It is still noteworthy that our observations may be rationalized by the significant homologies between the sequence of p72 (and other NDP-kinases) and sequences of protein kinases (Hemmerich et al., 1992).

p72 appears to be relatively abundant in the mucosal mast cell line RBL 2H3, yet it is found in lesser relative amounts in most other rat tissues (Figure 8). Differential reactivity of p72 isozymes from different tissues with the antibodies directed against p72 from RBL cells may render such a quantitative comparison of p72 abundance less reliable. However, the observed pattern was in part also confirmed on the mRNA level (Hemmerich et al., 1992). Immunologically related proteins that underwent autophosphorylation, were also identified both in murine and human tissues (Figures 8 and 9). These findings, along with the high degree of sequence conservation among all NDP-kinases isolated so far from organisms as different as humans, *Drosophila*, and *Dictyostelium*, further underscore the importance of these enzymes in fundamental cellular processes.

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