A Cromoglycate Binding Protein from Rat Mast Cells of a Leukemia Line Is a Nucleoside Diphosphate Kinase†

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ABSTRACT: Recently, we have shown that a membrane-permeant derivative of the antiasthmatic drug cromoglycate (CG) effectively inhibits the Fc_ϵ-receptor-mediated secretory response of rat mucosal mast cells (line RBL-2H3) at a stage preceding the transient rise in the cytoplasmic free calcium concentration [Hemmerich, S., Sijpkins, D., & Pecht, I. (1991) *Biochemistry 30*, 1523-1532]. In contrast to cromoglycate itself, which is membrane impermeant and ineffective in these cells, its *bis*-acetoxymethyl ester derivative (CG/AM) can diffuse across the plasma membrane into the cytosol, where it is hydrolyzed into the impermeant CG dianion, which presumably may interact with intracellular components involved in the $Fc_\epsilon R$ signal transduction pathway. In order to identify cytosolic components involved in the stimulus–secretion coupling that interact with this drug, we coupled CG to an insoluble matrix. This matrix was indeed effective in the affinity isolation from RBL cells of a cytosolic protein that exhibits an apparent molecular mass of 18 kDa on reducing SDS gels. This protein was purified to homogeneity and then fragmented, and the amino acid sequence of three resultant peptides was determined. Using the corresponding synthetic oligonucleotides, we cloned and sequenced a cDNA that encodes the full-length 18-kDa polypeptide (p18). The protein sequence deduced from this cDNA is identical to that of rat nucleoside diphosphate kinase [Kimura, N., Shimada, N., Nomura, K., & Watanabe, K. (1990) *J. Biol. Chem. 265*, 15744-15749] and highly homologous (88%) to the human NM23 gene product whose expression is associated with reduced metastatic potential, as well as with the Drosophila awd gene product (77% sequence identity). The p18 isolated from RBL cells was indeed shown to catalyze phosphate transfer from nucleoside triphosphates to nucleoside diphosphates. This activity was inhibited by cromoglycate ($I_{50} \approx 2$ mM). The amino acid sequence of p18 and its enzymatic activity indicate that this cromoglycate binding protein is a nucleoside 5′-diphosphate kinase, which, through control of the cytoplasmic pool of nucleoside triphosphates (e.g., GTP), may affect the transduction of the Fc_ϵ-receptor-mediated signal.

Disodium 1,3-bis[(2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane [commonly known as disodium cromoglycate (DSCG),[1] Cromolyn, Intal, or Lomudal], is a drug widely used for the treatment of allergic asthma (Cox, 1967; Cox et al., 1970). Unlike many other antiallergic drugs that act distal to the step of mediator release from mast cells and basophils, DSCG has been shown to inhibit both the antigen-induced $^{45}Ca^{2+}$ uptake by, and mediator secretion from, rat peritoneal mast cells (Foreman et al., 1977; Cox, 1967). However, DSCG has been found ineffective on cells of the line RBL-2H3 (Barsumian et al., 1981) that are a widely used model in the study of type I Fc_ϵ-receptor ($Fc_\epsilon RI$)-mediated secretion and closely resemble rat mucosal mast cells (Seldin et al., 1986). Recently, we have synthesized the di(acetoxymethyl) ester of cromoglycate and shown it to passively diffuse through the plasma membrane into the cytosol, where it is converted into the CG dianion by enzymatic hydrolysis. CG was found to accumulate in the cytoplasm of the RBL cells treated with CG/AM to concentrations that are 40-fold its respective extracellular concentration and effectively [I_{50} = 2 mM cytoplasmic CG (Hemmerich et al., 1991)] inhibit both the $^{45}Ca^{2+}$ uptake and mediator secretion caused by immunological stimulation, i.e., clustering the $Fc_\epsilon RI$. By contrast, mediator secretion induced by Ca^{2+} ionophores was not impaired in these cells. These observations suggested cromoglycate to be useful as ligand in probing for proteins that are involved in the $Fc_\epsilon RI$–secretion coupling.

In this paper, we report the isolation and primary structure of an NDP-kinase that was isolated from RBL cytosol by affinity chromatography with cromoglycate as ligand. In the following paper in this issue, we report the oligomeric structure of this enzyme and studies on its autophosphorylation.

Materials and Methods

Materials. 1,3-Bis[2′-carboxylatochromon-5′-yl)oxy]-2-(6″-aminocaproamido)propane (aminocaproamidocromolyn) was prepared in a 10-step synthesis starting from resorcinol and β,β′-dibromoisopropylammonium bromide as described earlier (Hemmerich & Pecht, 1988). Disodium 1,3-bis[2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane (DSCG) was a generous gift of Fisons plc, Loughborough, England. Sepharose–poly[(*N*-hydroxysuccinimidyl)caproate] (activated CH-Sepharose no. 17-0490-01), Sephadex, and molecular weight markers for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Pharmacia, Uppsala, Sweden. Protease inhibitors, RNase, DNase, Triton X-100, and dithiothreitol (DTT) were from Sigma, St.

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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, an 18-kDa polypeptide from RBL cells that exhibits NDP-kinase activity; RBL, rat basophilic leukemia cells of the line 2H3; SDS-PAGE: sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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Louis, MO. Materials for polyacrylamide gel electrophoresis were from Sigma and Bio-Rad, Richmond, CA. Nonradioactive nucleotides were from Boehringer, Mannheim, F. R.G., or Sigma, and all radiolabeled compounds were from New England Nuclear, Boston, MA. All enzymes used in the cDNA cloning were from New England Biolabs (NEB), Boston, MA. Sequenase was purchased from United States Biochemicals, Cleveland, OH. Vectors and primers were from Pharmacia or NEB. The rat kidney cDNA expression library in λgt11 was purchased from ClonTech, Palo Alto, CA.

Cells. Mucosal type mast cells of the rat basophilic leukemia subline 2H3 (RBL-2H3) have been cultured in our laboratory for the last decade and originated in the National Institutes of Health, Bethesda, MD (Barsumian et al., 1981). The cells were grown to confluency in stationary flasks using minimal essential medium supplemented with 10% fetal calf serum, 2 mM sodium glutamate, and antibiotics (MEM-FCS). Larger numbers of RBL-2H3 cells were grown in bulk fermentor culture and were a kind gift of the Gesellschaft für biotechnologische Forschung, Braunschweig, F.R.G.

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); TBST, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20; SSC, 150 mM NaCl, 15 mM sodium citrate.

Sodium Dodecylsulfate–Polyacrylamide Electrophoresis. SDS–PAGE was done with the discontinuous buffer system described by Laemmli (1970), and proteins were visualized by Coomassie Brilliant Blue. The R_F values of the markers were plotted versus the logarithm of their molecular mass, thus yielding a calibration curve. For detection of radiolabeled polypeptides the stained gels were later dried and exposed to Agfa Curix or Kodak XAR-5 film at −70 °C.

Affinity Matrix Preparation. Aminocaproamidocromolyn was reacted with Sepharose–poly(*N*-hydroxysuccinimidyl caproate) according to the protocol recommended by the manufacturer at a ratio of 50 μmol of ligand per gram of freeze-dried matrix and 8 mM ligand concentration. Typically 18 ± 2 μmol of ligand per milliliter of gel-matrix was found to be immobilized (determined as the difference between total ligand and that in the filtrate: [ligand] = $A_{323nm}/8500\ M^{-1}\ cm^{-1}$).

Isolation of p72 from RBL Cells. Analytical scale preparation: 10^8 cells were surface radioiodinated as described earlier (Hemmerich & Pecht, 1988) and lysed in 1 mL of BBS–Ca^{2+} supplemented with 0.5% Triton X-100, leupeptin (5 μg/mL), pepstatin (5 μg/mL), aprotinin (0.22 TIU/mL), and 1 mM PMSF. The lysate was cleared by centrifugation (1 h at 15 000 rpm), and the supernatant was loaded onto a cromoglycate agarose affinity column (1-mL bed volume) equilibrated in BBS–Ca^{2+} and 0.5% Triton X-100. Following extensive washing with BBS-Ca^{2+} and 0.5% Triton X-100, the column was eluted with 3 mL of 20 mM DSCG in BBS–Ca^{2+} and 0.5% Triton X-100. Five 0.66-mL fractions were collected. Aliquots of those were analyzed by SDS–PAGE and autoradiography.

Large-scale isolation: RBL-cells harvested from a 15-L fermentor (typically 240-g cell mass) were blended and subsequently sonicated in a 10-fold volume of cold HTE 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). This sonicate was spun at first 15 min at 3000 rpm, and then the supernatant was cleared by centrifugation at 12 500 rpm for 1 h and subsequently fractionated by $(NH_4)_2SO_4$. While the 40% cut was discarded, both the 57.5% and 90% cuts were redissolved in parallel in 1 mL per gram of original cell mass of BBS–EDTA supplemented with the above proteolytic inhibitors at the indicated concentrations. These solutions were dialyzed overnight against BBS–EDTA and then supplemented with 3 mM $CaCl_2$ and loaded on affinity columns containing cromoglycate agarose (15-mL bed volume) equilibrated in BBS–Ca^{2+}. Following extensive washing with BBS–Ca^{2+}, the columns were eluted each with 10 void volumes of a gradient of 0–20 mM DSCG in BBS–CA^{2+} and 11 fractions collected. Samples of these fractions were analyzed by SDS–PAGE. Fractions containing the same protein (either the 18-, 34-, or 36-kDa protein bands) were pooled separately and dialyzed against BBS. Further purification of p18, p34, and p36 to close to 100% electrophoretic purity was achieved by repeating the described affinity procedure on long thin (90 mm × 4 mm diameter) columns of cromoglycate-Sepharose which were eluted very slowly (0.5 mL/h) with 20 mL of a gradient of 0–10 mM DSCG in BBS–Ca^{2+}. The fractions containing the different proteins were pooled separately, dialyzed against PBS and concentrated on a Centricon ultramembrane (30 000-Da cut off), yielding 20 mg/mL stock solutions, which were aliquoted into 40-μL portions, quickly frozen in liquid N_2, and stored at −70 °C. At the end of each purification step, an aliquot of the resulting solution was analyzed for protein concentration according to Lowry et al. (1951) and for NDP-kinase activity as follows.

Nucleoside Diphosphate Kinase Assays. NDP-kinase activity was measured using a coupled pyruvate kinase–lactate dehydrogenase assay as described by Agarwal et al. (1978) with minor modifications. The assays were performed at room temperature in a 1-mL reaction mixture containing 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 6 mM $MgCl_2$, 0.1 mM phosphoenolpyruvate, 0.1 mg/mL NADH, 0.5 mM ATP, 0.1 mM dTDP, 2 units of pyruvate kinase, and 2.5 units of lactate dehydrogenase. The reaction was started by addition of 10 μL of appropriately diluted sample [1:10 for the crude cell extract and $(NH_4)_2SO_4$ cut; 1:1000 for affinity-purified protein]. NADH oxidation which reflects the ADP formation by the NDP-kinase was followed by the decrease in absorbance at 334 nm.

Alternatively, the enzymatic activity of the purified protein was assayed in a reaction mixture consisting of 5 μL of buffer (100 mM Na/HEPES, pH 8.0, 200 mM NaCl, 20 mM $MgCl_2$), 1 μL of 20 mM ATP and 1 μL of [γ-^{32}P]ATP (10 μCi, >5000 Ci/mmol), 2 μL of 10 mM NDP, and 1 μL of purified p18 (5 μg/mL). Following 30 min of incubation at 30 °C, samples were taken (1 μL) and mixed with 10 μL of 5 mM EDTA (pH 7.4), and 1 μL of this mixture was examined by TLC (on PEI-cellulose in 0.75 M KH_2PO_4, pH 3.65). The dry plate was exposed to X-ray film, and the radioactive spots were cut out and counted.

Amino Acid Sequencing. The highly purified protein samples were treated with 20 mg/mL CNBr in 70% HCOOH (final concentrations). The digest was then subjected to reverse-phase HPLC, and the best-resolved peptides were sequenced in a microsequencer of Applied Biosystems, Pasadena, CA.

Design and Synthesis of Oligonucleotide Probes. Nondegenerate oligonucleotides coding for three CNBr-fragments of p18 were synthesized in an automatized oligonucleotide synthesizer of Applied Biosystems, Pasadena, CA. For each peptide, a pair of oligonucleotides was designed, one of which

coded directly for the N-terminus and the other inversely for the C-terminus with an overlap region of 9–11 base pairs. This design enabled labeling of the oligonucleotides both by polynucleotide kinase mediated 5′-end phosphorylation with [γ-^{32}P]ATP and DNA-polymerase-mediated template directed fill-in with [α-^{32}P]deoxynucleoside triphosphates.

Identification of mRNA Coding for p18. Cellular RNA from 10^9 cells or 2 g of tissue was extracted into a solution of 3 M LiCl and 7.5 M urea (Auffray & Rougeon, 1980). Then RNAs (25 μg per lane) were fractionated by electrophoresis through 1% agarose/formaldehyde gels and transferred to BA85 nitrocellulose sheets (Schleicher & Schüll, Dassel, F.R.G.). The blots were baked at 80 °C for 2 h and prehybridized in solution A [25% formamide, 0.6 M NaCl, 75 mM sodium citrate, 65 mM KH_2PO_4, 5 mM EDTA, pH 7.2, 0.2% SDS, Ficoll, poly(vinylpyrrolidin) (PVP), BSA at 1 mg/mL, and denatured salmon sperm DNA at 100 μg/mL] for 6 h at 42 °C. Hybridization was carried out overnight at 42 °C with fresh solution A containing 5×10^6 cpm/mL of dissociated complementary ^{32}P-labeled oligonucleotides. Blots were washed (three times) in 2 × SSC/0.5% SDS at 42 °C and exposed to X-ray film.

Cloning of p18 cDNA. Phages carrying a rat kidney cDNA library in λgt11 (Clontech, Palo Alto, CA; cloning site: *Eco*RI) were mixed with *Escherichia coli* Y1088, plated, and lifted in duplicate onto BA85 nitrocellulose colony/plaque hybridization filters (Schleicher & Schüll, Dassel, F.R.G.). Filters were denatured, neutralized, baked at 80 °C for 2 h, and probed with ^{32}P-labeled oligonucleotides essentially as described for the Northern blots above.

Expression of p18 as a Fusion Protein with β-Galactosidase. E. coli Y1090 were plated, and then the plate was placed over an ordered grid and infected at marked positions with the 17 purified recombinant phages that hybridized with the oligonucleotide coding for peptide p18/32. p18–β-galactosidase fusion proteins were induced with isopropyl β-D-galactopyranoside (IPTG) as described by Young and Davis (1985). Colonies were lifted in duplicate onto BA85 nitrocellulose colony/plaque hybridization filters. Those were quenched in 10% fetal calf serum (FCS) in TBST buffer and then probed with rabbit IgG specific for p18 (Hemmerich & Pecht, 1992) at a concentration of 10 μg/mL in TBST, 20% FCS, and 0.1% NaN_3. Following TBST washes (three times), the filters were incubated in TBST containing 20% FCS and 2×10^5 cpm/mL radioiodinated p18 ([^{125}I]p18; Hemmerich & Pecht, 1992). The filters were then washed three times in TBST, dried, and exposed to X-ray film.

DNA Sequencing. Sequencing of single-stranded recombinant M13 phage DNA was performed by the dideoxynucleotide chain-termination method (Sanger et al., 1977) on both strands with deoxyadenosine 5′-([α-^{35}S]thio)triphosphate and T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland, OH).

Results

Isolation of an 18-kDa Polypeptide by Affinity Chromatography of RBL Cell Lysate or Cytosol on Immobilized Cromoglycate. The CG-Sepharose matrix illustrated in Figure 1 was prepared to serve as tool for the isolation of CG-binding proteins from RBL-2H3 cells by affinity chromatography. In initial analytical scale experiments, whole lysates of surface radioiodinated RBL cells were adsorbed onto the CG-Sepharose matrix, and then the column was eluted with 20 mM DSCG (in BBS–Ca^{2+} buffer, 0.5% Triton X-100). Analysis of the eluate by reducing SDS–PAGE and autoradiography revealed a relatively abundant small protein (≈18 kDa, p18)

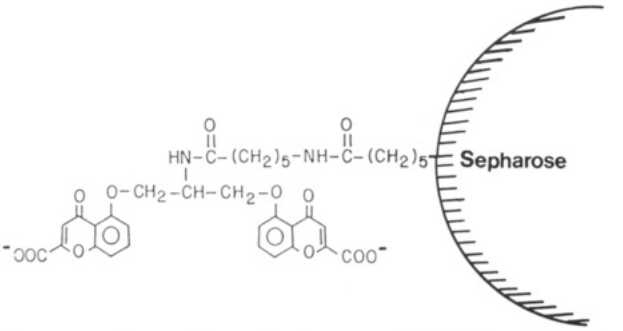

FIGURE 1: Cromoglycate-Sepharose. 1,3-Bis[2′-carboxylatochromon-5′-yl)oxy]-2-(6″-aminocaproamido)propane was reacted with Sepharose-poly[$(CH_2)_5$COO–succinimide)] to form cromoglycate–NHCO$(CH_2)_5$NHCO$(CH_2)_5$–Sepharose that is schematically illustrated.

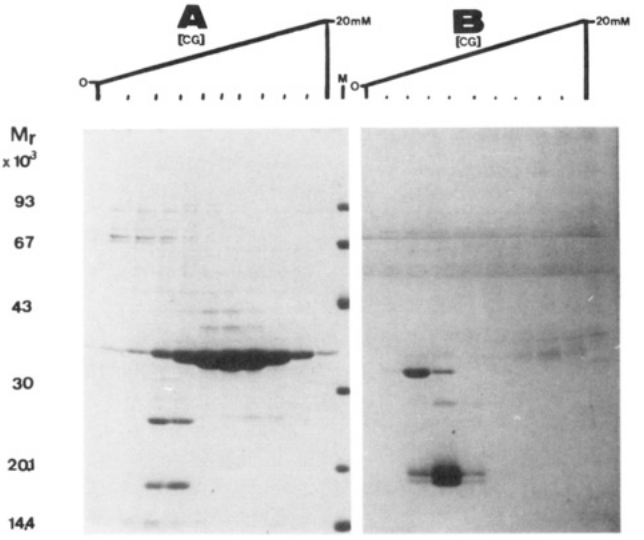

FIGURE 2: Isolation of cytoplasmic CG-binding proteins. RBL cytosol was fractionated with $(NH_4)_2SO_4$, and then both the fraction still soluble in 40% but not in 57.5% saturated $(NH_4)_2SO_4$ solution (A) and in parallel the 90% $(NH_4)_2SO_4$ cut (B) were loaded onto affinity columns containing Sepharose derivatized with cromoglycate. Following extensive washing with BBS–Ca^{2+}, the columns were eluted each with 10 void volumes of a 0–20 mM disodium cromoglycate gradient in BBS–Ca^{2+}. Samples of the 2 × 11 eluate fractions were subjected to SDS–PAGE under reducing conditions. The figure depicts the Comassie Brilliant Blue stained gels.

accompanied by two larger polypeptides of 34- and 36-kDa apparent molecular mass (p34 and p36). Since these three bands were nonradioactive, we assumed them to be components of the cytoplasm rather than the plasma membrane. This notion was further substantiated by the finding that a detergent extract (0.5% Triton X-100 in BBS–Ca^{2+}) of an RBL cell particulate fraction was found void of p18, p34, and p36 (data not shown). Hence, in later large-scale isolations, only RBL cell cytosol was used. Efficient resolution of these three proteins was achieved by combining $(NH_4)_2SO_4$ fractionation of the cytosol prior to the column step and elution of the columns with a 0–20 mM gradient of DSCG in BBS–Ca^{2+} (Figure 2). Thus p36 purified predominantly from the cytosol fraction, which was still soluble in 40% but insoluble in 57.5% (of saturating concentration) $(NH_4)_2SO_4$ solution, while p18 and p34 copurified from the 90% $(NH_4)_2SO_4$ cut. Traces of p18 also copurified with p36 from the 57.5% $(NH_4)_2SO_4$ cut along with small amounts of a 25-kDa band that may represent an additional CG-binding protein but was not further pursued (Figure 2). The elution peaks of p34, p18, and p36 centered at 5, 7, and 12 mM DSCG, respectively, the two former usually showing considerable overlap. Complete resolution

of these polypeptides was achieved by one further affinity chromatography of the pooled eluate on long thin columns packed with cromoglycate-Sepharose. In a typical isolation starting from 240 g of cell pellet that was found to have a specific NDP-kinase activity of ~0.6 μmol of ATP/(min·mg of protein), the $(NH_4)_2SO_4$ step achieved an initial 3–4-fold purification of p18. The p18 preparation resulting from the first affinity purification was found to exhibit a specific NDP-kinase activity of 550 μmol of ATP/(min·mg of protein). The second and final affinity chromatography step yielded ~15 mg of p18 with a specific NDP-kinase activity of 900 μmol of ATP/(min·mg), ~5 mg of p 34, and ~40 mg of p36. With respect to p18, the entire procedure was found to represent a 1500-fold purification with 70% overall yield. Thus, with 2 g of pellet being roughly equivalent to 10^9 cells, a lower limit for the abundance of p18, p34, and p36 was determined as 4×10^6, 6.6×10^5, and 5.5×10^6 copies per cell, respectively.

Identification of p18, p34, and p36 by Partial Amino Acid Sequencing. The amino-termini of all three proteins were found blocked. Therefore peptide fragments generated with CNBr were analyzed by Edman degradation followed by solid-phase sequencing. The sequences of three p36 fragments were all found to be practically identical to those found in various mammalian lactate dehydrogenases (LDH), while three p34 peptides were all >90% identical to rat NAD(P)H quinone oxidoreductase. At that time, no homologies between p18 peptides and any known protein sequence present in the data banks could be resolved.

Molecular Cloning of the p18 cDNA. Three CNBr fragments of p18 yielded the following sequences: p18/23, ValTrpGluGlyLeuAsnValValLysThrGlyArg; p18/32, LysPheLeuArgAlaSerGluGluHisLeuLysGlnHisTyrIle-AspLeuLysAspArgProPhePhe; p18/49, LeuGlyGluThrAsn-ProAlaAspSerLysProXxxValIleArgGlyAspPhe.

We assumed that methionine residues were present at the amino termini of all three peptides. On the basis of the obtained sequences and published codon usage frequencies in the rat (Maruyama et al., 1986), we synthesized two partially overlapping nondegenerate oligonucleotides corresponding to each peptide. Inosine was used to replace the bases corresponding to the unknown residue in peptide p18/49.

To select a probe for screening of cDNA libraries, all three pairs of oligonucleotides were radiolabeled with ^{32}P as described under Materials and Methods and separately used in Northern blot analysis of total RNA from various rat tissues. The probe corresponding to peptide p18/32 hybridized to an 800–850 bp transcript in RNA extracted from RBL cells and other rat tissues (Figure 3). The other two pairs of probes yielded faint signals that could not be correlated with the relative abundance or with the expected size of the transcript.

We therefore employed the pair of probes corresponding to peptide p18/32 for selection of the p18 cDNA. Since mRNA coding for p18 had been found in rat kidney (Figure 3), a rat kidney cDNA expression library in λgt11 phage was screened, under moderate stringency conditions, with oligonucleotide probes corresponding to peptide p18/32. Seventeen positive clones were obtained when approximately 10^6 independent phage plaques were screened. These clones were purified, expanded, and checked for expression of p18 β-galactosidase fusion proteins by screening replica of IPTG-induced phage-infected colonies of *E. coli* Y-1090 (Young & Davis, 1985) with polyclonal rabbit antibodies specific for p18 (Hemmerich & Pecht, 1992). Three clones scored positive in this assay. The cDNA inserts of two of these clones (clone 12 d, ~640 bp; clone 156, ~550 bp) were subcloned into an M13 phage vector and their nucleoside sequences determined by the dideoxynucleoside chain termination method (Sanger et al., 1977).

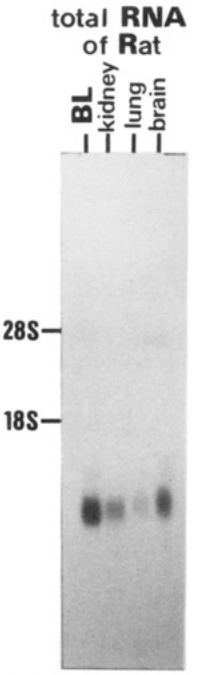

FIGURE 3: Hybridization of rat RNA with the oligonucleotide probe derived from peptide p18/32. Northern blots of 25 μg of total RNA of RBL, rat kidney, rat lung, and rat brain were hybridized with ^{32}P-labeled oligonucleotides derived from peptide p18/32. The positions of the 28S and 18S ribosomal RNAs were used to calibrate the blot. The probe was found to bind to an 820 bp message.

The complete nucleoside sequences of these cDNAs are shown in Figure 4a. The 631 bp sequence of clone 12 d contains a long open reading frame of 456 bp and short 5′ and 3′ untranslated sequences. The nucleoside sequence GXXATGG containing the first in-frame ATG (bases 60–66) agrees with the concensus sequence for translation initiation (Kozak, 1984). A potential polyadenylation signal (AATAAA) is found close to the cDNA 3′ end. The deduced open reading frame codes for a 152 amino acid polypeptide with a molecular mass of 17.286 Da. The sequences of the three cyanogen bromide-generated peptides were all found included in the open reading frame (underlined in Figure 4a), thus confirming the assignment of the cDNA to the p18 protein isolated from RBL-2H3 cells. In addition, the amino acid composition determined for purified p18 (data not shown) agreed with that deduced for the expected protein encoded by the cloned cDNA.

p18 is Rat NDP-Kinase. Comparison of the p18 sequence with sequences present in computerized data bases showed it to be identical to that of rat NDP-kinase (Kimura et al., 1990) and remarkably similar to the human NM23 (Rosengard et al., 1989) and *Drosophila* awd proteins (Biggs et al., 1988), of which the latter has also been shown to possess NDP-kinase activity (Biggs et al., 1990). This observation led us to examine the capacity of p18 to catalyze the transfer of terminal phosphate from [γ-^{32}P]ATP, to CDP, GDP, and UDP as acceptors. As shown in Figure 5, purified p18 catalyzed the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to all nucleoside diphosphates tested and produced the respective [γ-^{32}P]NTPs. The specific activity of p18 was found to be very similar for all three substrates used and was in the range of 900 μmol of NTP/(min·mg of protein). The exchange reaction was dependent on the amount of protein added and on the presence

a

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 1 CGGTTCTCGTCTCTGCTCTCTTCGCTCTCCGCTGGCACCAGCCGACCCACCAGCTTTCGGACATGGCCAA  70
                                                                MetAlaAs

71 CCTCGAGCGTACCTTCATTGCCATCAAGCCAGATGGCGTGCAGCGCGGCCTGGTGGGCGAGATCATCAAG 140
                    CGCATCAAGCCAGATGGCGTGCAGCGCGGCCTGGTGGGCGAGATCATCAAG
   nLeuGluArgThrPheIleAlaIleLysProAspGlyValGlnArgGlyLeuValGlyGluIleIleLys

141 CGATTCGAGCAGAAGGGGTTCCGCCTGGTGGCCATGAAGTTCCTTCGGGCTTCTGAGGAACACCTGAAGC 210
    CGATTCGAGCAGAAGGGGTTCCGCCTGGTGGCCATGAAGTTCCTTCGGGCTTCTGAGGAACACCTGAAGC
    ArgPheGluGlnLysGlyPheArgLeuValAlaMetLysPheLeuArgAlaSerGluGluHisLeuLysG

211 AGCACTATATAGACCTGAAAGACCGTCCTTTCTTCCCGGGGCTGGTGAAGTACATGAACTCAGGGCCTGT 280
    AGCACTATATAGACCTGAAAGACCGTCCTTTCTTCCCGGGGCTGGTGAAGTACATGAACTCAGGGCCTGT
    lnHisTyrIleAspLeuLysAspArgProPhePheProGlyLeuValLysTyrMetAsnSerGlyProVa

281 GGTGGCCATGGTCTGGGAGGGGCTCAATGTGGTGAAAACAGGCCGATGGATGCTGGGGGAGACCAATCCA 350
    GGTGGCCATGGTCTGGGAGGGGCTCAATGTGGTGAAAACAGGCCGATGGATGCTGGGGGAGACCAATCCA
    lValAlaMetValTrpGluGlyLeuAsnValValLysThrGlyArgValMetLeuGlyGluThrAsnPro

351 GCTGATTCGAAGCCAGGCACCATTCGTGGGGATTTCTGCATTCAAGTTGGCAGGAACATCATTCACGGCA 420
    GCTGATTCGAAGCCAGGCACCATTCGTGGGGATTTCTGCATTCAAGTTGGCAGGAACATCATTCACGGCA
    AlaAspSerLysProGlyThrIleArgGlyAspPheCysIleGlnValGlyArgAsnIleIleHisGlyS

421 GTGATTCAGTGGAGAGTGCCGAGAAAGAGATCGGTCTATGGTTTAAGCCCGAAGAACTGATTGACTATAA 490
    GTGATTCAGTGGAGAGTGCCGAGAAAGAGATCGGTCTATGGTTTAAGCCCGAAGAACTGATTGACTATAA
    erAspSerValGluSerAlaGluLysGluIleGlyLeuTrpPheLysProGluGluLeuIleAspTyrLy

491 GTCTTGTGCCCATGACTGGGTGTATGAGTAGACGCGGAGAAACCAGAGTCCTTTTCAGCACTACTGATGG 560
    GTCTTGTGCCCATGACTGGGTGTATGAGTAGACGCGGAGAAACCAGAGTCCTTTTCAGCACTACTGATGG
    sSetCysAlaHisAspTrpValTyrGluEnd

561 GGTTCCTGGACACAGCTCTTCATCCCACTGACTGGATGGACCATCTTTTCTAAAACAATAAAGACTTTGCG
    GGTTCCTGGACACAGCTCTTCATCCCACTGACTGGATGGACCATCTTTTCTAAAACAATAAAGACTTTGGACG
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b

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p18   1  MANLERTFIAIKPDGVQRGLVGDIIKRFEQKGFRLVAMKFLRASEEQLKQHYIDLKDRPF
NM23  1  ...C.....................E.............GL..MQ...DL..E..V.......
awd   1 MA..K.....MV............K..E.......K...L..TW..K.L.EK..A..SA...

p18  61 FPGLVKYMNSGPVVAMVWEGLNVVKTGRWMLGETNPADSKPGTIRGDFCIQVGRNIIHGS
NM23 61 .A.......H.....................V.................................
awd  62 .....N.........P.............Q...A......L.....................

p18  121 DSVESAEKEIGLWFKPEELIDYKSCAHDWVYE
NM23 121 ...............H....V..T...QN.I..
awd  122 .A........A...NEK..VTWTPA.K..I..
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FIGURE 4: (a) Nucleotide sequence of cDNA 12d and 15b. The 631 bp long oligonucleotide sequence of the cDNA insert of phage clones 12d (upper line) contains a 456 bp open reading frame that encodes a 152 amino acid polypeptide whose deduced amino acid sequence is shown beneath (lower line) using the three-letter code. In cDNA 15b (middle line) the N-terminal region of p18 was found truncated. The p18 peptide sequences determined directly by Edman degradation and the putative polyadenylation signal are underscored. (b) Comparison of the amino acid sequence of the rat p18 with the human *NM23* (Rosengard et al., 1989) and *Drosophila awd* sequences (Biggs et al., 1990). Variations from the sequence of p18 are indicated. Identities are represented by dots. The consensus single-letter code for amino acids is used.

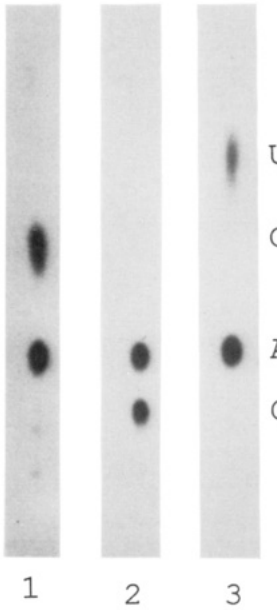

FIGURE 5: Nucleoside-5′-diphosphate kinase activity of p72. The assay was carried out by a 30-min incubation of purified p72 with the respective nucleoside diphosphate at 30 °C, as detailed under Materials and Methods. The reaction products were then separated on an PEI-cellulose plate by thin-layer chromatography in 0.75 M KH_2PO_4 (pH 3.65) buffer. An autoradiogram (2-h exposure) of the plate is presented. Lane 1, CDP; lane 2, GDP; lane 3, UDP.

of Mg^{2+} ions in the millimolar range.

Discussion

Affinity chromatography with RBL cytosol on CG-derivatized Sepharose was shown to yield three different cytoplasmic proteins that on reducing SDS gels exhibit apparent molecular masses of 18, 34, and 36 kDa. Since these proteins were eluted by DSCG at millimolar concentration in high ionic strength buffer (0.2 M sodium borate, 0.16 M NaCl), their interaction with the matrix is certainly due to their affinity for cromoglycate rather than to ion exchange phenomena. The amino acid sequence of the 18-kDa polypeptide (p18) as deduced from the open reading frame of a respective cDNA (Figure 4a) is identical to the amino acid sequence of rat nucleoside diphosphate kinase reported very recently (Kimura et al., 1990). It is highly homologous to the amino acid sequences of the human NM23 (17.14 kDa; Rosengard et al., 1989) and *Drosophila* awd (16 kDa) proteins (Biggs et al., 1988) as well as to the sequences reported recently for the *Dictyostelium discoideum* Gip17 protein (16.8 kDa; Lacombe et al., 1990) and the 16-kDa GTP-binding protein from *Myxococcus xanthus* (Muñoz-Dorado et al., 1990a). These proteins possess very similar molecular masses, and, except for NM23, have all been shown to have nucleoside-5′-diphosphate kinase activity (Biggs et al., 1990; Muñoz-Dorado et al., 1990b; Lacombe et al., 1990). The notion of p18 being an NDP-kinase is supported further by its biochemical activity: p18 was shown to effectively transfer [^{32}P]phosphate from [γ-^{32}P]ATP to CDP, GDP, or UDP (Figure 5). Furthermore, the structural features and autophosphorylation activity of p18 presented in the following paper are characteristic for this family of enzymes.

The deduced amino acid sequence of p18 contains motifs typical for nucleotide-binding proteins as well as of protein kinases, including consensus sequences for a nucleotide-binding site and kinase/ATPase catalytic site (marked with asterisks in Figure 4b) (Wierenga & Hol, 1983; Hunter, 1986; Hanks et al., 1988). In p18 the first glycyl residue of the nucleotide-binding site consensus sequence is replaced by a glutamyl residue. Replacement of a glycine in the GlyXGlyXXGly stretch is also found in other kinases (e.g., phosphorylase kinase, Ca^{2+}–calmodulin dependent kinase) and ATPases (e.g., F_1 ATP synthase/ATPase). In p18 this may account for the its apparent lack of specificity with respect to the pyrimidine/purine of its nucleotide substrate. The overlined amino acid sequence in Figure 4b is 80% homologous to one of the phosphorylation sites in casein (Brignon et al., 1977). However, the SerSerSer in the latter is substituted by SerAspSer in p18. Since we observed that p18 phosphorylates itself predominantly on carboxyl residues and is capable, albeit at very low stoichiometry, of phosphorylating casein (Hemmerich & Pecht, 1992), we speculate that this aspartate may be the site of the autophosphorylation, which occurs on p18 transiently during its catalytic cycle.

The rat NDP-kinase isolated by our CG-ligand affinity chromatography protocol was shown to be a component of the cytoplasm well soluble in aqueous buffered solution. Apparently there is no association between CG-binding p18 and cellular membranes, since, using the same CG-affinity matrix,

we failed to isolate p18 from a detergent-solubilized extract of the particulate fraction of RBL cells. Thus our findings do not support the existence in RBL cells of membrane-associated NDP-kinase, which Kimura & Shimada (1988) reportedly identified in rat liver. However, this apparent contradiction needs further efforts to be resolved; thus association with a membrane might mask the CG-binding site of p18, therefore rendering its isolation by CG-affinity chromatography impossible. Alternatively, different cell types might harbor different subspecies of NDP-kinase.

The study described in this and the following paper emerged from our research of signal transduction in RBL cells. We have reported earlier that cromoglycate that had been introduced into the cytoplasm of RBL cells or rat peritoneal mast cells as its lipid-soluble bisacetoxymethyl ester (which in the cell is rapidly hydrolyzed into the dianion by cytoplasmic esterases) effectively uncoupled $^{45}Ca^{2+}$ influx and the ensuing exocytosis from $Fc_\epsilon RI$ aggregation. At similar concentrations, CG was shown in vitro to inhibit the initial step of the NDP-kinase action of p18 that is the formation of phosphorylated enzyme intermediate ($I_{50} \approx 2$ mM with 4 μM purified p18 and ATP as substrate; Hemmerich et al., 1991). Hence it is conceivable that NDP-kinase plays a role in coupling the immunological stimulus to secretion in mast cells and probably in other signal transduction cascades. In support of this notion, NDP-kinases were suggested to be involved in the activation of GTP-binding proteins (G proteins) by in situ phosphorylation of the liganded GTP (Lacombe et al., 1990; Kimura & Shimada, 1988). Moreover, intracellular application of the nonhydrolyzable GTP analogue guanosine 5′-[γ-thio]triphosphate to serosal mast cells was sufficient for inducing degranulation (Neher, 1988), while guanosine 5′-[β-thio]triphosphate was shown to selectively activate calcium signaling in these cells (zur Mühlen et al., 1991). Thus, modulation of the NDP-kinase activity of p18 could in turn significantly affect G protein mediated cellular responses, including exocytosis. Very recently, the notion of direct regulation of G proteins, e.g., $p21^{ras}$, by NDP-kinase has resurfaced with the discovery of significant homology between the product of the *prune* gene in *Drosophila* and ras GTPase-activating proteins (GAPs; Teng et al., 1991). The *prune* mutation has been shown to have a highly specific, complementary lethal interaction with the conditional dominant *killer of prune* mutation of a *Drosophila* NDP-kinase gene ($awd^{K\text{-}pn}$; Biggs et al., 1988, 1990). Thus the observed structural homology between the product of the *prune* locus to (mammalian) GAPs may imply a direct interaction between ras-like GTPases and NDP-kinase. This proposal suggests a pivotal role for NDP-kinase in the regulation of G protein mediated signal transduction pathways.

However, our evidence for a role of p18 in the signal transduction pathway coupling secretion to $Fc_\epsilon RI$ aggregation in RBL cells is still limited at this stage, and alternatives to our proposal should also be considered. At physiological pH, cromoglycate is an amphiphilic weakly chelating dianion. Thus its rather high cytoplasmic concentrations required for effective inhibition of secretion (≥2 mM) may cause a range of secondary effects that could synergize to produce the observed uncoupling of the signal transduction pathway. Furthermore, other rare cromoglycate binding proteins, which we may have failed to detect in our experiments, could rather be responsible for the observed effects. Further work is needed to resolve these hypotheses and elucidate the role of NDP-kinase in different signal transduction cascades.

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REFERENCES

Agarwal, R. P., & Parks, R. E. (1978) *Methods Enzymol. 51*, 376–386.
Auffray, C., & Rougeon, F. (1980) *Eur. J. Biochem. 107*, 303–314.
Barsumian, E. L., Isersky, C., Petrino, M. G., & Siraganian, R. P. (1981) *Eur. J. Immunol. 11*, 317–323.
Biggs, J., Tripoulas, N., Hersperger, E., Dearolf, C., & Shearn, A. (1988) *Genes Dev. 2*, 1333–1343.
Biggs, J., Hersperger, E., Steeg, P. S., Liotta, L. A., & Shearn, A. (1990) *Cell 63*, 933–940.
Brignon, G., Ribadeau-Dumas, B., Mercier, J.-C., & Das, B. G. (1977) *FEBS Lett. 76*, 274–279.
Cox, J. S. G. (1967) *Nature (London) 216*, 1328–1329.
Foreman, J. C., Hallett, M. B., & Mongar, J. L. (1977) *Brit. J. Pharmacol. 59*, 473P.
Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42–52.
Hemmerich, S., & Pecht, I. (1988) *Biochemistry 27*, 7488–7498.
Hemmerich, S., & Pecht, I. (1992) *Biochemistry* (following paper in this issue).
Hemmerich, S., Sijpkins, D., & Pecht, I. (1991) *Biochemistry 30*, 1523–1532.
Hunter, T. (1986) in *The Enzymes* (Boyer, P. D., & Krebs, E. G., Eds.) Vol. 17, pp 191–246, Academic Press, Orlando, FL.
Kimura, N., & Shimada, N. (1988) *J. Biol. Chem. 263*, 4647–4653.
Kimura, N., Shimada, N., Nomura, K., & Watanabe, K. (1990) *J. Biol. Chem. 265*, 15744–15749.
Kozak, M. (1984) *Nucleic Acids Res. 12*, 857–872.
Lacombe, M.-L., Wallet, V., Troll, H., & Veron, M. (1990) *J. Biol. Chem. 265*, 10012–10018.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowry, O. N., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–268.
Maruyama, T., Gojobori, T., Aota, S., & Ikemura, T. (1986) *Nucleic Acids Res. 14*, 151–197.
Muñoz-Dorado, J., Inouye, M., & Inouye, S. (1990a) *J. Biol. Chem. 265*, 2702–2706.
Muñoz-Dorado, J., Inouye, M., & Inouye, S. (1990b) *J. Biol. Chem. 265*, 2707–2712.
Neher, E. (1988) *J. Physiol. 395*, 193–214.
Pearce, F. L., Befus, A. D., Gauldie, J., & Bienenstock, J. (1982) *J. Immunol. 128*, 2481–2486.
Rosengard, A. M., Krutzsch, H. C., Shearn, A., Biggs, J. R., Barker, E., Margulies, I. M. K., King, C. R., Liotta, L. A., & Steeg, P. S. (1989) *Nature (London) 342*, 177–180.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Teng, D. H. F., Engele, C. M., & Venkatesh, T. R. (1991) *Nature (London) 353*, 437–440.
Wierenga, R. K., & Hol, W. G. J. (1983) *Nature (London) 302*, 842–844.
Young, R. A., & Davis, R. W. (1985) in *Genetic Engineering*, Vol. 7, pp 25–42, Plenum Press, New York.
Zur Mühlen, F., Eckstein, F., & Penner, R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 926–930.