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Molecular Analysis of Mutations in a Patient with Purine Nucleoside Phosphorylase Deficiency

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Summary

Purine nucleoside phosphorylase (PNP) deficiency is an inherited autosomal recessive disorder resulting in severe combined immunodeficiency. The purpose of this study was to determine the molecular defects responsible for PNP deficiency in one such patient. The patient's PNP cDNA was amplified by PCR and sequenced. Point mutations leading to amino acid substitutions were found in both alleles. One point mutation led to a Ser-to-Gly substitution at amino acid 51 and was common to both alleles. In addition, an Asp-to-Gly substitution at amino acid 128 and an Arg-to-Pro substitution at amino acid 234 were found in the maternal and paternal alleles, respectively. In order to prove that these mutations were responsible for the disease state, each of the three mutations was constructed separately by site-directed mutagenesis of the normal PNP cDNA, and each was transiently expressed in COS cells. Lysates from cells transfected with the allele carrying the substitution at amino acid 51 retained both function and immunoreactivity. Lysates from cells transfected with PNP alleles carrying a substitution at either amino acid 128 or amino acid 234 contained immunoreactive material but had no detectable human PNP activity. In summary, molecular analysis of this patient identified point mutations within the PNP gene which are responsible for the enzyme deficiency.

Introduction

Purine nucleoside phosphorylase (PNP; E.C.2.4.2.1) deficiency is a rare form of severe combined immunodeficiency. It was first described, in 1975 by Giblett et al. (1975), in a 5-year-old girl with T-cell immunodeficiency. Subsequently, 32 other patients, including the patient studied in the present report, have been identified (Markert et al. 1987*a;* Markert 1991). Children with PNP deficiency typically have profoundly deficient T-cell function and either normal, decreased, or autoreactive B-cell function. In addition to recurrent infections and failure to thrive, some of these children also suffer from autoimmune disease, neurologic impairment, and malignancies (Markert 1991).

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PNP-deficient patients die in the first or second decade of life, usually from infections (Ammann et al. 1978; Carapella-de Luca et al. 1978; Virelizier et al. 1978; Corrales et al. 1983; McGinniss et al. 1985), malignancies (Stoop et al. 1977; Watson et al. 1981), or graft-versus-host disease following nonirradiated-blood transfusions (Stoop et al. 1977; Strobel et al. 1989).

Human PNP is a symmetric trimer composed of three identical 32,153-dalton subunits, each with a substrate-binding site (Agarwal and Parks 1969; Zannis et al. 1978; Williams et al. 1987). PNP reversibly catalyzes the phosphorolysis of the purine nucleosides, (deoxy)inosine and (deoxy)guanosine, to their respective purine bases and the corresponding ribose-1-phosphate. In T cells, the absence of PNP activity is thought to lead to an accumulation of deoxyguanosine triphosphate, an accumulation which inhibits the enzyme ribonucleotide reductase (Carson et al. 1977; Mitchell et al. 1978; Ullmann et al. 1979; Kredich and Hershfield 1989). This inhibition blocks DNA

synthesis, thereby preventing the cellular proliferation required for an immune response.

PNP deficiency is inherited in an autosomal recessive fashion. The PNP gene is located on chromosome 14 (Ricciuti and Ruddle 1973) and comprises six exons spaced over approximately 9 kb of DNA (Williams et al. 1984, 1987). Several different mutant PNP proteins have been described. These have been characterized with regard to altered migration in isoelectric focusing, resistance to sulfhydryl oxidation, and heat instability (Siegenbeek van Heukelom et al. 1976; Gudas et al. 1978; Wortmann et al. 1979; McRoberts and Martin 1980). To date, the genetic mutation responsible for enzyme inactivity has been identified in only one homozygous patient (Williams et al. 1987). In that patient, a single-base-pair change which resulted in an amino acid substitution was detected. We describe the sequence of two mutant PNP alleles from a second PNP-deficient patient, TP. In these alleles we identify three unique point mutations (different from the one previously described), two of which result in the loss of enzymatic activity. Coupled with the knowledge of the crystallographic structure (Ealick et al. 1990), these observations let us speculate about the role of certain amino acids in PNP structure and function.

Material and Methods

Cell Lines

B-lymphoblastoid cell lines were established from Ficoll-Hypaque-purified peripheral mononuclear cells by Epstein-Barr virus transformation (Katsuki and Hinuma 1975) in the presence of 1 μg cyclosporin A/ml (Anderson and Gusella 1984). The identity of each cell line studied was confirmed by HLA typing, courtesy of Dr. Francis Ward (Duke University), and by a radiochemical assay of PNP activity.

PNP Assay

Extracts from the B-lymphoblastoid cell lines, prepared by freezing and thawing, were chromatographed on a G25 Sephadex spun column according to a method described by Greenberg et al. (1989). These cell extracts were assayed for PNP activity essentially according to a method described by Fox et al. (1977), except that ^{14}C-hypoxanthine (product) was separated from substrate (^{14}C-inosine) on cellulose thin-layer plates (PolygramCel 400 UV_{250}; Mackerey-Nagel) which were developed in water.

Protein Levels

Protein levels of the B-lymphoblastoid and COS cell lysates were determined using the BioRad protein assay, Bradford (1976) method.

RNA and DNA Purification

RNA was isolated from the patient's cell line by the technique of Chirgwin et al. (1979; Markert et al. 1987*b*), by guanidinium isothiocyanate extraction followed by purification using cesium chloride gradient centrifugation. Poly(A+) RNA was prepared using oligo[dT] column chromatography (Avis and Leder 1972). DNA was purified by the method of Gustafson et al. (1987).

Northern RNA Analyses

Approximately 3.5 μg of Poly(A+) RNA was applied to each lane of a 2.2 M formaldehyde 1% agarose gel (Thomas 1983). The RNA was transferred to a Pall Biodyne A membrane (ICN Radiochemicals, Irvine, CA) and was processed according to a method described by Markert et al. (1987*b*).

Probes

The plasmid, pPNP1, a nearly full-length (1,418 bp) PNP cDNA in the plasmid pBR322, was provided by Dr. David Martin (Genentech, Inc.) (Williams et al. 1984). The PNP cDNA probe used for Southern analyses was a 587-bp *Pst*I restriction fragment derived from pPNP1. This fragment encompassed the 3′ 139 bp of exon 2, all of exons 3 and 4, and the first 168 bp of exon 5 of the PNP cDNA. The adenosine deaminase control probe, 117-7, obtained from Drs. John Hutton and Dan Wiginton (Cincinnati), extends between the *Bam*HI sites at positions 14373 and 14784 of the ADA gene (Wiginton et al. 1986). This position is 5′ of exon 2 of the ADA gene. The actin control probe, pHFβA.1, contains the 3′ untranslated region of the human fibroblast cytoplasmic β-actin cDNA (Gunning et al. 1983) and was provided by Dr. Larry Kedes (University of Southern California). The probes were labeled with ^{32}P to a specific activity of greater than 10^8 cpm/μg (Feinberg and Vogelstein 1983, 1984).

cDNA Synthesis, Cloning, and Sequencing

cDNA was prepared by the method of Gubler and Hoffman (1983). The PNP cDNA was amplified from the total cDNA by PCR (Perkin Elmer Cetus) (Saiki et al. 1985) by a sense primer from −79 to −60 and an

antisense primer from +1182 to +1201 of the PNP cDNA (the numbering is with respect to the initiation of translation in the normal PNP cDNA) (Williams et al. 1984). This amplified cDNA was cloned (from the *Bam*HI site at −58 to the blunted 3′ end) into the plasmid, pGEM3Zf− (Promega). PNP cDNA for both alleles was sequenced by the double-stranded (Wang et al. 1988) dideoxynucleotide chain-termination method (Sanger et al. 1977). Mutations which led to restriction-site changes were confirmed by Southern analysis of genomic DNA (Southern 1975). The sequences of both alleles were confirmed by sequencing patient PNP cDNAs which had been cloned from an independent PCR amplification.

Transfections

Site-directed mutagenesis (Taylor et al. 1985) of the normal PNP cDNA was performed for each point mutation by the Amersham oligonucleotide-directed in vitro mutagenesis system, version 2. The directions in the technical manual were followed. In brief, a mutant oligomer was synthesized and phosphorylated with T4 polynucleotide kinase. This was annealed to single-strand template (made from normal PNP cDNA in pGEM3Zf− plasmid [Promega]). A mutant DNA strand incorporating thionucleotides was synthesized using the mutant oligomer as a primer. The nonmutant template was removed by nicking with *Nci*I enzyme and treatment with exonuclease III. The second strand was then synthesized with DNA polymerase I and ligated with T4 DNA ligase. *Escherichia coli* were transformed with the resulting double-stranded plasmid containing the mutated cDNA insert. Mutant PNP cDNAs were confirmed by sequencing (as above) and then were cloned into the *Bam*HI site of the pRK5 expression vector (Didsbury et al. 1989), obtained from Dr. John Didsbury (Duke University). This vector contains the simian virus 40 enhancer and cytomegalovirus promoter. The 5′ end of the PNP cDNA sequence thus was the *Bam*HI site at −58 with respect to the ATG at the initiation of translation. The 3′ end of the sequence was the final base pair in the normal PNP cDNA pPNP1 described above. The *Bam*HI site at the 3′ end was derived from the multiple cloning site of the vector used in the site-directed mutagenesis experiments. The cesium chloride–purified mutant cDNAs were transfected into COS cells by the DEAE-dextran method (McCutchan and Pagano 1968; Didsbury et al. 1989). In brief, 6-cm dishes of cells were incubated in 2 ml Dulbecco's modified Eagle medium (DMEM) with 10% NuSerum (Collaborative Research) and 0.1 mM chloroquine. DNA (3 μg) was added in 120 μl DEAE dextran (6.6 mg/ml; Pharmacia) in Tris-buffered saline pH 7.5. After 3 h at 37°C, the medium was aspirated, and the cells were treated at room temperature for 1 min with 1.5 ml of 10 g dimethyl sulfoxide/100 ml in PBS (PBS = 137 mM sodium chloride, 2.7 mM potassium chloride, 0.88 mM potassium phosphate, 6.6 mM sodium phosphate, pH 7.5). Cells were then washed twice in PBS and were then returned to medium (DMEM with 10% FCS and 50 mM HEPES). Forty-eight hours after transfection, cells were washed with PBS and removed from the plates with 0.04 g trypsin/100 ml, 0.5 mM EDTA (Gibco), in 137 mM sodium chloride, 5.4 mM potassium chloride, 5.5 mM glucose, 4.2 mM sodium bicarbonate for 5 min at 37°C. Cells from each 60-mm plate were resuspended at a concentration of 10^7 cells/100 μl in 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM 2-mercaptoethanol; were subjected to three rounds of alternate freezing and thawing in dry ice/ethanol; and were stored in liquid nitrogen.

Enzyme Activity

Equivalent amounts of protein from each lysate were applied to a Tris/EDTA/borate pH 8.8 agarose gel (Innovative Chemistry, Marshfield, MA). The samples were electrophoresed in a temperature-controlled (4°C) electrophoresis chamber (Innovative Chemistry) at 160 V for 45 min. PNP activity was detected by overlaying the gel with approximately 12 ml of a mixture containing 6 mM inosine, 7 mM sodium arsenate, 33 mM Tris pH 8, 0.017 units xanthine oxidase/ml, 0.7 mM nitro blue tetrazolium, 0.14 mM phenazine methosulfate, 0.5 g agarose/100 ml. PNP was detected as a purple spot after incubation in the dark for 1 h at 37°C.

Isoelectric Focusing

Lysates (approximately 80 μg of protein each) were applied to a 1-mm slab isoelectric focusing gel of 5.5% acrylamide, 2.2% ampholytes (Resolytes pH 3.5–10; Hoefer), 0.1% glycerol. The upper (cathode) buffer was 0.02 M NaOH; the lower (anode) buffer was 0.02 M acetic acid. The gel was run for 2 h at 20 W. After the gel had run, enzyme activity was detected as described above by overlaying the gel with a mixture containing substrate, or immunoreactivity was detected by the immunoblotting procedure (see below).

Immunoblot

Immunoreactivity was assessed by applying lysates (approximately 80 μg of protein each) to isoelectric focusing gels or to SDS-PAGE with 10% acrylamide in the lower gel. The protein was transferred from the gel to Immobilon-nitrocellulose (Millipore) by electroblotting for 2 h at 70 V in a Hoefer TE42 apparatus. The Immobilon was pretreated by being dipped in water and then soaking for 5 min in transfer buffer containing 39 mM glycine, 48 mM Tris, 1.3 mM SDS, 6.2 M methanol, pH 8.3. After transfer, the membrane was stained with India ink. Rabbit anti-PNP IgG, a gift from Dr. William Osborne (University of Washington, Seattle) (Osborne et al. 1977) was added at a 10^{-5} dilution with 5 g Carnation nonfat dry milk/100 ml in TBS-T (TBS-T = 20 mM Tris pH 7.6, 137 mM sodium chloride, 0.1 g Tween 20/100 ml) to block nonspecific binding. The blot was hybridized overnight at room temperature with the primary antibody, was washed three times in TBS-T, and hybridized for 2 h at room temperature with the secondary antibody, [^{125}I] donkey F(ab')$_2$ anti-rabbit IgG (Amersham). The hybridized blot was again washed at room temperature in TBS-T and was autoradiographed at −70°C for 10–14 d.

Results

Clinical History and Cell-Line Data

The clinical history, immune function studies, and PNP levels in the patient, TP, have been described elsewhere (Markert et al. 1987*a*). PNP activity levels in the HLA-typed B-lymphoblastoid cell lines were 18 nm/h/mg protein and 1,900 nm/h/mg protein for patient TP and her mother, respectively (normal range 2,496–8,276 nm/h/mg protein; n = 10). These results are consistent with those previously reported for the red-cell-hemolysate PNP activity levels.

To study the enzyme activity and immunoreactivity of PNP in B-cell lines derived from the patient and her mother, lysates were applied to isoelectric focusing gels (fig. 1). PNP activity was detected by overlaying the gel with the substrate linked to a color reaction. Duplicate gels were analyzed by immunoblotting. PNP in cells derived from the patient's mother ran to a similar position as did normal human PNP (fig. 1*A*); however, the enzyme activity appeared to be less than that of the control, consistent with the PNP activity levels reported above. On immunoblotting, lysates of cells derived from the mother revealed one band corresponding to normal PNP, whereas lysates derived from the patient had no immunoreactive material (fig. 1*B*). Material from the father was not available.

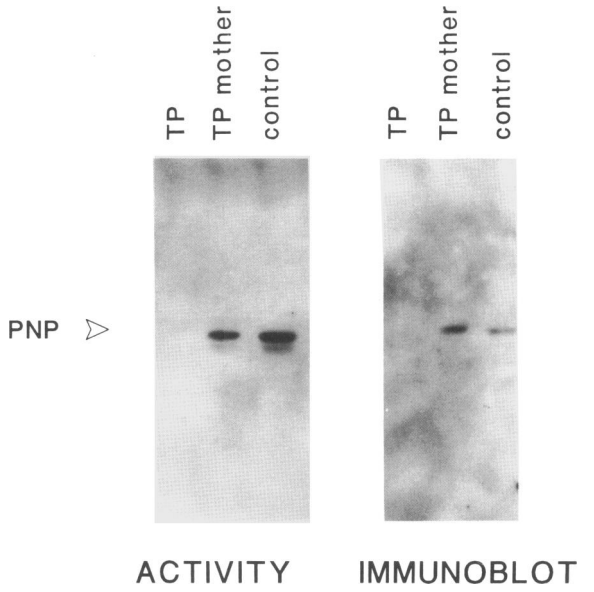

Figure 1 PNP activity and immunoreactivity in cell lysates from members of the TP family. In the left-hand panel, lysates containing approximately 80 μg of protein were applied to an isoelectric-focusing gel. After electrophoresis, the gel was overlaid with the PNP substrate linked to a color reaction. This panel shows both the absence of PNP activity in lysates of B-cell lines derived from the patient and the presence of PNP activity in the lysates of cells derived from her mother and from a normal control. The gels were oriented with the cathode at the top. In the right-hand panel, a duplicate isoelectric-focusing gel was blotted to a membrane and was probed for immunoreactive PNP protein. This shows the absence of immunoreactive protein in the lysates of cells derived from the patient.

PNP mRNA Levels

Figure 2 shows RNA blot hybridization (Northern blot) of poly(A +) RNA isolated from the lymphoblastoid cell lines established from patient TP and her mother. As shown in figure 2*A*, ^{32}P-labeled full-length PNP cDNA hybridized to mRNA of patient TP and her mother at the same position as it did in the normal control. As a control, the membrane was stripped and probed with a β-actin cDNA (fig. 2*B*). All samples hybridized to the actin probe at the expected position (approximately that of 18S RNA). The size of β-actin mRNA is thought to be 1,761 nucleotides plus a poly A tail (Ponte et al. 1984); and the size of PNP mRNA is thought to be 1,500 nucleotides plus a poly A tail (Jonsson et al. 1991). The PNP mRNA appears to

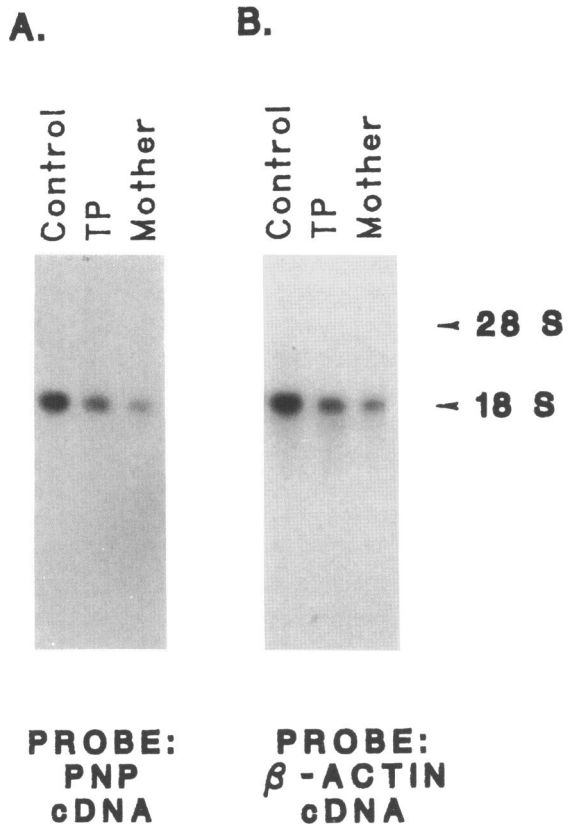

Figure 2 Northern blot analysis of the TP family. Poly(A+) RNA was probed with either a ^{32}P-labeled PNP cDNA probe (*A*) or a β-actin cDNA probe (*B*). The gel shown in panel *A* has been stripped and reprobed in panel *B*. The position of 18S and 28S rRNA which was run in parallel is indicated.

be only slightly smaller than the actin mRNA. The relative intensities of the bands with the PNP probe are similar to those with the actin probe, indicating that unequal amounts of poly(A+) RNA had been loaded in each lane. Thus, the relative amounts of PNP mRNA in cell lines derived from patient TP and her mother were similar to that in control cell lines.

cDNA Sequencing

PNP cDNA from patient TP was amplified by PCR, cloned, and sequenced; two mutant PNP alleles were identified. Figure 3 shows a schematic representation of the mutations and polymorphisms in the patient's two alleles. It is noteworthy that both alleles share four polymorphisms and one base-pair change resulting in an amino acid substitution. Two of the polymorphisms, the double A in the 3′ untranslated region and the two tandem copies of a 10-bp sequence in the 5′

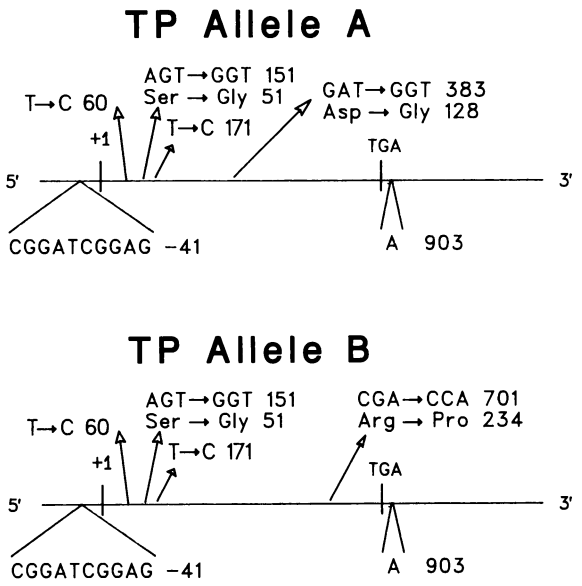

Figure 3 Two mutant PNP cDNAs in patient TP. Arrows indicate the polymorphisms and the point mutations leading to amino acid substitutions found in both alleles. +1 = position of the ATG for the start of translation; and TGA = position of the end of translation.

flanking region, have been reported elsewhere (Williams et al. 1984, 1987). The other two polymorphisms, at bp 60 and bp 171, have not been previously reported. The nucleotide change leading to an amino acid substitution which is common to both alleles occurs at nucleotide 151 (AGT to GGT) in exon 2 and results in a change from Ser to Gly at position 51 in the protein. In allele A, the base change at nucleotide 383 (GAT to GGT) in exon 4, a base change which leads to an Asp-to-Gly substitution at amino acid position 128, results in the loss of an *Eco*RV restriction site. A Southern blot (fig. 4) confirms this change and demonstrates that allele A was inherited from the mother. In allele B, the base change at nucleotide 701 (CGA to CCA) in exon 5, a base change which leads to an Arg-to-Pro substitution at amino acid position 234, results in the loss of a *Taq*I restriction site. A Southern blot (not shown) confirms this change, but, because paternal blood was not available for this study, we could not determine whether this change was inherited from the father or was a spontaneous mutation.

Functional Studies

In order to determine which, if any, of the point mutations were responsible for the deficiency in pa-

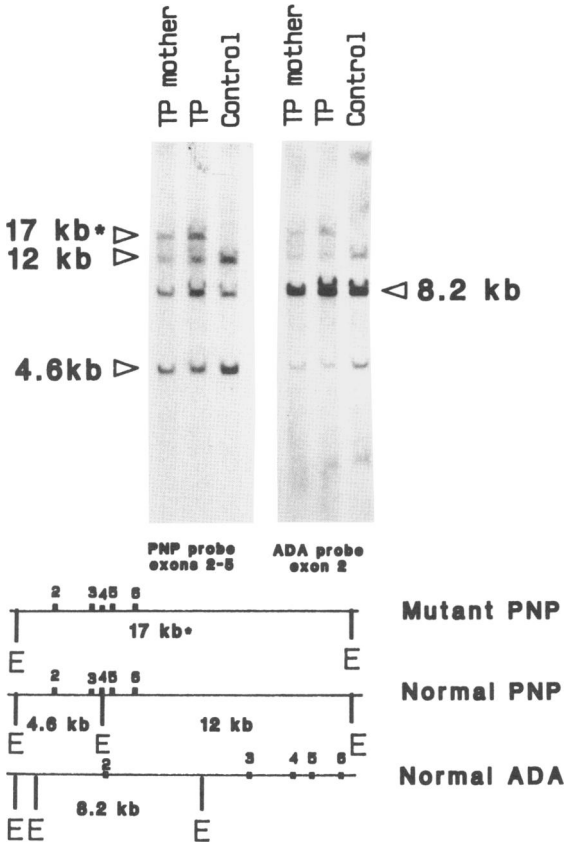

Figure 4 Southern analysis of genomic DNA, in examination of the point mutation at position 383. DNA was derived from the individuals indicated. DNA was digested with *Eco*RV, electrophoresed on an agarose gel, and transferred to a membrane. The left-hand panel shows the membrane after hybridization with a radiolabeled PNP cDNA fragment containing part of exon 2, all of exons 3 and 4, and part of exon 5. With the mutant PNP allele found in patient TP and her mother, the loss of an *Eco*RV site in exon 4 results in the appearance of the larger, 17-kb fragment. The right-hand panel shows the same membrane after it was stripped and reprobed with a genomic clone derived from the exon 2 region of the adenosine deaminase gene. Only the band of the appropriate size was detected, showing that the DNA had been digested fully. The band at 8.2 kb in the left-hand panel likely represents DNA cross-reactive with the PNP probe.

tient TP, we expressed the normal cDNA as well as each mutant cDNA in vitro in COS cells. To create the mutant cDNAs, we used site-directed mutagenesis to create the specific mutation in a full-length normal PNP cDNA. Creation of the mutant was verified by sequencing across the site of the mutation. The mutant cDNA was cloned into an expression vector. As controls, the normal PNP cDNA and an antisense PNP cDNA were cloned into the expression vector. These were transfected into the COS cells by a DEAE dextran method. The cells were harvested at 48 h, and lysates were examined by immunoblot and PNP-activity analysis.

Electrophoresis of the cell lysates separated the human and monkey PNP proteins. Enzyme detection by a color reaction showed that mutant gene products containing amino acid substitutions either at position 128 or at position 234 had no enzymatic activity (fig. 5*A*). Only the monkey PNP activity could be detected in these lysates. The mutant gene product containing the amino acid substitution at position 51 retained enzyme activity; bands were detected at the positions of both normal human and normal monkey PNP migration. Figure 5*B* shows an immunoblot done after the lysates were subjected to SDS-PAGE. The antibody used does not react significantly with monkey PNP; only trace amounts of immunoreactive material are found in untransfected COS lysates or COS lysates from the antisense transfection experiments. Lysates from cells transfected with the construct containing the substitution at amino acid position 51 were immunoreactive as well as functionally active. Lysates from cells transfected with constructs containing either the mutation at amino acid position 128 or the mutation at amino acid position 234 contained immunoreactive PNP.

Discussion

We investigated the molecular defect in PNP-deficient patient TP. This patient's enzyme deficiency has been documented previously by enzyme assays of red blood cells, and we confirmed the deficiency by the same assay of the B-lymphoblastoid cell line. Northern analysis was used to categorize the type of defect which led to a loss of PNP enzymatic function in our patient. The detection of a normal amount of full-length PNP mRNA in patient TP suggested that at least one of her PNP alleles carried a point mutation. We were able, in fact, to show that both alleles carried point mutations which affected amino acid sequence. We were able to show by site-directed mutagenesis that the mutations in patient TP's PNP alleles disrupted PNP function. By Southern analysis, we determined that allele A was inherited from the patient's mother.

Ealick et al. (1990) have elucidated the crystallographic structure of the wild-type PNP enzyme. The core of the PNP subunit consists of an eight-stranded mixed beta-pleated sheet and a five-stranded mixed beta-pleated sheet which form a distorted barrel structure. This barrel is surrounded by seven alpha-helices.

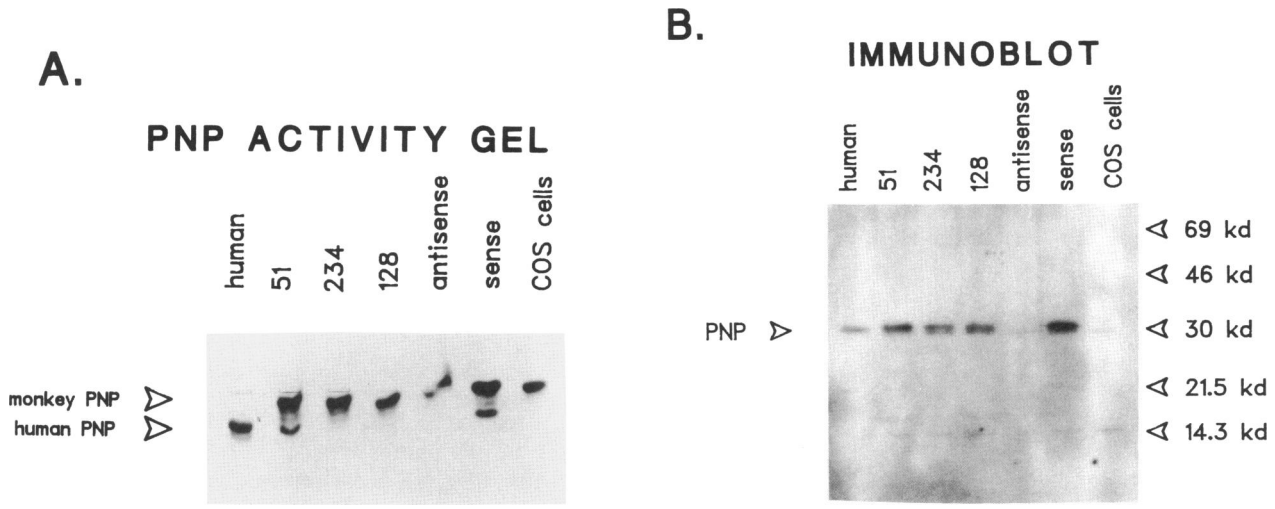

Figure 5 Structure/function analyses. Panel *A* shows the PNP enzymatic activity of various COS cell lysates, and panel *B* shows the immunoreactivity of the same lysates. One to two microliters of each lysate was electrophoresed on an agarose gel and then was overlaid with a solution containing PNP substrate (*A*). In addition, 5 μl (approximately 80 μg of protein) was electrophoresed on SDS-PAGE and then was transferred to a membrane and probed with rabbit anti-PNP IgG and an ^{125}I-labeled anti-rabbit IgG (*B*). The lysates are as follows: Lane 1, Human control cells. Lane 2, COS cells transfected with a vector containing PNP cDNA carrying the mutation at bp 151 (amino acid position 51, Ser to Gly). Lane 3, COS cells transfected with a vector containing PNP cDNA carrying the mutation at bp 701 (amino acid position 234, Arg to Pro). Lane 4, COS cells transfected with a vector containing PNP cDNA carrying the mutation at bp 383 (amino acid position 128, Asp to Gly). Lane 5, COS cells transfected with a vector containing anti-sense PNP cDNA. Lane 6, COS cells transfected with a vector containing sense PNP cDNA. Lane 7, COS cells. Gels were oriented with the cathode at the top. For both the activity gel and the immunoblot, the same banding pattern was seen on lysates made from separate transfection experiments. Approximately equal amounts of protein were added to each lane.

The Ser-to-Gly substitution at amino acid position 51 is in one of the connecting loops between two beta-pleated sheets and is quite removed from the substrate-binding site. These two amino acids are similar in size and charge. It is not surprising, then, that the PNP protein with Gly at amino acid position 51 retains enzymatic activity and immunoreactivity. As this mutation was common to both alleles, it is possible that this change represents a polymorphism within the population.

In contrast, the Asp-to-Gly substitution at amino acid position 128 affects the charge of the molecule and results in loss of enzyme activity. The mutation in allele B leads to an Arg-to-Pro substitution at amino acid position 234. This particular amino acid is in a four-amino-acid sequence which makes a sharp turn between an alpha-helix and a beta-pleated sheet (Ealick et al. 1990). The positive charge on the arginine is probably important for stabilizing this sequence and for allowing proper folding. A proline would undoubtedly disrupt this sharp turn.

Since we detect no immunoreactive PNP in the patient cell lysates but do detect it in the COS lysates, we believe that both mutants may be quickly degraded within the cell. These mutant proteins are found in the COS cells because their expression is driven at high levels off our vector. The finding of immunoreactive PNP in lysates from cells transfected with the PNP alleles mutant at either residue 128 or residue 234 is similar to Williams et al.'s (1987) finding that PNP carrying a substitution from Glu to Lys at amino acid position 89 lacked enzymatic activity but remained immunoreactive although the protein focused at a more basic pI than did normal PNP.

We compared this PNP-deficient patient's mutations with point mutations found in other diseases. A review of published point mutations in disease genes has shown that 63% of point mutations are transitions (Cooper and Krawczak 1990). In this patient, four of the five mutations were transitions. Cooper and Krawczak's (1990) review also showed that 32% of all point mutations in disease genes occurred in a CpG sequence. These mutations (CG to TG or CG to CA) are consistent with the methylation-mediated deamination of 5-methyl cytosine (Cooper and Youssoufian 1988; Cooper and Krawczak 1990). As CpG sequences

tend to be hot spots for mutation, mutations at these sites are often found in many individuals. None of the point mutations described in the present paper occurred at a CpG sequence.

In PNP deficiency, one must consider the effect of trimer formation on enzyme activity. It has been suggested, although not formally proved, that trimer formation is necessary for enzyme activity (Ealick et al. 1990). Heterotrimers have been found in parents of children with PNP deficiency. These trimers contain one or two normal subunits mixed with two or one mutant subunits. Osborne et al. (1977) showed that parents of one PNP-deficient child expressed some immunoreactive PNP (14% of total) which migrated at a position in native gels which is consistent with trimers composed of mixtures of mutant and normal subunits. The PNP activity of the parents was between one-quarter and one-third of normal. One can postulate that the mixed heterotrimers were responsible for the parents' PNP activity being less than half of normal. This finding contrasts with our results. The two mutations (substitutions at amino acid positions 128 and 234) which cause lack of enzyme activity do not result in accumulation of any immunoreactive material in the patient's or mother's cells. On isoelectric-focusing gels, PNP from the patient's mother runs as a single normal band. In isoelectric focusing, heterotrimers containing normal and mutant subunits should have been detected by the presence of an additional band(s) located closer to the cathode than the normal trimer (Williams et al. 1987). Presumably, the mutant PNP is rapidly degraded. Perhaps because of this, the PNP activity of maternal cell lysates is approximately half of normal, not less than this.

It is interesting to correlate the known PNP mutations with the clinical data on patients. Although early reports suggested that B-cell function was normal or increased in PNP deficiency, more recent studies have shown that B-cell function can be disrupted as well (Markert 1991). This is the case with patient TP. She has had normal B-cell counts but significantly depressed immunoglobulin levels (Markert et al. 1987*a*). In contrast, the only other patient whose molecular defect has been characterized had normal B-cell function (Giblett et al. 1975). The immunologic differences between these patients may be secondary to genes other than PNP which vary between the two patients.

In summary, TP is only the second patient whose PNP deficiency has been characterized at the molecular level. We identified one point mutation which does not disrupt the protein's enzymatic activity and immunoreactivity and which may reflect a polymorphism within the population. In addition, we found two new point mutations within the PNP gene which result in proteins without enzymatic function. Although immunoreactivity was found in lysates of cells transfected with the mutant alleles, the patient's cells had no detectable immunoreactive PNP protein. This implies that these mutant proteins may be rapidly degraded in the cell.

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