Identification of a New Pyk2 Isoform Implicated in Chemokine and Antigen Receptor Signaling*

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Pyk2 is a protein tyrosine kinase that links G-protein-coupled receptors, inflammatory cytokines, and extracellular stimuli that elevate intracellular calcium concentration with activation of the mitogen-activated protein kinase pathways and regulation of ion channel functions. Here we describe the identification, cloning, and characterization of a new isoform of Pyk2 (Pyk2-H) that is generated by alternative RNA splicing. Pyk2-H is mainly expressed in hematopoietic cells including T-cells, B-cells, and natural killer cells. Engagement of T-cell or B-cell antigen receptors leads to rapid tyrosine phosphorylation of Pyk2-H. Pyk2-H is also activated in response to the chemokines RANTES and macrophage inflammatory protein-1β in T cells. In addition, we show that glutathione S-transferase fusion proteins containing the carboxyl termini of Pyk2 and Pyk2-H bind to a different set of tyrosine-phosphorylated proteins in thymus lysates. Specific expression of Pyk2-H and its activation by antigens or chemokines in hematopoietic cells may contribute toward the generation of cell type-specific signals involved in host immune responses.

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‡The abbreviations used are: FAK, focal adhesion kinase; Pyk2, proline-rich tyrosine kinase 2; Pyk2-H, proline-rich tyrosine kinase 2-hematopoietic isoform; RANTES, regulated on activation normal T-cell expressed and secreted; GST, glutathione S-transferase; FCS, fetal calf serum; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

antigen receptors, integrin receptors, and chemokine receptors in hematopoietic cells (9–12).

We and others have observed that in some cells of hematopoietic origin Pyk2 protein migrates in SDS-PAGE gels as a doublet (9, 11). This observation suggested that different Pyk2 isoforms may be expressed in hematopoietic cells. In this report we describe the identification, cloning, and initial characterization of an isoform of Pyk2, that is generated by alternative RNA splicing, and is mainly expressed in hematopoietic cells (Pyk2-H). Using isoform-specific anti-Pyk2 antibodies we show that Pyk2-H is expressed in primary T and B lymphocytes and in natural killer cells. We demonstrate that Pyk2-H and not Pyk2 is rapidly activated in response to stimulation with chemokines and following activation of the T-cell or B-cell antigen receptors suggesting a potential role in host immune responses.

EXPERIMENTAL PROCEDURES

Reagents—All tissue culture media and antibiotics were obtained from Life Technologies, Inc. The In vitro mutagenesis kit was from CLONTECH, GeneAmp XL PCR Kit from Perkin-Elmer, TA cloning kit from Invitrogen, anti-mouse CD3 antibodies from PharMingen, goat anti-hamster IgG from Boehringer Mannheim, affinity purified goat anti-mouse IgM F(ab′)2 fragment, and rabbit anti-goat IgG antibodies (F(ab′)2 specific) were from Jackson ImmunoResearch Laboratories, LipofectAMINE was from Life Technologies, Inc., RANTES and macrophage inflammatory protein-1β were from PeproTech. Murine brain and thymus cDNAs were provided by Zoran Bogdanovic (New York University, New York). A murine genomic clone containing Pyk2 sequences (phage clone P1) was purchased from Genome Systems, Inc. BALB/c mice were obtained from the Laboratory Animal Facility of the NYU Medical Center.

Cell Culture—Human promyelocytic leukemia HL-60 cells, human T cell leukemia Jurkat cells, human erythroleukemia K562 cells, rat mast/basophilic leukemia RBL cells, human erythroleukemia Mo7 cells, and human preneugakaryocyte leukemia CHRF cells were maintained in RPMI containing 10% fetal calf serum (FCS). Rat phaeochromocytoma PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 7% FCS and 7% horse serum. Human DU6 cell line was obtained from Derya Untumaz (Skirball Institute, New York) and were cultured in RPMI containing 10% FCS and supplemented with 10 units/ml human IL2 (Chiron) as described previously (12). Lysates of human platelets were obtained from Carl-Henrik Heldin (Ludwig Institute, Uppsala, Sweden). Lysates of purified natural killer cell population were provided by Angela Gismondi (University of Rome, Rome, Italy). Natural killer cells were obtained from human peripheral mononuclears isolated from buffy coats by gradient centrifugation and passage through nylon wool columns. Nylon non-adherent cells were further purified by negative cell sorting using a mixture of anti-CD5 and anti-CD14 monoclonal antibodies (13). Murine thymocytes were isolated as described previously (9). Briefly, single cell suspensions were collected from mice at 6 weeks of age and maintained in RPMI medium supplemented with 5% FCS. B cells were depleted by addition of sheep anti-mouse immunoglobulin-coated magnetic beads (Dynabeads, Dynal Inc.). For each experiment, 10⁶ cells/ml were stimulated for the indicated times by addition of anti-CD3 antibodies (50 μg/ml). Following stimulation the cells were rapidly centrifuged using Picofuge (Stratagene) and washed once in cold phosphate-buffered saline and the pellet was lysed in lysis buffer. Enriched populations of B cells were obtained from murine splenocytes by depletion of T cells with anti-

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Thy1, anti-CD4, and anti-CD8 antibodies as described elsewhere (14). For stimulation, B cells (approximately 5 × 10^6/ml) were incubated in serum-free RPMI containing goat anti-mouse IgM (50 μg/ml) for 5 min and then added with anti-CD3 IgG antibodies for 15 min at 37 °C. DU6 T cells were starved in RPMI containing 0.5% FCS and then stimulated with 0.5 μg/ml anti-CD3 antibody for 5 min. Following 15 min of stimulation, cell lysates were subjected to immunoprecipitation using polyclonal antibodies against Pyk2 or against GST fusion proteins containing different domains of Pyk2 have been generated (Fig. 1A). All our anti-Pyk2 antibodies were specific for Pyk2 and did not recognize the deletion variant (14). Polyclonal antibodies against Pyk2 were raised in rabbits injected with the GST fusion protein containing the following amino acids of human Pyk2: residues 684 to 1009 (rabbit number 598), residues 684 to 762 (rabbit number 600), residues 739 to 780 (rabbit number 601), or with the peptides corresponding to the last 17 residues of Pyk2 (rabbit number 623). Anti-Pyk2 antibodies (Ko and N-18) were previously described (2). Mouse monoclonal anti-paxillin and anti-Cas antibodies were obtained from Transduction Laboratories. Mouse monoclonal anti-phosphotyrosine antibodies (4G10) were obtained from Dr. Craig Davis or rabbit polyclonal anti-phosphotyrosine antibodies (rabbit number 72) were used as described previously (12, 15, 16). Cell Lysis, Immunoprecipitations, and Immunoblotting—Cells lysis, immunoprecipitation, and immunoblotting were performed as described previously (6, 15). For immunoprecipitation, antibodies against Pyk2 (numbers 598, 600, and 638) were cross-linked to protein A beads (Zymed) with dimethylpimelimidate (Pierce) (5, 6). For the experiments shown in Figs. 1, 3, 4, and 6, different tissues were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% sodium deoxycholate, 1% Nonidet P-40, 1.5 mM MgCl2, 1 mM EDTA, 0.2 mM EGTA, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 25 μM zinc chloride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Different sections of rat brains were provided by Bernardo Rudy (NYU, New York) and lysed in modified RIPA buffer. Identification of Autophosphorylation Sites in Pyk2 and Pyk2-H—For exogenous substrate phosphorylation, equal amounts of Pyk2 proteins were immunoprecipitated from 293T cells transiently transfected with vector alone (pRK5) or vector containing Pyk2 or Pyk2-H. The immunoprecipitates were washed three times with lysis buffer, once with kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM MnCl2, 5 mM MgCl2), and incubated with 50 μl of kinase buffer containing 1 μCi of [γ-32P]ATP (1000 Ci/mM, New England Science Products Inc.) for 10 min at room temperature. The reaction was stopped by addition of 3 × sample buffer (Bio-Rad) and resolved on 7% SDS-PAGE gels. Fold increase in phosphorylation of poly(Glu-Tyr) was determined by quantitation with a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics). For identification of autophosphorylation sites, 293T cells were transiently transfected with pRK5 containing Pyk2, Pyk2-H, or mutant forms in which the trans-oligonucleotide (GAATTGTTACATCGGGTGGCCGCGG-CATGGG) were used to delete 126 base pairs of Pyk2 using a CLON-TECH kit. The deletion was confirmed by DNA sequencing. In order to obtain exon-intron boundaries around the spliced exon, a mouse genomic clone of Pyk2 (P11) was subjected to the DNA sequencing. The mutagenic oligonucleotide (CACAACCTGCGTTCAGGAGGAGGACTTCATCCAAC) and the trans-oligonucleotide (GAATTGATATCGGGTGGCCGCGG-CATGGG) were used to delete 126 base pairs of Pyk2 using a CLONTECH kit. The deletion was confirmed by DNA sequencing. In order to obtain exon-intron boundaries around the spliced exon, a mouse genomic clone of Pyk2 (P11) was subjected to the DNA sequencing. The mutagenic oligonucleotide (CACAACCTGCGTTCAGGAGGAGGACTTCATCCAAC) and the trans-oligonucleotide (GAATTGATATCGGGTGGCCGCGG-CATGGG) were used to delete 126 base pairs of Pyk2 using a CLONTECH kit. The deletion was confirmed by DNA sequencing. In order to obtain exon-intron boundaries around the spliced exon, a mouse genomic clone of Pyk2 (P11) was subjected to the DNA sequencing. The mutagenic oligonucleotide (CACAACCTGCGTTCAGGAGGAGGACTTCATCCAAC) and the trans-oligonucleotide (GAATTGATATCGGGTGGCCGCGG-CATGGG) were used to delete 126 base pairs of Pyk2 using a CLONTECH kit. The deletion was confirmed by DNA sequencing. In order to obtain exon-intron boundaries around the spliced exon, a mouse genomic clone of Pyk2 (P11) was subjected to the DNA sequencing. The mutagenic oligonucleotide (CACAACCTGCGTTCAGGAGGAGGACTTCATCCAAC) and the trans-oligonucleotide (GAATTGATATCGGGTGGCCGCGG-CATGGG) were used to delete 126 base pairs of Pyk2 using a CLONTECH kit. The deletion was confirmed by DNA sequencing.
Alternative Splicing of Pyk2 in Hematopoietic Cells

In order to compare and analyze the properties of Pyk2 and Pyk2-H proteins, we have raised rabbit polyclonal antisera against a GST fusion protein containing the amino acid sequence corresponding to the exon that is spliced out from Pyk2 to generate Pyk2-H (number 638). 293T cells were transiently transfected with mammalian vectors containing the Pyk2 or Pyk2-H genes in human embryonic 293T cells. Lysates from the transfected cells were subjected to immunoprecipitation with antisera numbers 638 or 600 followed by SDS-PAGE and immunoblotting with antibodies number 623, that recognize both Pyk2 and Pyk2-H (Fig. 3A). Antisera number 638 recognizes Pyk2, but not Pyk2-H, while antisera 600 immunoprecipitated both Pyk2 and Pyk2-H from transfected 293 cells, indicating that these antisera recognize the Pyk2, but not the Pyk2-H isoform.

Expression Pattern of Pyk2 and Pyk2-H in Hematopoietic and Neuronal Cells—We next compared the pattern of Pyk2 and Pyk2-H protein expression in the thymus and spleen by using isoform-specific 638 antibodies that could distinguish Pyk2 from Pyk2-H, or number 600 antibodies that recognize both isoforms. Lysates from murine brain, spleen, and thymus were subjected to immunoprecipitation with antisera 638 or with antisera 600 followed by SDS-PAGE and immunoblotting with antibodies number 623 (Fig. 3B). Similar amounts of Pyk2 were immunoprecipitated from brain lysates with either antibodies 600 or 638, while expression of Pyk2-H was approximately 10-fold higher than expression of Pyk2 in the spleen and thymus (Fig. 3B). To test whether Pyk2 and Pyk2-H are co-expressed in the same cell or whether each isoform is present in a different cell type present in these tissues, we explored the expression pattern of Pyk2 and Pyk2-H in various hematopoietic cell types by using the same immunoprecipitation and immunoblotting strategy. We found that Pyk2-H is expressed in T cells, B cells, and natural killer cells, while Pyk2 is expressed in platelets, mast/basophilic cell line (RBL), megakaryocytic (CHRF), and erythroleukemic cell lines (Mo7, K562) (Fig. 3C). Both Pyk2 and Pyk2-H are present in human T-cells leukemia Jurkat cells and human promyelocytic leukemia HL-60 cells (Fig. 3C).

Pyk2-H Is Activated by Chemokine and Antigen Receptors in T and B Cells—Given the fact that Pyk2-H is present in primary T and B cells we further tested whether Pyk2-H participates in signaling pathways that are part of the host immune response, such as antigen and chemokine receptors signaling pathways. We have therefore examined the status of Pyk2-H phosphorylation in response to antigens and inflammatory cytokines in primary hematopoietic cells. Murine B cells were stimulated with goat anti-mouse IgM (50 µg/ml) and anti-goat IgG antibodies. The stimulated cells were lysed, subjected to immunoprecipitation with anti-Pyk2 antibodies (numbers 600 or 638) followed by immunoblotting with antibodies against phosphoryrosine or anti-Pyk2 (number 623). Murine thymocytes were stimulated with anti-CD3 monoclonal antibodies for 5 min at 37 °C, lysed, and subjected to immunoprecipitation with anti-Pyk2 antibodies (numbers 600 and 638). The precipitates were resolved on 7% SDS-PAGE gels and immunoblotted with anti-phosphotyrosine antibodies or anti-Pyk2 (number 623) antibodies. The experiment presented in Fig. 4A shows that activation of T-cell or B-cell antigen receptors leads to a significant increase in tyrosine phosphorylation of Pyk2-H (Fig. 4A). This experiment also confirms that Pyk2-H and not Pyk2 is

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**Fig. 1. Expression pattern of Pyk2 isoforms in brain and hematopoietic tissues.** A, schematic diagram of antibodies against Pyk2 used in these studies; B, approximately 1.5 mg of total tissue lysates of mouse brain and thymus were immunoprecipitated with anti-Pyk2 antibodies (number 600) or preimmune antisera and stained with Commassie Brilliant Blue. The arrows indicate two isoforms of Pyk2 present in lysates of spleen and thymus and heavy chains of IgG. C, lysates of different sections of rat brains, or lysates of mouse brain, spleen, and thymus were immunoprecipitated with anti-Pyk2 antibodies (number 600) followed by immunoblotting with antibodies against Pyk2 (KD). D, lysates of different murine tissues were immunoprecipitated with anti-Pyk2 antibodies (number 600) followed by immunoblotting with antibodies against Pyk2 (KD). The arrows indicate the p106 and p110 forms of Pyk2. The separation of p106 Pyk2 from the p110 isoform in liver and thymus is better seen in the shorter exposure.

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alternative abundance of the p106 Pyk2 isoform versus the full size p110 Pyk2 that were detected by immunoprecipitation analysis of lysates from thymus (Fig. 1C). Deduced amino acid sequences of the reaction products indicates that the short PCR product represents an in-frame deletion of 126 base pairs which eliminates 42 amino acid from the proline-rich region of Pyk2. Fig. 2B shows a comparison of the nucleotide and amino acid sequences of the proline-rich region of Pyk2 and of the shorter proline-rich region of the Pyk2 isoform cloned from thymus cDNA. To confirm that the shorter form of Pyk2 is generated by alternative RNA splicing, we have cloned the corresponding genomic regions of murine Pyk2. The exon that is spliced out (126 base pairs) in the shorter form of Pyk2 is preceded by a 2.2-kilobase intron and followed by a 4.8-kilobase intron in the mouse Pyk2 gene (Fig. 2C). Since the shorter form of Pyk2 was cloned from and is predominantly expressed in hematopoietic tissues this form was named Pyk2-H.

Isoform-specific Anti-Pyk2 Antibodies (Number 638) Recognize Pyk2 and Not Pyk2-H—In order to compare and analyze the properties of Pyk2 and Pyk2-H proteins we have raised rabbit polyclonal antisera against a GST fusion protein containing the amino acid sequence corresponding to the exon that is spliced out from Pyk2 to generate Pyk2-H (number 638). 293T cells were transiently transfected with mammalian vectors containing the Pyk2 or Pyk2-H genes in human embryonic 293T cells. Lysates from the transfected cells were subjected to immunoprecipitation with antisera numbers 638 or 600 followed by SDS-PAGE and immunoblotting with antibodies number 623, that recognize both Pyk2 and Pyk2-H (Fig. 3A). Antisera number 638 recognizes Pyk2, but not Pyk2-H, while antisera 600 immunoprecipitated both Pyk2 and Pyk2-H from transfected 293 cells, indicating that these antisera recognize the Pyk2, but not the Pyk2-H isoform.

Expression Pattern of Pyk2 and Pyk2-H in Hematopoietic and Neuronal Cells—We next compared the pattern of Pyk2 and Pyk2-H protein expression in the thymus and spleen by using isoform-specific 638 antibodies that could distinguish Pyk2 from Pyk2-H, or number 600 antibodies that recognize both isoforms. Lysates from murine brain, spleen, and thymus were subjected to immunoprecipitation with antisera 638 or with antisera 600 followed by SDS-PAGE and immunoblotting with antibodies number 623 (Fig. 3B). Similar amounts of Pyk2 were immunoprecipitated from brain lysates with either antibodies 600 or 638, while expression of Pyk2-H was approximately 10-fold higher than expression of Pyk2 in the spleen and thymus (Fig. 3B). To test whether Pyk2 and Pyk2-H are co-expressed in the same cell or whether each isoform is present in a different cell type present in these tissues, we explored the expression pattern of Pyk2 and Pyk2-H in various hematopoietic cell types by using the same immunoprecipitation and immunoblotting strategy. We found that Pyk2-H is expressed in T cells, B cells, and natural killer cells, while Pyk2 is expressed in platelets, mast/basophilic cell line (RBL), megakaryocytic (CHRF), and erythroleukemic cell lines (Mo7, K562) (Fig. 3C). Both Pyk2 and Pyk2-H are present in human T-cells leukemia Jurkat cells and human promyelocytic leukemia HL-60 cells (Fig. 3C).

Pyk2-H Is Activated by Chemokine and Antigen Receptors in T and B Cells—Given the fact that Pyk2-H is present in primary T and B cells we further tested whether Pyk2-H participates in signaling pathways that are part of the host immune response, such as antigen and chemokine receptors signaling pathways. We have therefore examined the status of Pyk2-H phosphorylation in response to antigens and inflammatory cytokines in primary hematopoietic cells. Murine B cells were stimulated with goat anti-mouse IgM (50 µg/ml) and anti-goat IgG antibodies. The stimulated cells were lysed, subjected to immunoprecipitation with anti-Pyk2 antibodies (numbers 600 or 638) followed by immunoblotting with antibodies against phosphoryrosine or anti-Pyk2 (number 623). Murine thymocytes were stimulated with anti-CD3 monoclonal antibodies for 5 min at 37 °C, lysed, and subjected to immunoprecipitation with anti-Pyk2 antibodies (numbers 600 and 638). The precipitates were resolved on 7% SDS-PAGE gels and immunoblotted with anti-phosphotyrosine antibodies or anti-Pyk2 (number 623) antibodies. The experiment presented in Fig. 4A shows that activation of T-cell or B-cell antigen receptors leads to a significant increase in tyrosine phosphorylation of Pyk2-H (Fig. 4A). This experiment also confirms that Pyk2-H and not Pyk2 is
exclusively expressed in murine thymocytes and B cells (Fig. 4A).

We have previously observed that stimulation of chemokine receptors by HIV envelope proteins or chemokines leads to rapid tyrosine phosphorylation of Pyk2 in several hematopoietic cells (12). We therefore examined whether activation of chemokine receptors in the human T cell line (DU6) leads to tyrosine phosphorylation and activation of Pyk2-H. DU6 cells were stimulated with 0.5 mM RANTES and macrophage inflammatory protein-1β for 30 s, lysed, and subjected to the same immunoprecipitation/immunoblotting procedures. Immunoprecipitated Pyk2-H is rapidly tyrosine phosphorylated following stimulation of DU6 cells by chemokines (Fig. 4B). Taken together, these experiments demonstrate that in primary T and B cells Pyk2-H is activated upon engagement of chemokine and antigen receptors (Fig. 4, A and B).

**Splicing Event in Pyk2-H Does Not Change Its Catalytic Activity**—Since it has been observed that deletions in the carboxyl-terminal domain of Pyk2 might lead to changes in enzyme activity (18), we further examined whether structural changes in the carboxyl terminus of Pyk2-H might have altered activation of this kinase as compared with kinase activity of Pyk2. We have compared in vitro kinase activities of Pyk2 and Pyk2-H toward the exogenous substrate (poly(Glu-Tyr)) or by measuring phosphorylation of tyrosine 402, a major autophosphorylation site of Pyk2. Immunoprecipitated Pyk2-H and Pyk2 were subjected to in vitro kinase reaction, run to SDS-PAGE gels, and intensity of 32P-labeled poly(Glu-Tyr) were compared. The experiments show that Pyk2-H and Pyk2 ex-
their mutant forms where tyrosine 402 was mutated to phenylalanine (Pyk2-H-Y402F and Pyk2-Y402F) to a tryptic digest followed by separation using reverse-phase HPLC. Three phosphorylated peptides were detected in preparations of Pyk2-H and Pyk2. Phosphorylated peptides obtained from Pyk2-H-Y402F and Pyk2-Y402F proteins have only two peaks, suggesting that the first peak observed in Pyk2-H or Pyk2 separations corresponds to peptide containing autophosphorylated tyrosine 402. Given the fact that more than 80% of labeled 32P was incorporated in that single peptide in both Pyk2 and Pyk2-H preparations, we concluded that tyrosine 402 is a major autophosphorylation site of Pyk2-H and Pyk2. Together, these results suggest that structural changes in the carboxyl terminus of Pyk2 did not have a major affect on the kinase activity of Pyk2-H.

**The Carboxyl Terminus of Pyk2-H and Pyk2 Bind Different Tyrosine-phosphorylated Proteins**—The carboxyl-terminal domain of Pyk2 contains two proline-rich regions that could be responsible for binding to proteins containing SH3 or WW domains (2). Given the fact that half of the first proline-rich region is deleted by a splicing event in Pyk2-H (Fig. 2A), we further examined whether this region could mediate specific interactions of Pyk2 with different cellular proteins. In order to screen for proteins that could bind differently to Pyk2 or Pyk2-H, the carboxyl termini of Pyk2 and Pyk2-H or the exon that is spliced out in Pyk2-H were expressed as GST fusion proteins (Fig. 6A) and used to precipitate proteins from thymus lysates. The precipitates were separated on 6% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated antibodies. The region encoded by the exon that is spliced out in Pyk2-H recognized similar patterns of tyrosine-phosphorylated proteins to those recognized by GST alone (Fig. 6B). Analysis of precipitated proteins with GST fusion proteins containing the entire carboxyl terminus of Pyk2 or Pyk2-H has revealed several common tyrosine-phosphorylated proteins with molecular mass of 62–70 kDa and a tyrosine-phosphorylated protein of approximately 115 kDa that bind specifically to the carboxyl terminus of Pyk2 but not to the carboxyl terminus of Pyk2-H (Fig. 6B). Using specific antibodies we demonstrate that paxillin is the 62–70-kDa-associated protein with Pyk2 and Pyk2-H (Fig. 6B). A weak association of p130Cas with Pyk2 was previously shown in B cells (11). We therefore examined the possibility whether tyrosine-phosphorylated p115 could be p105Hef1 or p130Cas. By using anti-Cas antibodies that recognize both p105Hef1 and p130Cas (Fig. 6B) we could not detect p130Cas or p105Hef1 that co-precipitated with the carboxyl termini of both Pyk2 or Pyk2-H in thymus lysates, suggesting that tyrosine-phosphorylated associated p115 protein is distinct from p105Hef1 or p130Cas (Fig. 6C). We therefore conclude that the carboxyl termini of Pyk2-H and Pyk2 bind both common and unique target proteins in lysates from activated thymus cells.

**DISCUSSION**

In this report, we describe identification and cloning of a novel Pyk2 isoform that is generated by alternative RNA splicing in the carboxyl terminus of Pyk2. Pyk2-H is mainly expressed in hematopoietic tissues and cells, with the highest expression in lymphocytes of the spleen and thymus, where immunoprecipitated Pyk2-H could be detected by Coomasie Brilliant Blue staining (Fig. 1B). In addition, expression of Pyk2-H appears to be restricted to specific hematopoietic cells, such as thymocytes, B cells, and natural killer cells, while Pyk2 is present in platelets, megakaryocytes, mast cells, and neuronal cells. We have also observed the presence of Pyk2-H in the heart, liver, and lung, although to a much lower level than in the thymus and spleen. Since the liver, heart, and lung contain

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**Fig. 3.** Protein expression of Pyk2 and Pyk2-H in different tissues and cells. Lysates from: A, Pyk2 or Pyk2-H transfected 293 cells; B, mouse brain, spleen, and thymus; or C, lysates of T cell line (DU6), thymocytes, splenic B cells, natural killer cells (NK), neoplastic Jurkat T-cells, promyelocytic HL-60 cells, basophilic leukemia RBL cells, megakaryocytic CHRF cells, erythroleukemic K562 and Mo7, pheochromocytoma PC12 cell lines, and platelets were immunoprecipitated with different antibodies against Pyk2 (numbers 600 or 638) and immunoblotted with anti-Pyk2 antibodies (number 623). The arrows indicate two isoforms of Pyk2.

**Fig. 4.** Pyk2-H is activated upon engagement of antigen and chemokine receptors in T and B cells. A, tyrosine phosphorylation of Pyk2-H in T or B cells. Left, splenic B cells were incubated with medium alone (−) or stimulated (+) with medium containing goat anti-mouse IgM (Fab′), and anti-goat IgG for 20 min at 37 °C. Right, murine thymocytes were either left unstimulated (−) or stimulated (+) with the anti-CD3 antibodies for 3 min at 37 °C. Lysates of these cells were immunoprecipitated with antibodies against Pyk2 (numbers 600 or 638) and analyzed by immunoblotting with anti-Tyr(P) (4G10) or anti-Pyk2 (number 623) antibodies. B, Pyk2-H is rapidly tyrosine phosphorylated in response to chemokines. DU6 cells were incubated with medium alone (−) or medium containing goat anti-mouse IgM (Fab′) and anti-goat IgG for 20 min at 37 °C, lysed, and subjected to immunoprecipitation with antibodies against Pyk2 (numbers 600 or 638) and analyzed by immunoblotting with anti-Tyr(P) (4G10) or anti-Pyk2 (number 623) antibodies.


display similar in vitro catalytic activities, measured by in vitro phosphorylation of poly(Glu-Tyr) (Fig. 5A). In order to compare the autophosphorylation sites of Pyk2 and Pyk2-H we have subjected the 32P-labeled autophosphorylated Pyk2-H, Pyk2, or
blood and since several cell lines derived from these organs express either only Pyk2 or neither of the Pyk2 isoforms (data not shown) we believe that Pyk2-H detected in immunoprecipitates from these organs is most likely due to circulating hematopoietic cells, rather than specific cells of these tissues.

Since the expression of Pyk2-H is restricted to specific hematopoietic cell lineages, whereas Pyk2 is expressed mainly in neuronal cells, an alternative splicing event may provide specificity in signaling between Pyk2 and Pyk2-H in hematopoietic versus neuronal cells. The role of different spliced isoforms of tyrosine kinases and phosphatases, such as Fyn and CD45, in hematopoietic cells have been previously reported (19, 20). Alternatively spliced isoform of Fyn in cells of hematopoietic lineages (FynT) was shown to have different signaling properties in T lymphocytes as compared with the brain Fyn (FynB) isoform (19). It has been demonstrated that structural changes in the amino-terminal part of kinase domains of FynT and FynB lead to differences in their abilities to promote antigen receptor-triggered calcium fluxes (21). In addition, a specific role for different spliced isoforms of FAK, a Pyk2-related tyrosine kinase, has also been demonstrated (22–24). Activation of an alternatively spliced form of FAK by anandamide appears to link cannabinoid G-protein-coupled receptors and tyrosine phosphorylation in hippocampal slices (22). Furthermore, the presence of the carboxyl-terminal isoform of FAK, called FRNK, was suggested to act as an endogenous regulator of FAK functions and formation of focal adhesions (23). In addition, a putative homologue of FAK, FakB, has been identified and shown to be differentially regulated from FAK in T cell and B cells (24). FakB was shown to participate in signaling by antigen receptors and associate with T-cell receptor-linked protein tyrosine kinase ZAP 70 in lymphocytes (24).

We and others have previously demonstrated that Pyk2 isoform present in T cells is selectively phosphorylated by Fyn, but not Lck, following engagement of T cell receptors (8, 9) and that stimulation of chemokine receptors by chemokines or HIV
envelope proteins leads to rapid tyrosine phosphorylation of Pyk2 isoform in several hematopoietic cells (12). Here we demonstrate that Pyk2-H, and not Pyk2, is the main isoform that is activated by antigen or chemokine receptors in primary hematopoietic cells. We have demonstrated that several cell lines such as Jurkat or HL-60 cells express both isoforms. However, the functional differences between Pyk2 and Pyk2-H in these cells are not well understood. For example, we have observed that activation of T-cell receptors in Jurkat T-cells or chemokine receptors in HL-60 cells lead to tyrosine phosphorylation of both Pyk2 isoforms (data not shown). Since Jurkat and HL-60 cells are neoplastic cell lines and since Pyk2 and Pyk2-H are normally present in different primary cells the biological importance of co-activation of Pyk2 and Pyk2-H in Jurkat and HL-60 cells is rendered less conclusive.

In addition, a little is known about the molecular mechanisms by which Pyk2 and Pyk2-H may signal differently. The spliced exon of Pyk2 contains 42 amino acids enriched in proline, serine, and threonine residues (7 proline, 6 serine, and 3 threonine), suggesting that this region may mediate protein-protein interactions, may be a target of proline-directed serine/threonine kinases, or might lead to changes in the intrinsic tyrosine kinase of Pyk2. In this report we show that structural changes in the carboxyl terminus of Pyk2-H did not have a major effect on Pyk2-H kinase activity toward exogenous substrate or toward autophosphorylated tyrosine 402. However, the carboxyl termini of Pyk2 and Pyk2-H may mediate both common and differential interactions with cellular proteins. GST fusion proteins containing the carboxyl termini of Pyk2-H or Pyk2 bind several common tyrosine-phosphorylated proteins in lysates of the thymus (Fig. 6B). An identified protein that binds to GST fusion proteins containing the carboxyl termini of Pyk2 and Pyk2-H, but not to the GST fusion protein containing the region encoded by the exon that is spliced out in Pyk2-H is the protein paxillin. It has been demonstrated that Pyk2 constitutively associates with paxillin in several hematopoietic cell lines and our results show that the splicing event in the proline-rich domain of Pyk2-H did not lead to structural changes that could affect binding of Pyk2-H to paxillin (13, 25). In addition, we have observed a p115 tyrosine-phosphorylated protein that preferentially binds to GST fusion protein containing the carboxyl terminus of Pyk2 and not Pyk2-H (Fig. 6B). A weak co-precipitation of p130Cas with Pyk2 was shown in B cell lines (11) and Pyk2 was shown to phosphorylate p130Cas and p105Hef1 (26). However, we have not been able to show co-precipitation of the carboxyl termini of Pyk2 or Pyk2-H with p105Hef1 or p130Cas in thymus lysates (Fig. 6B). Although the identity and function of p115 tyrosine-phosphorylated protein that is associated with the carboxyl terminus of Pyk2 is currently unknown, the identification of the proteins that associate specifically with the residues of the spliced exon of Pyk2 or that bind specifically to the shorter carboxyl terminus of Pyk2-H will shed light on the role this region plays in the control of Pyk2 functions.

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FIG. 6. The carboxyl termini of Pyk2-H and Pyk2 bind common and unique tyrosine-phosphorylated proteins. A, GST alone or GST fusion proteins containing the spliced exon (GST-SE), the carboxyl terminus of Pyk2 (GST-Pyk2-CT) or Pyk2-H (GST-Pyk2H-CT) were purified from bacterial lysates, separated on SDS-PAGE gel, and the purity of GST fusion proteins was estimated by staining with Commassie Brilliant Blue. In addition, we have observed a p115 tyrosine-phosphorylated protein that preferentially binds to GST fusion protein containing the carboxyl terminus of Pyk2-H or Pyk2-H (GST-Pyk2H-CT), washed, and separated on 6% SDS-PAGE gels. Precipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine (number 72), anti-paxillin and anti-p130Cas antibodies. p105Hef-1 and p130 Cas were immunoprecipitated from thymus lysates with anti-p130Cas antibodies (IP Cas) or without antibodies (−) and immunoblotted with anti-p130Cas antibodies.
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