The Human PIM-1 Gene Product Is a Protein Serine Kinase

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Abstract

The human PIM-1 gene, a homologue of murine retroviral insertion site pmpl-1, is overexpressed in a subset of hematolymphoid malignancies. Deduced amino acid sequence of PIM-1 complementary DNA predicts it to be a protein kinase. In vitro transcription coupled translation of the putative 313-amino acid open reading frame yields a M, 34,000 protein; an immune complex kinase assay of the wild-type PIM-1 and not a site-directed mutant, in which the invariant Lys67, has been changed to Arg, demonstrates autophosphorylating activity on serine residues. Thus, PIM-1 is a protein serine kinase with a possible role in neoplastic transformation.

Introduction

The murine pim-1 locus serves as a preferred integration site for the slow transforming Moloney leukemia virus in the virally induced T-cell lymphomas; integration adjacent to the pim-1 gene is accompanied by its enhanced transcription. The 2.6-kilobase pim-1 transcript is also detected in normal hematolymphoid tissues (spleen, thymus, and fetal liver) (1). The human homologue PIM-1 is transcribed in a subset of myeloid leukemias and B-cell lymphomas of which the myeloblastic cell line K562 expresses the highest levels (2–4). The deduced 313-amino acid open reading frame of PIM-1 cDNA isolated from K562 cells exhibits hallmarks of a protein kinase. Homology between murine and human PIM-1 is 94% at the amino acid level with the protein kinase domain dispersed between amino acid residues 36 through 293. A priori, it is not evident whether PIM-1 is a tyrosine or serine/threonine-specific kinase protein. The presence of Tyr198 homologous to the autophosphorylation site Tyr418 of c-src raises the possibility that it is a tyrosine kinase (3, 5). A recent alignment of catalytic domains from 65 members of the protein kinase family with conserved features has led to the identification of ten distinct subdomains; construction of a phylogenetic tree based on this scheme suggests evolutionary and possible functional interrelationships between the protein kinases (6). Two important conclusions drawn from this analysis are of interest to the present study: (a) pim-1 can be assigned to a subfamily of serine/threonine-specific kinases with no close relatives; (b) Tyr418 of c-src is not a unique and consistent characteristic of tyrosine kinases. As a first step toward understanding the role of PIM-1-encoded protein kinase activity in normal and transformed hematolymphoid cells, we have characterized its in vitro kinase activity and demonstrate that the hPIM-1 gene encodes a serine-specific autophosphorylating activity.

Materials and Methods

Plasmid Construction. The open reading frame of 939 nucleotides present in K562 human PIM-1 cDNA plasmid pc1 (3) was amplified by PCR (7) with 5’ primer TGATCAATGCTTTGTGCTAAAAAT-CAACTCG containing the initiation ATG and 3’ primer GGTACCTTATGTGCTGGCCCCGCGACAG spanning the stop codon TAG and the amplification product cloned into the blunted-ended EcoRV site of blue-script vector SK+ (Stratagene) downstream from the T7 promoter to give wild-type plasmid SKPIM. Site-directed mutagenesis of Lys67 to Arg was performed by taking advantage of the unique Mscl and BglII sites around codons 65 and 128 of SKPIM. The sense primer ACTGCGCGGTCGACACCGAGCATC containing a Mscl site TGGCCA and AGA at codon 67 instead of AAA, and an antisense primer GAAGTCGAAGAGATCTTGCAACGG containing the BglII recognition site AGATCT were used to amplify a 215-base pair fragment which was digested with Mscl and BglII and ligated to wild-type SKPIM plasmid previously cut with Mscl and BglII and treated with bacterial alkaline phosphatase. The site-directed mutation was confirmed by Sanger's dideoxy sequencing method.

In Vitro Transcription and Translation. The SKPIM plasmids were linearized with EcoRI to generate sense transcripts from the T7 promoter. Antisense transcripts were synthesized from the T3 promoter on HindIII linearized plasmid (Fig. 1). Transcription was performed essentially as described by Melton et al. (8) using a Boehringer Mannheim in vitro transcription kit. In vitro translation was carried out using rabbit reticulocyte translation system (Bethesda Research Laboratories). In vitro transcribed RNA (200–500 ng) was translated either with cold or [35S]methionine (1000 cpm/mmol), in a total volume of 30 μl. A typical reaction using 45 μCi of [35S]methionine yielded 3–5 × 104 cpn of trichloroacetic acid-precipitable protein.

Antisera and Immunoprecipitation. PIMC was conjugated either with bovine serum albumin or KLH via diethyl carbodiimide to give the respective conjugates PIMC-bovine serum albumin and PIMC-KLH, and rabbits immunized with each of the conjugates were monitored for antibodies every 4 weeks by enzyme-linked immunosorbent assay. High titer antibodies were obtained with PIMC-KLH. Immunoprecipitations were carried out as described by Kessler (9). In vitro-translated product (30 μl) was preclariﬁed with protein A-Sepharose beads and immunoprecipitated with 6 μl of 1:10 diluted antiserum or preimmune serum at 4°C for 2 h and the immune complex was adsorbed on protein A-Sepharose beads for 1 h. Beads were washed three times with RIPA buffer (0.1% NaCl-0.01% sodium phosphate (pH 7.0)-Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 1% Trasylol) and the products were subjected to 12.5% SDS-PAGE.

In Vitro Kinase Assay and Phosphoamino Acid Analysis. PIM-1 protein from in vitro translation with unlabeled methionine or whole cell lysate (K562 and HL60) was immunoprecipitated with PIMC-KLH antibodies, adsorbed on protein A-Sepharose beads, and suspended in a buffer containing 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 0.1% Triton X-100, 25 mm MgCl2, 5 mm vanadate, and 1 mm sodium fluoride. Kinase reaction was initiated by the addition of 10 μCi [γ-32P]ATP (6000 Ci/mmol). At the end of a 10-min incubation at room temperature, the reaction was arrested by adding EDTA to 5 mm, the products were solubilized in Laemmli loading buffer and resolved on a 12.5% SDS-PAGE, and the gel was dried and autoradiographed. Two dimensional thin layer electrophoresis of the phospho-protein hydrolysate was performed as described by Cooper et al. (10).
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Fig. 1. Construction of SKPIM plasmid. The 313 amino acid open reading frame (ORF) from PIM-1 cDNA pCl (3) was amplified by polymerase chain reaction with 5' and 3' primers including the initiation ATG and stop TAG codons, and the product was cloned into the EcoRV site of bluescript vector SK+ as described in “Materials and Methods.” The orientation of SKPIM was determined to be T7 promoter-PIM (5’-3’) T3 promoter.

Results

SKPIM Plasmid Construction and in Vitro Transcription. The PIM-1 cDNA clone PC1 contains 350 nucleotides of 5’-untranslated leader sequence and 642 nucleotides of 3’-untranslated sequence. In order to optimize conditions for in vitro transcription, SKPIM was constructed by amplifying sequences encoding only the open reading frame (Fig. 1). Incorporation of erroneous nucleotides during the PCR reaction was eliminated by limiting the number of amplifications; thus the product of 15 cycles of amplification of the 939-nucleotide open reading frame was subcloned into the EcoRV site of bluescript vector SK+. In vitro transcription of EcoRI-linearized plasmid with T7 RNA polymerase yielded a single species of 0.98-kilobase mRNA.

In Vitro Translation and Immune Complex Kinase of PIM-1. The 980-base pair RNA yielded a single species of Mr 34,000 PIM-1 protein upon in vitro translation with the rabbit reticulocyte system. Fig. 2a (Lane 5) shows detection of this protein from the translation reaction by PIMCKLH antisera. Immunoprecipitation of the translation product from antisense RNA (Fig. 2a, Lane 2) or blocking the immune complex formation with excess PIMC-KLH peptide (Fig. 2a, Lane 6) abolished detection of the Mr 34,000 PIM-1 protein. In a number of different experiments, the amount of PIM-1 protein recovered by immunoprecipitation was 30–40% of the total protein synthesized in vitro. These results help establish the specificity of PIMC-KLH antibodies as well as the in vitro transcription-coupled translation conditions. Immune complex kinase assay of the Mr 34,000 protein synthesized in vitro would define the protein kinase activity of PIM-1. In order to eliminate possible contribution due to coprecipitating kinase activities, a site-directed mutant of SKPIM plasmid, which encodes Arg at codon 67 instead of the highly conserved Lys was constructed as described in “Materials and Methods.” Lys, served as the best target to generate a loss of function mutant because of the extensive studies carried out on other protein kinases. Homologous residues in v-src and epidermal growth factor receptor react with the ATP analogue p-fluorosulfonyl-5'-benzoyladenosine which inhibits the protein kinase activity, raising the

Fig. 2. a, in vitro transcription-coupled translation of SKPIM plasmid. Antisense (Lanes 1–3) and sense (Lanes 4–6) transcripts transcribed from SKPIM plasmid was translated using the rabbit reticulocyte system with [35S]methionine and immunoprecipitated with either preimmune serum (Lanes 1 and 4) or immune serum (Lanes 2, 3, 5, and 6). Samples in Lanes 3 and 6 were immunoprecipitated in the presence of 100 nm PIMC-KLH peptide. Lane 7 denotes in vitro translation in the absence of exogenous RNA; 6000 cpm were loaded per lane, resolved on 12.5% SDS-PAGE, fixed, and dried, and the autoradiograph was exposed overnight. b, immune complex kinase assay of in vitro-synthesized human PIM-1 protein. Immunoprecipitations were performed on in vitro-synthesized wild-type (Lanes 1 and 2) or two different Arg mutant human PIM-1 (Lanes 3–6). Samples in Lanes 1, 3, and 5 were immunoprecipitated with PIMC-KLH antibodies and samples in Lanes 2, 4, and 6 received preimmune serum. The Immune complex kinase assay was performed as described in “Materials and Methods”: 10,000 cpm were loaded per lane and the autoradiograph was exposed overnight. Center ordinate, positions of molecular weight markers in thousands.

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possibility that the invariant Lys is involved in mediating phosphotransfer reaction. Site-directed mutation of invariant Lys abolishes enzymatic function in a number of protein kinases including v-src, v-mos, v-fps, epidermal growth factor receptor, insulin receptor, and platelet-derived growth factor receptor (6). Fig. 2b depicts SDS-PAGE analysis of an immune complex kinase reaction of wild-type (Fig. 2b, Lane 1) or two different mutant clones with Arg67 substitution (Fig. 2b, Lanes 3 and 5). The presence of a single major species of phosphorylation around M, 34,000 (pp34 PIM) is suggestive of a PIM-1-mediated autophosphorylating activity. The fuzziness of the pp34 band could be due to multiple species of phosphorylated PIM-1 proteins. No phosphoproteins were detected in the mutant protein immune complex kinase (Fig. 2b, Lanes 3 and 5) demonstrating loss of catalytic activity due to the replacement of Lys67 with Arg.

Immune Complex Kinase Assay of PIM-1 in Whole Cell Lysates. Because the PIMC-KLH antibodies recognized in vitro synthesized PIM-1 protein with high specificity, the ability of PIM-1 protein immunoprecipitated from whole cell extracts to mediate autophosphorylation could be studied. High levels of PIM-1 mRNA seen in K562 cells correlated well with abundant PIM-1 autophosphorylating activity as denoted by significant phosphorylation of a M, 34,000 protein, pp34 (Fig. 3, Lanes 2–4). Addition of PIM-1 protein synthesized by in vitro transcription coupled translation reaction to the immune complex generated from whole cell lysate does not significantly augment the protein kinase activity as seen by a lack of difference between

Fig. 3. Immune complex kinase assay of human PIM-1 from whole cell lysates. Five x 10^7 K562 (Lanes 1–4) or HL60 (Lanes 5, 6) cells were lysed in 250 μl of lysis buffer and aliquots were treated with preimmune (Lanes 1 and 5) or PIMC-KLH antiserum (Lanes 2–4 and 6). An immune complex kinase assay was performed as described in “Materials and Methods.” Lane 2 contained a mixture of in vitro-synthesized and whole cell PIM-1 immunoprecipitates; 10,000 cpm per lane were loaded in all the lanes except Lane 3 which received 5,000. Arrowhead, position of pp34. Right, resolution of the 92,000–14,000 molecular weight markers.

Discussion

The PIM-1 gene is preferentially expressed in a subset of myeloid leukemias and B-cell lymphomas including Burkitt’s. Unlike the mouse homologue, it does not appear to be overexpressed in human T-cell lymphomas (3). Recent experiments on transgenic mice overexpressing PIM-1 in the appropriate tissues demonstrate a low incidence of spontaneous T-cell lymphomas; however, induction of a high incidence of T-cell lymphomas by chemical or viral agents in these animals is accompanied by overexpression of c-myc gene, possibly due to a cooperativity between these two oncoproteins (11, 12). In humans, the possible cooperation between PIM-1 and the translocated c-MYC gene product remains to be elucidated. c-myc serves as a substrate for casein kinase II, a nuclear serine/threonine kinase (13). Immunochemical studies of human PIM-1 have localized it to the cytoplasm (4) indicating a role for its activity in phosphorylating cytoplasmic or integral membrane proteins (14).

An earlier report on the in vitro autophosphorylating activity of K562 PIM-1 protein suggested it to be tyrosine specific (15). The anomaly between our results and those of Telerman et al.
could be due to: (a) the assay conditions used in their studies. The first part of the indirect assay involved detection of $^{35}$S methionine-labeled PIM-1 protein from K562 cells by immunoprecipitation, resolution on SDS-PAGE, fixation, and treatment of the gel with dimethyl sulfoxide-PPO for autoradiography. In the next step, a slice corresponding to the human PIM-1 protein was cut out from the SDS-polyacrylamide gel, rehydrated, and allowed to autophosphorylate by the addition of $^{32}$P]ATP, subjected to SDS-PAGE, fixed, dried, and autoradiographed. Phosphoamino acid analysis on a single dimension thin layer chromatography of the PIM-1 protein thus phosphorylated revealed a product which was interpreted to be phosphoserine. The effect of dimethyl sulfoxide-PPO treatment on the mobility of phosphoserine that may be kinased under these conditions is not known; (b) lack of specificity of antisera which was raised against a fusion protein containing part of the kinase domain (amino acids 128–197). Direct human PIM-1 immune complexes from K562 cell lysates did not demonstrate detectable protein kinase activity. Addition of bacterial TrpE fusion protein encoding amino acids 128–197 to this complex restored phosphotransfer activity. Thin layer chromatography of autophosphorylated PIM-1 protein under our experimental conditions revealed phosphoserine and not phosphothreonine (data not shown).

Protein kinases form a major class of critical gene products mediating cytoplasmic and nuclear signals. Initially, functional assays led to identification of protein kinases with known biochemical characteristics (16). More recently, a number of novel means including (a) isolation of cellular homologues of v-onc genes, (b) low stringency oligonucleotide screening of cDNA libraries, (c) screening of expression cDNA libraries with antiphosphoamino acid antibodies, (d) isolation of genes specific for cell cycle arrest in yeast, and (e) molecular cloning of proviral integration (reviewed in Refs. 14 and 16) have resulted in a large increase in the number of molecularly cloned protein kinases. The specificity of phosphotransfer remains to be confirmed in a number of these cases. Although the new theoretical alignment described by Hanks et al. (6) makes it possible to assign newly identified kinases to specific subfamilies, emergence of subfamilies unidentified thus far cannot be ruled out. An example of such a possibility was reported recently in the case of a Saccharomyces cerevisiae protein kinase, YPK1, which displays a high degree of sequence homology to protein serine kinases and yet mediates phosphotransfer to tyrosine on synthetic peptides and is phosphorylated on Tyr in vivo (17).

Finally, in the past decade, immune complex kinase assays of in vitro translated v-src and v-mos RNAs facilitated characterization of their activity prior to molecular cloning of their cellular homologues (18, 19). With the advent of PCR, specific amplification of catalytic domain sequences coupled with in vitro transcription, translation, and immune complex kinase assay described in this report provides an easy approach to characterize protein kinase activities as their genes are isolated.

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