We have previously reported that the Huntingtin interacting protein 1 (HIP1) gene is fused to the platelet-derived growth factor β receptor (PDGFβR) gene in a patient with chronic myelomonocytic leukemia. We now show that HIP1/PDGFBβR oligomerizes, is constitutively tyrosine-phosphorylated, and transforms the murine hematopoietic cell line, Ba/F3, to interleukin-3-independent growth. A kinase-inactive mutant is neither tyrosine-phosphorylated nor able to transform Ba/F3 cells. Oligomerization and kinase activation required the 55-amino acid carboxy-terminal TALIN homology region but not the leucine zipper domain. Tyrosine phosphorylation of a 130-kDa protein and STAT5 correlates with transformation in cells expressing HIP1/PDGFBβR and related mutants. A deletion mutant fusion protein that contains only the TALIN homology region of HIP1 fused to PDGFβR is incapable of transforming Ba/F3 cells and does not tyrosine-phosphorylate p130 or STAT5, although it is itself constitutively tyrosine-phosphorylated. We have also analyzed cells expressing Tyr → Phe mutants of HIP1/PDGFBβR in the known PDGFβR SH2 docking sites and report that none of these sites are necessary for STAT5 activation, p130 phosphorylation, or Ba/F3 transformation. The correlation of factor-independent growth of hematopoietic cells with p130 and STAT5 phosphorylation/activation in both the HIP1/PDGFBβR Tyr → Phe and deletion mutational variants suggests that both STAT5 and p130 are important for transformation mediated by HIP1/PDGFBβR.

Chronic myelomonocytic leukemia (CMML) is a type of myelodysplastic syndrome characterized by dysplastic monocytes, variable bone marrow fibrosis, and progression to acute leukemia. The t(5;12)(q33;p13) is a recurring cytogenetic abnormality associated with CMML and results in expression of a fusion protein, TEL/PDGFBβR, containing the amino-terminal portion of TEL (ETV6) and the transmembrane and tyrosine kinase domains of platelet-derived growth factor β receptor (PDGFβR) (1).

The TEL/PDGFBβR fusion has constitutive kinase activity mediated by the TEL pointed (PNT) self-association motif, transforms the murine hematopoietic cell line Ba/F3 to IL-3-independent growth and induces hematopoietic malignancies in murine models of leukemia (2–5). The kinase activity, as well as the PNT oligomerization motif, is necessary for transformation. These structure-function relationships are similar to those observed in other transforming tyrosine kinase fusions such as TPR/MET (6), BCR/ABL (7), TEL/ABL (2), and TEL/JAK2 (8, 9). Each of these require an oligomerization domain to constitutively activate the partner tyrosine kinase and hence transform cells.

We have identified another cytogenetic abnormality associated with the clinical phenotype of CMML, t(5;7)(q33;q11.2), that results in expression of the transforming fusion protein HIP1/PDGFBβR (10). Huntington interacting protein 1 (HIP1) is 116-kDa protein that binds Huntington, the protein product of the gene mutated in Huntington disease (11, 12). All but the 18 carboxyl-terminal amino acids of HIP1 are fused to the transmembrane and tyrosine kinase domains of the PDGFβR in this novel fusion protein.

The function of HIP1 is unknown, but it contains evolutionarily conserved sequences. The yeast homologue, SLA2P, is essential for yeast growth as well as assembly and function of the cortical cytoskeleton (13). There is also a C. elegans homologue of unknown function. Finally, HIP1 has a leucine zipper motif and the carboxyl terminus is homologous to TALIN, a cytoskeletal protein implicated in cell-substratum as well as cell-cell interactions (15).

Several signaling pathways are employed by the activated native PDGFβR kinase. The signaling intermediates, including SRC family members, phosphatidylinositol 3-kinase (PtdIns 3-kinase), RasGTPase-activating protein, and phospholipase Cγ (PLCγ), may also be activated by HIP1/PDGFBβR. Certain tyrosine residues when phosphorylated are designated SH2 docking sites, since they are binding sites for these SH2 domain-containing signaling proteins. Two tyrosine phosphorylation sites (Tyr579 and Tyr581) (Fig. 1) associate with the SRC family tyrosine kinases (SRC, Fyn, and Yes) (16, 17), and this association leads to mitogenesis in NIH 3T3 cells. The 85-kDa regulatory subunit of PtdIns 3-kinase binds to phosphorylated Tyr778 and Tyr781 in the kinase insert domain of the PDGFβR, and PLCγ binds to phosphorylated Tyr1069 and Tyr1091 (18). Both are required for full mitogenic activity in HepG2 cells (19).
Here we have dissected the role of HIP1 in HIP1/PDGFβR transformation by deletion mutational analysis of the HIP1 moiety. We have also determined the relevance of the mitogenic properties of each of the constructs is shown below and 1021 demarcated by the vertical lines below the schematic. A summary of properties of each of the constructs is shown on the right. +, ability to oligomerize, phosphorylate tyrosine (kinase activity), or confer factor-independent growth to Ba/F3 cells (Transforming); −, does not have the designated property; nd, no data. TH, Talin homology region.

**EXPERIMENTAL PROCEDURES**

**Reconstruction of the Fusion cDNA for Expression Experiments**—The breakpoint was amplified from patient material using primers HIP1330F (5'-CCCTGAAACTGCTAAGAACCA-3') and PDGFβR 1806R, and the product was digested with BglI and NheI. The BglI–SacII fragment of the PDGFβR was isolated after BglI and SacII digestion of the PDGFβR cDNA and ligated to the NheI–BglI breakpoint fragment. This ligation reaction was amplified with primers containing the optimal Kozak consensus sequence at HIP1 amino acid positions 497, 509, 511, 716, 740, 751, 771, 1009, and 1021 demarcated by the vertical lines below the schematic. A summary of properties of each of the constructs is shown on the right. +, ability to oligomerize, phosphorylate tyrosine (kinase activity), or confer factor-independent growth to Ba/F3 cells (Transforming); −, does not have the designated property; nd, no data. TH, Talin homology region.

**Construction of Mutants of HIP1/PDGFβR**—The reconstructed cDNAs for HIP1/PDGFβR and HIP1/KI were subcloned into pcDNA3 (Invitrogen). H/P(del1)3 is the carboxyl-terminal truncation mutant used for the association studies; H/P(del1)3 has deletion of HIP1 sequences up to the EcoRI site; H/P(ATG497) through H/P(ATG752) have HIP1 sequences deleted up to amino acid designated in the parentheses; H/P(NI) has deletion of HIP1 sequences up to the NheI site; H/P(RI) is the R635K point mutant that makes the expressed protein devoid of tyrosine kinase (TK) activity; H/P(RI)(del)LZ has the 23-amino acid leucine zipper (LZ) deleted from H/P(RI); H/P(del)H has deletion of all HIP1 sequences up to the breakpoint; H/P(F2) has Tyr to Phe point mutations at PDGFβR positions 579 and 581; H/P(F8) has deletion of HIP1 sequences up to the breakpoint; H/P(F6) has Tyr to Phe point mutations at PDGFβR positions 579, 581, 716, 740, 751, 771, 1009, and 1021 demarcated by the vertical lines below the schematic. A summary of properties of each of the constructs is shown on the right. +, ability to oligomerize, phosphorylate tyrosine (kinase activity), or confer factor-independent growth to Ba/F3 cells (Transforming); −, does not have the designated property; nd, no data. TH, Talin homology region.

**Fig. 1. Structure and biological properties of the cloned fusion protein HIP1/P and related mutants.** HIP is the full-length construct. The predicted internal ATG translation start sites are marked in this part of the figure but apply to all of the mutants. H/P(del1)3 is the carboxyl-terminal truncation mutant used for the association studies; H/P(del1)3 has deletion of HIP1 sequences up to the EcoRI site; H/P(ATG497) through H/P(ATG752) have HIP1 sequences deleted up to amino acid designated in the parentheses; H/P(NI) has deletion of HIP1 sequences up to the NheI site; H/P(RI) is the R635K point mutant that makes the expressed protein devoid of tyrosine kinase (TK) activity; H/P(RI)(del)LZ has the 23-amino acid leucine zipper (LZ) deleted from H/P(RI); H/P(del)H has deletion of all HIP1 sequences up to the breakpoint; H/P(F2) has Tyr to Phe point mutations at PDGFβR positions 579 and 581; H/P(F8) has deletion of HIP1 sequences up to the breakpoint; H/P(F6) has Tyr to Phe point mutations at PDGFβR positions 579, 581, 716, 740, 751, 771, 1009, and 1021 demarcated by the vertical lines below the schematic. A summary of properties of each of the constructs is shown on the right. +, ability to oligomerize, phosphorylate tyrosine (kinase activity), or confer factor-independent growth to Ba/F3 cells (Transforming); −, does not have the designated property; nd, no data. TH, Talin homology region.

**HpaI and NheI, and the insert was ligated into HpaI/NheI-digested pcDNA3-HIP1/PDGFβR.**

In addition, HIP1 amino-terminal deletion mutants were constructed. The H/P(RI) deletion mutant was prepared by digestion of the wild type HIP1/PDGFβR construct in pcDNA3 with EcoRI followed by religation. H/P(NI) was made by digesting HIP1/PDGFβR in pcDNA3 with EcoRI and NheI, filling in the sticky ends with T4 DNA polymerase followed by religation. The H/P(del1)H mutant was constructed by amplifying the transmembrane and a portion of the PDGFβR cytoplasmic domain. The forward primer spanned the breakpoint, had a 5' end EcoRI site, and incorporated an ATG at the breakpoint with an optimal Kozak consensus sequence (5'–CAGTAACTGCCTGGTGCTGCAGGGCTGGAAGACCATGGG–3'). The reverse primer, TPSY1R (5’–ATGCTGCTCGCATACTGGCTGAGGCTGGCCTC–3’) encodes the PDGFβR sequence just 3’ of the unique SacII site. The ATG truncation mutants between the EcoRI and NheI sites were constructed using the same 3’–primer (TPSY1R), and the 5’–primers were engineered to have an EcoRI site and an ATG with an optimal Kozak consensus sequence at HIP1 amino acid positions 497, 599, 690, and 752. The 5’–primers were as follows: H/P(ATG497), 5’–CGGAAAGATTCAGAGGTGACCAAACAGGTGACCATGGG–3’; H/P(ATG599), 5’–GGGAAATTCCTGCTGCTGAGGGCTGGAGACC–3’; H/P(ATG690), 5’–GGGGACTGCTGCTGAGGGCTGGAGACC–3’; H/P(ATG752), 5’–AGCTCGAGATTTCCGAGCTGAGGAGACC–3’; H/P(ATG853), 5’–GCTGAGATTTCCGAGCTGAGGAGACC–3’; H/P(ATG853), 5’–GCTGAGATTTCCGAGCTGAGGAGACC–3’; H/P(ATG853), 5’–GCTGAGATTTCCGAGCTGAGGAGACC–3’; H/P(ATG853), 5’–GCTGAGATTTCCGAGCTGAGGAGACC–3’. The resultant PCR products were digested with EcoRI and SacII and ligated with EcoRI/SacII-digested H/P(RI) pcDNA3 vector.

To construct the F8 mutant of HIP1/PDGFβR, the wild type PDGFβR F8 mutant was used and has been described previously (19). An F6 mutant bearing mutations in Tyr579, Tyr581, Tyr716, Tyr740, Tyr751, Tyr771, Tyr1009 and Tyr1021 was constructed by digesting the mutated F8 PDGFβR in pcDNA3 (kindly provided by Andrius Kazlauskas) with SacII and EcoRI and replacing the released fragment with the HIP1/PDGFβR EcoRI/SacII 2-kilobase fragment (Fig. 1). To make the most membrane-proximal mutations that would convert this F6 mutant to HIP1/PDGFβR/F8, an antisense oligonucleotide was designed to encode Tyr→Phe mutations at positions Tyr579 and Tyr581 of the PDGFβR. This
oligonucleotide also contained the unique SacII site in the PDGFβR. This 3’-primer was then used with a sense oligonucleotide just 5’ of the unique NheI site of the HIP1 cDNA (nucleotides 2264–2284 of the HIP1 sequence) to amplify a PCR product using the “wild type” fusion cDNA as a template. The product was digested with SacII and NheI and subcloned into a NheI/SacII-digested HIP1/PDGFβR-bearing pcDNA3. The cDNA constructs for the indicated mutations of H/P were cloned into the pcDNA3 expression vector, and in vitro transcription/translation was performed according to the manufacturer’s instruction (Promega TNT kit) using radiolabeled [35S]methionine. One μg of each plasmid was added to the reaction and resulted in approximately equal amounts of each translated protein. For the immunoprecipitations, one-half of the reaction mixture was incubated with antiserum to the carboxyl terminus of the human PDGFβR (UBI), and immune complexes were collected with protein G-Sepharose. After fixing the gels containing the [35S]methionine-labeled and SDS-PAGE-separated proteins, the gels were treated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to film for 2 h. Predicted electrophoretic mobilities for H/P and related mutants were based on analysis of electrophoretic mobilities of native PDGFβR and TEL/PDGFβR, which migrate with apparent molecular masses of approximately 20–30 kDa larger than predicted by primary sequence.

**FIG. 2.** HIP1/PDGFβR and mutants associate in vitro. The cDNA constructs for the indicated mutations of H/P were cloned into the pcDNA3 expression vector, and in vitro transcription/translation was performed according to the manufacturer’s instruction (Promega TNT kit) using radiolabeled [35S]methionine. One μg of each plasmid was added to the reaction and resulted in approximately equal amounts of each translated protein. For the immunoprecipitations, one-half of the reaction mixture was incubated with antiserum to the carboxyl terminus of the human PDGFβR (UBI), and immune complexes were collected with protein G-Sepharose. After fixing the gels containing the [35S]methionine-labeled and SDS-PAGE-separated proteins, the gels were treated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to film for 2 h. Predicted electrophoretic mobilities for H/P and related mutants were based on analysis of electrophoretic mobilities of native PDGFβR and TEL/PDGFβR, which migrate with apparent molecular masses of approximately 20–30 kDa larger than predicted by primary sequence.
The resultant mutant (H/P(F2)) was then digested with SacII and RI, and the fragment that was released was used to replace the wild type sequence in the H/P(F6)MSCVneo mutant as well as to replace the wild type sequence in the H/P.MSCVneo construct. Briefly, the nonmutated RI/SacII fragment was excised, and the vectors were ligated with the RI/SacII Tyr579- and Tyr581-mutated fragment. The amplified fragment was sequenced.

In Vitro Transcription/Translation—In vitro transcription/translation was performed using a rabbit reticulocyte lysate kit (TNT system; Promega) according to the manufacturer's specifications. Proteins were labeled with [35S]methionine. One-half of the reaction was removed, and 2 µl of IgG specific for the C terminus of PDGFβR was used to immunoprecipitate translated products. The immunocomplexes were precipitated with protein G-Sepharose and washed three times with 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris HCl, pH 7.4, 1% Triton X-100). Total reaction mixtures and immunoprecipitates were resolved by SDS-PAGE, and the separated proteins were visualized by fluorography using Amplify (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunofluorescence—COS-1 cells were transfected with HIP1/PDGFβR using the DEAE-dextran method (20). The transfected COS-1 cells were subjected to indirect immunofluorescence antibody staining 48 h after transfection. At 24 h, cells were replated at 400,000 cells/ml onto glass coverslips. At 48 h, cells were fixed with 4% formaldehyde in phosphate-buffered saline and blocked in 5% milk in phosphate-buffered saline/1% Triton X-100 for 1 h, and then a 1:100 dilution of antiserum to the cytoplasmic tail of the PDGFβR (Pharmingen) was added for 1 h. After washing the cells, secondary fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was added for 30 min. The cells were then washed, incubated with 1 µg/ml Hoechst dye 33258 for 1 min, and washed again, and then the coverslips were mounted on glass slides. The cells were visualized with an Olympus fluorescence microscope and photographed with Kodak Royal Gold II 400 print film.

Stable Expression of HIP1/PDGFβR—The full-length fusion HIP1/PDGFβR, R634K mutant (HIP(KI)), H/P(RI)(del)LZ, H/P(F2), H/P(F8), and the larger amino-terminal HIP1 deletion constructs were subcloned into the pHIP1Neo vector (kindly provided by R. Hawley, University of Toronto). 293T cells (the kind gift of R. Mulligan, Harvard Medical School, Boston) were transfected via the calcium phosphate technique (20). 48-h supernatant (1 ml) was then added to 106 Ba/F3 cells (1 ml) in the presence of polybrene (4 µl) as described previously (21). Cells with stable expression were selected in the presence of G418 and IL-3 as described (4). When analyzing associated proteins, the immunoprecipitates were washed with Tris-buffered saline containing 0.5% Triton X-100 rather than the lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100).

When the Western blot was probed twice with anti-phosphotyrosine antibodies (4G10) followed by anti-PDGFβR tail antibodies, the blot was stripped of 4G10 antibody by incubating it at 50 °C for 30 min.
TABLE II

| Construct | No. of wells/total wells |
|-----------|--------------------------|
| H/P(ATG497) | 96/96 |
| H/P(ATG599) | 96/96 |
| H/P(ATG690) | 80/96 |
| H/P(ATG752) | 0/96 |
| H/P(ATG830) | 0/96 |
| H/P(F2) | 56/96 |
| H/P(F5) | 40/96 |

...in a solution of 2% SDS, 20 mM Tris, pH 6.8, 84 mM β-mercaptoethanol. The blot was then washed with phosphate-buffered saline four times for 10 min prior to staining with the secondary antibody.

**Electromobility Shift Assays (EMSAs)—**EMSAs were performed as described previously (22). In brief, nuclear extracts (6 mg) from control and activator of transcription (STAT) protein complexes (in cytoplasmic domain) (Fig. 3). HIP1/PDGFβR and all of the mutants were constitutively tyrosine-phosphorylated in stable Ba/F3 infectants, except for the kinase-inactive point mutant H/P(KI) (Fig. 3). HIP1/PDGFβR, H/P(RI)(del)LZ and H/P(NI) had predicted electrophoretic mobilities of 220, 170, and 100 kDa, respectively. The phosphorylated band at 180 kDa in the HIP1/PDGFβR and H/P(RI)(del)LZ lanes is probably the result of an alternative start site at ATG497, the first ATG after the EcoRI site of HIP1, because on longer exposures this band is recognized by the anti-PDGFβR antibodies (data not shown and Fig. 1).

Expression of the wild type and selected mutant proteins under control of the viral long terminal repeat was confirmed with antibodies directed against the PDGFβR carboxyl terminus (in cytoplasmic domain) (Fig. 3). HIP1/PDGFβR and selected mutants were tested in the IL-3-dependent, murine hematopoietic cell line, Ba/F3. The HIP1/PDGFβR constructs were subcloned into the murine eucaryotic retroviral vector MSCVneo. Viral supernatants were prepared by transient transfection of 293T cells and used to infect Ba/F3 cells (21).

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**RESULTS**

Oligomerization of HIP1/PDGFβR and Related Mutants—Full-length HIP1/PDGFβR (10) was used to construct related mutants including H/P(KI), which contains a point mutation, R634K, known to inactivate PDGFβR kinase activity; H/P(RI)(del)LZ, which contains a 23-amino acid deletion of the leucine zipper motif as well as deletion of amino-terminal sequences up to the EcoRI site; a series of HIP1 amino-terminal deletion mutants; and the Tyr → Phe mutations in the cytoplasmic domain of the HIP1 sequence (Fig. 1). These mutants were constructed by using the unique restriction enzyme sites EcoRI (H/P(RI)) and Nhel (H/P(NI)) to excise HIP1 sequences or amplifying HIP1 and PDGFβR sequences of the fusion protein by PCR. All mutants were confirmed by sequence analysis.

When selected constructs were translated in *vitro*, HIP1/PDGFβR and related mutants migrated at the expected sizes and were recognized by an antibody to the carboxyl terminus of the PDGFβR (Fig. 2). Utilization of the first ATG of the full-length HIP1/PDGFβR and truncated H/P(RI)(del)LZ yielded protein products with expected electrophoretic mobilities of 220 and 170 kDa, respectively (see legend to Fig. 2). H/P(NI) and H/P(del)H migrated as a 100-kDa doublet and a 90-kDa protein, respectively. The 100-kDa doublet is consistent with the 100-kDa doublet and a 90-kDa protein, respectively. The 100-kDa doublet is consistent with the orientation of HIP1 amino-terminal truncation mutants and PDGFβR Tyr → Phe mutants transform Ba/F3 cells to IL-3-independent growth The G418-resistant cells growing in IL-3 were seeded in 96-well trays with 2 × 10^4 cells/200 μl/well in RPMI 1640 and 10% fetal calf serum media with or without IL-3. Cells were assessed for viability (trypan blue).

In brief, nuclear extracts (6 mg) from control and activator of transcription (STAT) protein complexes (in cytoplasmic domain) (Fig. 3). HIP1/PDGFβR and all of the mutants were constitutively tyrosine-phosphorylated in stable Ba/F3 infectants, except for the kinase-inactive point mutant H/P(KI) (Fig. 3). HIP1/PDGFβR, H/P(RI)(del)LZ and H/P(NI) had predicted electrophoretic mobilities of 220, 170, and 100 kDa, respectively. The phosphorylated band at 180 kDa in the HIP1/PDGFβR and H/P(RI)(del)LZ lanes is probably the result of an alternative start site at ATG497, the first ATG after the EcoRI site of HIP1, because on longer exposures this band is recognized by the anti-PDGFβR antibodies (data not shown and Fig. 1).

Expression of the wild type and selected mutant proteins under control of the viral long terminal repeat was confirmed with antibodies directed against the PDGFβR carboxyl terminus (in cytoplasmic domain) (Fig. 3). HIP1/PDGFβR and selected mutants were tested in the IL-3-dependent, murine hematopoietic cell line, Ba/F3. The HIP1/PDGFβR constructs were subcloned into the murine eucaryotic retroviral vector MSCVneo. Viral supernatants were prepared by transient transfection of 293T cells and used to infect Ba/F3 cells (21).

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**Expression and Tyrosine Phosphorylation of HIP1/PDGFβR and Related Mutants—**Expression and transforming properties of HIP1/PDGFβR and selected mutants were tested in the IL-3-dependent, murine hematopoietic cell line, Ba/F3. The HIP1/PDGFβR constructs were subcloned into the murine eucaryotic retroviral vector MSCVneo. Viral supernatants were prepared by transient transfection of 293T cells and used to infect Ba/F3 cells (21).

Expression of the wild type and selected mutant proteins under control of the viral long terminal repeat was confirmed with antibodies directed against the PDGFβR carboxyl terminus (in cytoplasmic domain) (Fig. 3). HIP1/PDGFβR and all of the mutants were constitutively tyrosine-phosphorylated in stable Ba/F3 infectants, except for the kinase-inactive point mutant H/P(KI) (Fig. 3). HIP1/PDGFβR, H/P(RI)(del)LZ and H/P(NI) had predicted electrophoretic mobilities of 220, 170, and 100 kDa, respectively. The phosphorylated band at 180 kDa in the HIP1/PDGFβR and H/P(RI)(del)LZ lanes is probably the result of an alternative start site at ATG497, the first ATG after the EcoRI site of HIP1, because on longer exposures this band is recognized by the anti-PDGFβR antibodies (data not shown and Fig. 1).

**Transforming Properties of HIP1/PDGFβR and Related Mutants in Ba/F3 Cells—**Ba/F3 stable infectants of HIP1/PDGFβR and related mutants were seeded in 96-well trays at a concentration of 2 × 10^4 cells/well and assayed for IL-3-independent proliferation as described (4). HIP1/PDGFβR, H/P(RI) and H/P(RI)(del)LZ transformed Ba/F3 cells to IL-3-independent growth.

In contrast, the H/P(NI) mutant, which also oligomerizes and is constitutively tyrosine-phosphorylated, was incapable of conferring IL-3-independent growth. For control experiments, the kinase-inactive mutant H/P(KI) and insert-free MSCVneo did not confer IL-3-independent growth (Table I).

**Tyrosine Phosphorylation of Proteins in Transformed Cell Extracts—**Western blots of whole cell lysates of the stable cell lines with anti-phosphotyrosine antibodies identified a consistent and prominent 130-kDa phosphoprotein in the H/P- and H/P(RI)(del) LZ-transformed cells but not the vector, H/P(KI), or H/P(NI) nontransformed cell lines (Fig. 3, right panel). There were no other consistent differences in the phosphorylation patterns of transformed cells compared with nontransformed cells other than autophosphorylated HIP1/PDGFβR and the related mutant fusion proteins.

To further characterize HIP1 sequences required for p130 tyrosine phosphorylation, mutants were constructed with further amino-terminal truncations of H/P between the EcoRI and *Nhel* sites of the HIP1 sequence (Fig. 1). These mutants were then tested for transforming ability (Table II) and ability to associate with and phosphorylate p130 (Fig. 4). Extracts were immunoprecipitated with antiserum against the PDGFβR tail, followed by staining with anti-phosphotyrosine antibodies. The results showed that the prominent 130-kDa phosphoprotein associated exclusively with the fusion proteins that transform the Ba/F3 cell line (Fig. 4). Of particular note, p130 was not detected and did not associate with the nontransforming H/P variants: H/P(ATG752), H/P(ATG830), or H/P(NI) (Figs. 3 and 4 and data not shown).

Since the only consistent difference between transformed and nontransformed cell extracts was an increase in tyrosine phosphorylation of p130, p130 tyrosine phosphorylation may be
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relevant to transformation. To determine the identity of p130, proteins involved in PDGFR or IL-3 signal transduction that migrated in the 110–140-kDa molecular mass range were targeted. Extracts were immunoprecipitated with specific antibodies, and the immunoprecipitates and supernatants were blotted onto nitrocellulose. Proteins were detected with horseradish peroxidase-conjugated anti-phosphotyrosine 4G10 monoclonal antibody or anti-PDGFR antibody as labeled. A, lanes 1–4 contain H/P, H/P(F2), H/P(F8), and H/P(KI), respectively. The first three cell lines were grown in the absence of IL-3 and in the presence of G418 (lanes 1–3). The last cell line was grown in the presence of IL-3 and G418. B, lanes 1–7 in each blot contain H/P(AGT497), H/P(AGT599), H/P(AGT752), H/P(AGT599), H/P(AGT690), H/P(AGT752), H/P(AGT690), H/P(N1), and insert-free MSCVneo cells, respectively. The first three cell lines were grown in the absence of IL-3 and presence of G418 (lanes 1–3). The last three cell lines (lanes 4–6) were grown in the presence of IL-3 and G418.

To probe the role of PtdIns 3-kinase and PLCγ in more detail, tyrosine phosphorylation of the 85-kDa subunit of PtdIns 3-kinase and PLCγ were measured in the H/P(F8)-transformed cells. It was hypothesized that by analogy to the native PDGFR (19), the H/P(F8) mutant would not bind PtdIns 3-kinase or PLCγ. Ba/F3 cell lysates were subjected to immunoprecipitation with anti-PtdIns 3-kinase and anti-PLCγ antibodies and then immunoblotted with antibody against phosphorylase or the immunoprecipitating antibody (Fig. 5, A and B). Cells expressing the wild-type H/P or the H/P(F2) mutant had increased phosphorylation of both proteins that were expressed at similar levels in each of these cell lines. As hypothesized, the H/P(F8) mutant, which has Tyr → Phe point mutations in all of the known SH2 docking sites has lost its ability to associate with and phosphorylate PtdIns 3-kinase and phospholipase Cγ. This finding together with the fact that the H/P(F8) variant with mutations in all known SH2 docking sites is transforming, suggests that signaling via the inositol phosphate-containing intermediates is unnecessary for Ba/F3 transformation.

Constitutive STAT5 Activation Is Associated with H/P Transformation—Since the mutant fusion protein H/P(F8) transforms Ba/F3 cells but lacks all of the known SH2 binding sites and is incapable of activating PtdIns 3-kinase or PLCγ (Table II and Figs. 4 and 5), we analyzed other signal transduction pathways that might be activated by the PDGFR portion of the fusion protein. In addition to PtdIns 3-kinase and PLCγ, STATs are reported to be activated by native PDGFR (23). Hence, STAT1, -3, and -5, which are highly expressed in Ba/F3 cells, were analyzed for activation in transformed cells.

Electromobility shift assay using a γ-interferon-activated sequence probe that binds to multiple activated STAT proteins demonstrated constitutive STAT5 activation in the transformed cells by both H/P and H/P(F8) (Fig. 6A). A shifted
A doublet was detected using nuclear extracts from the various stably infected cell lines. The upper band of the doublet was constitutively supershifted with STAT5 antibody that recognizes both STAT5α and STAT5β. No supershift was detected when STAT1 or STAT3 antibodies were added to the binding reaction (data not shown). STAT5 was constitutively tyrosine-phosphorylated in transformed cells, consistent with the electromobility shift assay results (Fig. 6B). These data demonstrate that although the transforming H/P(F8) mutant does not activate PtdIns 3-kinase or PLCγ, it is a potent activator of STAT5.

Finally, immunofluorescent staining with the anti-PDGFβR antibody of HIP1/PDGFβR transiently transfected COS-1 cells showed the fusion protein was localized to the cytoplasm (Fig. 7). Similar data were obtained for various HIP1 truncation mutants and in stably transfected Ba/F3 cells (data not shown).

In summary, the HIP1 protein oligomerizes, has constitutive kinase activity, transforms Ba/F3 cells to IL-3-independent growth, and is localized to the cytoplasm. The properties of oligomerization, constitutive kinase activity, and transformation do not require the leucine zipper motif as a deletion mutant of the leucine zipper retains these properties. In contrast, a mutant containing only the 55 C-terminal amino acids of HIP1 fused to PDGFβR kinase (HIP(N1)) retains only the oligomerization and constitutive tyrosine kinase activity. When these 55 amino acids are deleted (HIP(del)H) oligomerization is abrogated. Taken together, these data demonstrate that the 55 carboxyl-terminal amino acids of HIP1 are sufficient for oligomerization and autophosphorylation but not for transformation of hematopoietic cells.

In addition, transformation by HIP1/PDGFβR and related mutants correlates with constitutive STAT5 activation and tyrosine phosphorylation of p130. These three properties require HIP1 amino acids 690–752 but do not require any of the known SH2 signal transducing molecule docking sites in the PDGFβR portion of the fusion. This suggests that in addition to tyrosine kinase activation, HIP1 sequences that function in ways other than self-association may be relevant for transformation of Ba/F3 cells to IL-3-independent growth and that both p130 and the STAT5 are important targets of HIP1/PDGFβR in mediating transformation.

**DISCUSSION**

HIP1/PDGFβR is a novel fusion protein associated with CML and t(5;7)(q33;q11.2). HIP1/PDGFβR oligomerizes, and transforms Ba/F3 cells to IL-3-independent growth. The transformed cells have a hyperphosphorylated p130 protein and constitutively activate STAT5. The kinaseinactive mutant HIP(KI) does not transform cells. As expected in HIP(KI) infected cells, neither it, p130, nor STAT5 are constitutively tyrosine-phosphorylated.

The contribution of HIP1 sequences to transformation and
kinase activation of HIP1/PDGFβR is more complex. To assess the role of HIP1 in these functions, deletion mutants of HIP1 were prepared, including H/P(RI), H/P(RI)(del)LZ, H/P(NI), stepwise deletions between the EcoRI and the NheI sites of the H/P fusion cDNA, and H/P(del)H. It was expected that the leucine zipper motif would be required for oligomerization and kinase activation by analogy with other transforming fusion proteins (2, 4, 6–9). In each of these examples, kinase activation is mediated by an oligomerization motif contributed by the non-tyrosine kinase fusion partner and is requisite for kinase activation and transformation. In the case of TPR/MET, the oligomerization motif is, in fact, a leucine zipper domain (6). Hence, it was surprising that deletion of the leucine zipper motif in HIP1/PDGFβR had no effect on oligomerization, kinase activation or transformation.

The H/P(RI), H/P(RI)(del)LZ, H/P(NI), and H/P(del)H deletion mutants were then constructed to identify HIP1 sequences sufficient for oligomerization, tyrosine kinase activation, and transformation. The H/P(NI) mutant contains only the 55 carboxyl-terminal amino acids of HIP1, including the TALIN homology region. H/P(NI) oligomerized and had tyrosine kinase activity, whereas the H/P(del)H mutant, which lacks any HIP1 sequences, did not oligomerize. These findings demonstrate that the carboxyl-terminal 55-amino acid domain containing the TALIN homology region are sufficient for oligomerization and tyrosine kinase activation. Self-association is a function that, to our knowledge, has not previously been attributed to TALIN.

Surprisingly, we found that although H/P(NI) oligomerizes and has tyrosine kinase activity, it is incapable of transforming cells. In light of this, we made more refined HIP1 amino-terminal deletions between the EcoRI and the NheI sites. These data suggest a critical role in transformation and p130 tyrosine phosphorylation for HIP1 amino acids 690–752. Unfortunately, there is no amino acid sequence homology to known functional motifs in this amino acid 690–752 polypeptide to suggest a biological role.

There are several potential explanations for the lack of transformation but retention of constitutive tyrosine kinase activation by the H/P mutants that do not contain amino-terminal amino acids up to amino acid 752. One possible explanation for the lack of transformation by these H/P mutants is that there are subtle differences in tyrosine kinase specific activity that cannot be appreciated by assessment of autophosphorylation with anti-phosphotyrosine antibodies.

Another possibility is that there is an altered subcellular localization of the nontransforming HIP1 deletion mutants compared with HIP1/PDGFβR, leading to sequestration of the constitutive tyrosine kinase from substrates whose phosphorylation is necessary for transformation. HIP1/PDGFβR is predominantly localized to the cytoplasm of transiently transfected COS-1 cells (Fig. 7) and Ba/F3 cells (data not shown). Because Ba/F3 cells have scant cytoplasm, it is not possible to exclude association with the plasma membrane or cytoskeletal elements. Further analysis using confocal microscopy of hematopoietic cells stably transfected with HIP1/PDGFβR, the nontransforming mutants, and mutants containing myristoylation signals or nuclear localization signals may definitively characterize the role of subcellular localization in transformation of relevant cell types by the fusion protein.

**Fig. 6. STAT5 is activated in H/P-transformed cells.** A, electrophoreticity shift assays were performed on nuclear extracts of Ba/F3 cells infected with a variety of constructs that were either growing constitutively in the absence of IL-3 (lanes 1–3, 5, 7–10, 12, and 14) or in the presence of IL-3 (lanes 4, 6, 11, and 13). The MSCVneo and the H/P(NI) cell lines were “starved” of IL-3 for 6 h. Extracts were incubated with radiolabeled γ-interferon-activated sequence probe and separated on 6% PAGE. B, Ba/F3 cells were “starved” of IL-3 for 6 h (lane 1), maintained in 0.5 ng/ml IL-3 (lane 2), or constitutively grown in media lacking IL-3 (lanes 3–6). Cells were lysed and assayed for phosphorylation of STAT5 by immunoprecipitation of STAT5 and Western blot with 4G10 anti-phosphotyrosine antibodies (upper panel) or anti-STAT5 antibodies (lower panel).

**Fig. 7. Immunofluorescence localization of H/P.** COS-1 cells transfected with H/P were stained with antiserum to the tail of the PDGFβR (Pharmingen) and visualized with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (upper panel) or with the Hoechst 33258 nuclear stain (lower panel).
The third possibility is that sequences amino-terminal to the TALIN homology region could, in addition to relocation, mediate interaction with heterologous proteins involved in transformation. Candidates for the heterologous interaction include Huntingtin, HIP1 itself, and the unidentified 130-kDa tyrosine-phosphorylated protein found in transformed cells. Since most candidate proteins in the 110–140 kDa molecular mass range have been excluded, identification of the 130-kDa protein will probably require biochemical purification and sequence analysis.

Tyr to Phe mutational analysis of the PDGFβR portion of H/P demonstrates that PtdIns 3-kinase and PLCγ activation are dispensable for transformation in the Ba/F3 assay. These data contrast with the function of native receptor in HepG2 cells in which there is an absolute dependence on PLCγ and PtdIns 3-kinase activation for mitogenic signaling. STAT5 is activated by the native PDGFβR, is known to transmit mitogenic signals, and is activated by IL-3 stimulation of Ba/F3 cells. We have demonstrated that although H/P(F8) does not activate PLCγ or PtdIns 3-kinase, it retains the ability to activate STAT5 as assessed by Western blot analysis for phosphotyrosine and electromobility shift assay analysis. These data, together with the known activation of STAT5 in hematopoietic cells by BCR/ABL (22, 24, 25) and TEL/JAK (8, 14), suggest that STAT5 activation is a common pathway of transformation by tyrosine kinase-containing fusion proteins.

In summary, we have completed a detailed analysis of the transformation properties of H/P. HIP1 contributes at least two functional domains necessary for transformation: first, a critical oligomerization motif within the TALIN homology region required for kinase activation and transformation of hematopoietic cells and, second, a domain from amino acid 690 to 752 that is required for transformation and association and phosphorylation of p130. Furthermore, p130 phosphorylation and association correlates with transforming ability of H/P. PDGFβR contributes tyrosine kinase activity, which is necessary but not sufficient for transformation. Although PtdIns 3-kinase and PLCγ activation are dispensable for transformation, HIP shares a feature common to other tyrosine kinase fusion in its ability to activate STAT5. Identification of p130 and STAT5 target genes should provide further insight into the pathogenesis of CMML.

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