Both platelet-derived growth factor (PDGF) and interleukin-4 (IL-4) play major roles in cell proliferation, differentiation, chemotaxis, and other functional responses. Here, we demonstrate that Stat6, previously shown to be activated by only IL-4 and IL-3, becomes activated after PDGF stimulation of NIH 3T3 fibroblasts. PDGF BB, and to a lesser extent PDGF AA, rapidly induced DNA binding activity from NIH 3T3 cell lysates utilizing the immunoglobulin heavy chain germ line e promoter (Ie) that specifically binds to Stat6 in an electrophoretic mobility shift assay. DNA binding activity could be detected within 5 min and reached maximum levels at approximately 20 min in parental NIH 3T3 cells. An identical mobility shift and time course of PDGF-mediated Ie binding activity was more pronounced in lysates of NIH 3T3 transfectants overexpressing human Stat6 (NIH 3T3-Stat6). The observed radiolabeled Ie mobility shift was competed by unlabeled Ie as well as by the \( \beta \)-casein gene promoter but not by the interferon-\( \gamma \)-stimulated response element or the interferon-\( \gamma \) response region of the guanylate-binding protein gene. A Stat6-specific polyclonal antiserum also supershifted the PDGF-induced Ie mobility shift. After PDGF BB treatment, a 100-kDa tyrosine phosphorylated species was detected with anti-Stat6 immuneprecipitates. Cycloheximide had little effect on Stat6 tyrosine phosphorylation. In addition to Stat6, Stat5a, and Stat5b, PDGF BB also induced Jak1 tyrosine phosphorylation suggesting a potential pathway for Stat activation. Strikingly, the concurrent addition of IL-4 enhanced PDGF BB-induced Ie binding activity, Jak1 tyrosine phosphorylation, and [\( ^{3}H \)] thymidine incorporation. These results provide evidence that Stat6 and Jak1 are common elements in PDGF and IL-4 signaling pathways and suggest that IL-4 could play a role in potentiating certain known PDGF-induced biological responses.

Inflammation, immune responses, and wound repair involve the interplay of multiple growth factors and cytokines. Among these modulators and promoters of growth are platelet-derived growth factor (PDGF)\(^1\) and interleukin-4 (IL-4). PDGF is a major mitogen and chemotactic factor for mesenchymal cells such as fibroblasts and smooth muscle cells (1, 2). Although PDGF plays an important role in normal development, accumulating evidence suggests that its abnormal expression also contributes to a variety of diseases including cancer (1, 2). PDGF exists as disulfide-linked homo- or heterodimers composed of two polypeptide chains encoded by distinct genes, designated PDGF A and PDGF B. PDGF BB is the human homologue of the v-sis oncogene product (3–5). PDGF AA activates only the \( \alpha \) PDGF receptor, whereas PDGF BB activates both \( \alpha \) and \( \beta \) PDGF receptors.

IL-4 is a cytokine that plays an important role in the regulation of inflammatory and cell-mediated immune responses (6). T cells, mast cells, and basophils produce IL-4. IL-4 plays a major role in mediating differentiation effects of B cells. It causes transcription of unarranged constant regions for IgE and IgG\(_1\) leading to isotype class switching and subsequent synthesis of IgE (7) and IgG\(_1\) (8). IL-4 also induces or increases the expression of myosin heavy chain class I molecules (9) and CD23 (10) on B cells and the expression of its own receptor on lymphocytes (11). Recently, IL-4 has been shown to be the major regulator of the lymphokine-producing phenotype of CD4\(^+\) T lymphocytes (12). Evidence suggests that IL-4 modulates functional responses such as extracellular matrix production (13) and chemotaxis (14), rather than inducing pronounced proliferative effects in fibroblasts (13). IL-4 has also been demonstrated to regulate the morphology and cytoskeletal organization of human vascular endothelial cells (15). Some diseases such as scleroderma have been characterized by increased serum levels of IL-4 (16).

The IL-4 receptor was originally isolated as a single chain molecule with a large intracellular domain of over 500 amino acids (17–20). This receptor, commonly designated IL-4R\( \alpha \), possesses all the characteristics of the hematopoietin receptor superfamily. It has been demonstrated to become phosphorylated on tyrosine residue(s) upon IL-4 treatment (21). Unlike several members of the hematopoietin receptor family, IL-4R\( \alpha \) is ubiquitously expressed on cells of hematopoietic and nonhematopoietic origin. Recently, it has been shown that the IL-2\( \gamma \) chain, designated \( \gamma_c \), is a shared component in the IL-4 receptor complex (22, 23). IL-4 receptor activation has been demonstrated to result in tyrosine phosphorylation of Jak1, Jak3 (24, 25), IRS-1 (26), IRS-2/4PS (27), and Stat6, as well as IL-4 Stat, IL-4 NAF, or STI-IL-4 (28–31). Phosphorylation of specific tyrosine residues within the two GYKXF motifs present in IL-4R\( \alpha \) has been proposed to be crucial for binding to and activating Stat6 (28, 32).

The Stats (signal transducers and activators of transcrip-
tion) were recently identified as a novel family of transcription factors (33–35). Stat activation represents a novel signaling pathway that has been correlated with mitogenic and pleiotropic functional responses induced by a variety of growth factors, cytokines and interferons (33, 35, 36). Selective activation of a Stat, presumably through tyrosine and serine (37, 38) phosphorylation and dimerization, results in its translocation to the nucleus where it activates transcription (36). The Stats have been shown to bind with different affinities to a variety of DNA motifs related to interferon-γ-activated sites (GAS). Therefore, it is likely that these differences as well as differences in Stat expression determine the transcriptional sequence that is activated and subsequent proliferation or differentiation events that occur in a given cell in response to this activation (39). The role of Stats in signaling through noncytokine receptors such as the tyrosine kinase receptors remains to be determined. However, it has been demonstrated that epidermal growth factor activates Stats 1, 3, and 5 (40–43) and PDGF activates Stats 1 and 3 (44, 45). In this study, we demonstrate for the first time that a noncytokine growth factor, PDGF, readily activates Stat6. Furthermore, our results provide evidence that IL-4 can act to enhance PDGF-induced Ie binding activity, Jak1 activation, and fibroblast proliferation, suggesting that IL-4 might synergize with PDGF to enhance PDGF-mediated biological responses in vivo.

EXPERIMENTAL PROCEDURES

Materials—The sequence of one strand of the double-stranded 1e used for EMSA was 5'-CATCTAAGCTTCAGAAAGCCAGCC-3', 5'-GATCGACTTGTTTCATATTACTCTAAATCCA-3', 5'-GATCGACGGAAAGGGAAACCGAAACTGAA-GCC-3', 5'-GATCGACTTCTCTGGGAAT-3', 5'-GATCGACCTTCTGGGAAT-3', 5'-GATCGACCTTCTGGGAAT-3'. Sequences were 5'-GATCGACTTCTCTGGGAAT-3'. Anti-Stat6 serum used for supershift, immunoprecipitation, and immunoblot analysis was raised against amino acid residues 787–804 (NH2-GEDIFPPLLPTEDQTK-COOH). A second antiserum was used to confirm our immunoblot analysis was the kind gift of Dr. J. James Ihle and has been previously described (30). Anti-phosphotyrosine monoclonal antibody (mAb) and anti-Jak1 antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-Jak1 serum was used to confirm our immunoblot analysis was the kind gift of Dr. J. James Ihle and has been previously described (30). Anti-phosphotyrosine mAb was detected with anti-mouse anti-body conjugated to horseradish peroxidase (Amersham Corp.). Enhanced chemiluminescence (Amersham Corp.) was performed according to the manufacturer's protocol.

Mitogen Assay—[3H]Thymidine incorporation into NIH 3T3 cells was performed as described previously (26) with the following modifications. Briefly, quiescent NIH 3T3 cells in 96-well microtiter plates were treated with various concentrations of PDGF BB or AA in the presence or absence of 1 μM murine IL-4. Cells were incubated for 15 min with 500 μM of a 1:3 slurry of protein A-Sepharose CL4B was added for 1 h. Immunocomplexes were washed three times with RIPA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 100 μM dithiothreitol was added, and the samples were fractionated on 8% SDS-polyacrylamide gels. After electrophoretic transfer to Immobilon P membranes, filters were blocked in TTBS (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20), 3% nonfat milk. Membranes were then incubated with anti-Stat6 antisum (1:400) overnight in TTBS, 1% bovine serum albumin, and washed four times with TTBS. After incubation with 22176-μM protein A for 1 h, membranes were washed four times with TTBS, dried, and autoradiography performed.

RESULTS

PDGF AA and BB Induce Ie GAS Binding Activity—Both PDGF and IL-4 are involved in inflammation, immune response, growth promotion, and wound healing pathways. Using an antiserum raised against purified proteins that became phosphorylated on tyrosine residue(s) after PDGF stimulation of responsive cells, we cloned Stat6 from a protein expression library.3 Although Stat6 was previously shown to be activated only by the cytokines, IL-4 and IL-3 (30), this result implied that PDGF might utilize Stat6 in its signal transduction pathway. Thus, we tested whether PDGF activated Stat6 in PDGF-responsive NIH 3T3 fibroblasts.

Stat6 is readily distinguishable from the other Stats by its ability to bind a GAS-like element found in the Ig germ line gene (Ie). To determine whether PDGF BB induced Ie binding activity, cells were incubated for 15 min with 50 μM cyclic AMP (cAMP). Cells were washed and harvested, and [3H]Thymidine uptake was quantitated using a Beckman 5500 scintillation counter.

For EMSA, 5 μg of whole cell lysate was incubated with the 32P-Oligonucleotide (29–33) probe (29–33). The labeled probe was detected by autoradiography. The probe is 5’-GTATT-GATCGACTTGTTTCATATTACTCTAAATCCA-3’.

2 M. May and W. J. LaRochelle, unpublished observations.

3 M. May, J. H. Pierce, and W. J. LaRochelle, manuscript in preparation.

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activity, PDGF BB treatment led to rapid induction of Ie binding within 5 min (Fig. 1A). This activity reached a maximum between 10 and 20 min and diminished after 30 min. As shown in Fig. 1B, an identical time course was also observed for NIH 3T3 transfectants overexpressing the human Stat6 gene product (NIH 3T3-Stat6). Furthermore, the mobility of the PDGF BB-induced 32P-Ie binding activity from NIH 3T3-Stat6 transfectant lysates was identical to that of PDGF BB-stimulated parental NIH 3T3 cells. The amount of the DNA binding activity was much more pronounced in the transfectant than in the parental lines, further indicating that Stat6 was involved in this event.

PDGF AA also induced Ie binding activity in NIH 3T3 cells (Fig. 1C). The PDGF AA-induced mobility shift followed a similar initial time course to that of PDGF BB. However, the binding activity was reduced at least 10-fold and diminished much more rapidly relative to that of PDGF BB (Fig. 1C). PDGF AA also induced Ie binding activity to a greater degree in the NIH 3T3-Stat6 transfectants, consistent with its overexpression (Fig. 1D). The diminished 32P-Ie binding activity by PDGF AA compared with PDGF BB may be due to the inability of PDGF AA to activate the β PDGFR and/or the lower levels of α PDGFR compared with β PDGFRs in NIH 3T3 cells. The induction of the le mobility shift appeared to be specific for PDGF since epidermal growth factor was unable to induce a similar effect (data not shown).

PDGF-induced Ie Binding Activity Is Selectively Competed by Ie and βCAS but Not by ISRE or GRR Oligonucleotides—Stat6 in particular, is selective in its DNA binding capacity (28–31). Ramos cells, a human B cell line, treated with IL-4 readily induced Ie binding activity through Stat6 as observed by EMSA (Fig. 2A). IL-4-induced 32P-Ie binding was readily abrogated by incubation with a 100-fold excess of unlabeled Ie or the β-casein gene promoter (βCAS) but not by the interferon-α-stimulated response element (ISRE) or the interferon-γ response region (GRR) of the guanylate-binding protein gene. We next tested whether the PDGF-induced 32P-Ie binding could be competed with an excess of these different transcription factor binding elements. As shown in Fig. 2B, addition of excess unlabeled Ie or βCAS completely abolished PDGF BB-induced 32P-Ie binding activity. However, the induction of Ie DNA binding activity by PDGF BB treatment was not blocked by addition of excess oligonucleotides comprising ISRE and only marginally by GRR. An identical result was obtained using the NIH 3T3-Stat6 transfectant (Fig. 2C). Double-stranded oligonucleotides representing the GAS sites from the FcRI and FcεRIb genes, but not the Ly6, as previously reported (29, 31) blocked binding by greater than 80% (data not shown). The PDGF AA-induced 32P-Ie binding activity was also competed by Ie and βCAS but not by ISRE or GRR in NIH 3T3 and NIH 3T3-Stat6 transfectants (Fig. 2, D and E). We conclude that PDGF induced an identical 32P-Ie mobility shift and competition profile to that observed in IL-4-treated Ramos cells.

PDGF-induced Ie Binding Activity Is Supershifted by Anti-Stat6 Serum and Is Competed by Anti-phosphotyrosine—We tested our hypothesis that PDGF activated Stat6 by determining whether the PDGF-induced Ie DNA binding activity could be supershifted with an anti-peptide serum specific for Stat6. After treatment with PDGF BB, whole cell extracts from NIH 3T3-Stat6 transfectants were incubated with 32P-Ie followed by anti-Stat6 serum. As shown in Fig. 3A, the anti-Stat6 serum supershifted 32P-Ie, providing evidence that Stat6 was present in the DNA binding activity detected after PDGF stimulation of the transfectant. An identical result was obtained for PDGF AA-treated NIH 3T3-Stat6 transfectants (Fig. 3A). The mobility of the supershifted binding activity was also identical to that obtained with IL-4-treated Ramos cells (Fig. 3A). To confirm this result, we were also able to observe a PDGF-induced supershift of the Ie DNA binding activity with a second anti-peptide serum directed against a different Stat6 epitope but not with control antiserum (data not shown). We also supershifted 32P-Ie in extracts from the PDGF BB-treated parental NIH 3T3 cells (data not shown).

IL-4-induced Stat6 Ie binding activity has been shown to require tyrosine phosphorylation (28). We exploited the EMSA to determine whether the activation of Stat6 by PDGF BB, like that of IL-4, was associated with tyrosine phosphorylation. As shown in Fig. 3B, the PDGF BB-induced Ie DNA binding activity was competed by greater than 80% when incubated with anti-phosphotyrosine but not by a control monoclonal antibody. All of these results suggest that PDGF and IL-4 activate Stat6 32P-Ie binding activity in a similar manner.

Induction of Stat6 Tyrosine Phosphorylation by PDGF BB—As a complementary approach to analyze whether PDGF induced tyrosine phosphorylation of Stat6, whole cell lysates from untreated or PDGF-treated NIH 3T3-Stat6 transfectants...
were immunoprecipitated with the anti-phosphotyrosine, subjected to SDS-PAGE, and the separated proteins were subsequently immunoblotted with anti-Stat6 serum. As shown in Fig. 4A, a 100-kDa tyrosine-phosphorylated protein was readily observed in the PDGF BB-treated sample. No Stat6 was recovered in untreated NIH 3T3-Stat6 transfectants or control serum immunoprecipitates. In agreement with our EMSA results, detection of the 100-kDa tyrosine-phosphorylated protein reached a maximum between 10 and 20 min and diminished after 30 min.

To demonstrate that Stat6 activation was directly related to PDGF BB treatment, NIH 3T3-Stat6 transfectants were treated with cycloheximide followed by PDGF BB stimulation. As shown in Fig. 4B, the time course of Stat6 phosphorylation was not significantly affected by cycloheximide treatment. Thus, PDGF BB-mediated Stat6 phosphorylation appeared to be independent of protein synthesis, providing evidence that PDGF was not indirectly activating Stat6.

To confirm that Stat6 was directly phosphorylated on tyrosine in response to PDGF BB treatment, whole cell lysates from PDGF BB-stimulated NIH 3T3-Stat6 transfectants were immunoprecipitated with anti-Stat6 serum, subjected to SDS-PAGE, and the resolved proteins were immunoblotted with anti-phosphotyrosine. As shown in Fig. 5A, a 100-kDa tyrosine-phosphorylated protein was readily detected. Stat6 tyrosine phosphorylation was not detected in untreated NIH 3T3-Stat6 transfectants, even though the immunoprecipitates of PDGF BB-treated or untreated NIH 3T3-Stat6 transfectants contained similar levels of Stat6 protein (Fig. 5B). PDGF BB was also found to induce tyrosine phosphorylation of Stat5a and 5b (Fig. 5A and B). All of these results demonstrate the tyrosine-phosphorylated nature of PDGF BB-activated Stat6 as well as Stat5a and 5b.

PDGF BB Induction of Jak1 Tyrosine Phosphorylation—The
phosphorylation and activation of Stat6 has been associated with IL-4 activation of Jak1 and Jak3 (24, 25), the latter of which is thought to be predominantly expressed in hematopoietic cells (49). Our observation that Stat6 as well as other Stats were activated in response to PDGF BB stimulation suggested that Jak1 may be involved in these responses. Therefore, whole cell lysates from PDGF BB-stimulated NIH 3T3-Stat6 transfectants were immunoprecipitated with anti-phosphotyrosine mAb (anti-PY) and subjected to SDS-PAGE as indicated under "Experimental Procedures." Resolved proteins were transferred to Immobilon P (IP) membranes, immunoblotted with anti-Stat6 serum followed by 125I-protein A, and visualized by autoradiography. The position of Stat6 is indicated by the arrow, and the positions of the molecular mass markers are indicated in kilodaltons.

Fig. 4. PDGF BB-mediated tyrosine phosphorylation of Stat6 complexes and effect of cycloheximide treatment. NIH 3T3-Stat6 transfectants were treated with PDGF BB in the absence (panel A) or presence of cycloheximide (CHX, panel B) for 0, 5, 10, 20, 30, 45, or 60 min as indicated. Whole cell lysates were then immunoprecipitated with anti-phosphotyrosine mAb (anti-PY) and subjected to SDS-PAGE as indicated under “Experimental Procedures.” Resolved proteins were transferred to Immobilon P membranes, immunoblotted with anti-Stat6 serum followed by 125I-protein A, and visualized by autoradiography. The position of Stat6 is indicated by the arrow, and the positions of the molecular mass markers are indicated in kilodaltons.

Fig. 5. PDGF BB induction of Stat6 as well as Stat5a and 5b tyrosine phosphorylation. NIH 3T3-Stat6 transfectants were incubated in the absence or presence of PDGF BB for 20 min. Whole cell lysates were then immunoprecipitated with anti-Stat5a, Stat5b, or Stat6 sera and subjected to SDS-PAGE as indicated under “Experimental Procedures.” Resolved proteins were transferred to Immobilon P membranes, and replicas were immunoblotted with anti-phosphotyrosine mAb (anti-PY, panel A) or anti-Stat5a, Stat5b, or Stat6 sera as indicated (panel B). Bound primary antibody was detected by anti-mouse antibody or anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence (Amersham Corp.).

phosphorylation and activation of Stat6 has been associated with IL-4 activation of Jak1 and Jak3 (24, 25), the latter of which is thought to be predominantly expressed in hematopoietic cells (49). Our observation that Stat6 as well as other Stats were activated in response to PDGF BB stimulation suggested that Jak1 may be involved in these responses. Therefore, whole cell lysates from PDGF BB-stimulated NIH 3T3-Stat6 transfectants were immunoprecipitated with anti-Jak1 serum, subjected to SDS-PAGE, and the resolved proteins were immunoblotted with anti-Stat6 antibody. As shown in Fig. 6A, a 130-kDa tyrosine-phosphorylated species was readily detected in Jak1 immunoprecipitates. Jak1 tyrosine phosphorylation was not detected in untreated NIH 3T3-Stat6 transfectants, despite the fact that the immunoprecipitates of PDGF BB-treated or untreated NIH 3T3-Stat6 transfectants contained nearly identical amounts of Jak1 (Fig. 6B). We conclude that PDGF BB activation of Jak1 suggests a potential pathway for activation of Stat6 as well as Stat5a and Stat5b in NIH 3T3 fibroblasts.

IL-4 Enhances PDGF BB Induction of Ie Binding Activity and Jak1 Tyrosine Phosphorylation—While these results demonstrated that tyrosine-phosphorylated Stat6 and Jak1 were common elements in both IL-4 and PDGF BB signal transduction pathways, they did not address whether IL-4 and PDGF BB cooperated in the activation of either signaling molecule in NIH 3T3 fibroblasts. To analyze whether IL-4 affected PDGF BB-induced Ie binding activity, we stimulated NIH 3T3 cells transfected with an expression vector containing the murine IL-4Rα cDNA (NIH 3T3-IL-4R) with IL-4, PDGF BB, or both IL-4 and PDGF BB. Whole cell extracts were prepared and assayed for the induction of 32P-Ie DNA binding activity by EMSA. While extracts from untreated fibroblasts did not contain any Ie binding activity, PDGF BB treatment resulted in a 2-fold greater induction of Ie binding compared with IL-4 treatment alone (Fig. 7A). Strikingly, treatment of NIH 3T3-IL-4R transfectants with both PDGF BB and IL-4 led to almost a 4-fold increase in the induction of Ie binding above that of IL-4. Immunoprecipitation of Stat6 and immunoblotting with anti-phosphotyrosine also demonstrated an increase in Stat6 tyrosine phosphorylation (data not shown). Similar, albeit less pro-
PDGF Activation of Jak1, Stat5a, Stat5b, and Stat6

**Fig. 6.** PDGF BB induction of Jak1 tyrosine phosphorylation. NIH 3T3-Stat6 transfectants were incubated in the absence or presence of PDGF BB for 20 min. Whole cell lysates were then immunoprecipitated with anti-jak1 serum and subjected to SDS-PAGE as indicated under “Experimental Procedures.” Resolved proteins were transferred to Immobilon P membranes and immunoblotted with anti-phosphotyrosine mAb (anti-PY, panel A) or anti-Jak1 serum as indicated (panel B). Bound primary antibody was detected by anti-mouse antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence (Amersham Corp.). The position of Jak1 anti-rabbit antibody conjugated to horseradish peroxidase (anti-PY, panel A) or anti-Jak1 serum as indicated under “Experimental Procedures.” Resolved proteins were transferred to Immobilon P membranes and immunoblotted with anti-phosphotyrosine mAb (anti-PY, panel A) or anti-Jak1 serum as indicated (panel B). Bound primary antibody was detected by anti-mouse antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence (Amersham Corp.). The position of Jak1

**Fig. 7.** Effect of IL-4 on PDGF BB-induced \[^{32}P\]-Ie binding activity and Jak1 tyrosine phosphorylation. NIH 3T3-IL-4R transfectants (panel A, left) or NIH 3T3-IL-4R-Stat6 transfectants (panel A, right) were untreated or treated with 500 ng/ml IL-4, 100 ng/ml PDGF BB, or both 500 ng/ml IL-4 and 100 ng/ml PDGF BB for 20 min as indicated. Panel A, whole cell lysates from NIH 3T3-IL-4R were then incubated with \[^{32}P\]-Ie for 15 min and assayed by EMSA as described under “Experimental Procedures.” Gels were exposed for 3 h and visualized by autoradiography. Panel B, top, whole cell lysates from NIH 3T3-IL-4R-Stat6 transfectants were immunoprecipitated with anti-Jak1 serum as indicated under “Experimental Procedures.” Resolved proteins were transferred to Immobilon P membranes and immunoblotted with anti-phosphotyrosine mAb. Bound primary antibody was detected by anti-mouse antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence. Panel B, bottom; Jak1 immunoprecipitates from panel B, top, were probed with anti-Jak1 serum. Bound primary antibody was detected by anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence. A PDI 4200e scanning densitometer with QS30 software was used to quantitate the induction of Ie binding activity and Jak1 tyrosine phosphorylation. The positions of the induced Ie mobility shift and tyrosine phosphorylated Jak1 are indicated by open arrowheads.

**Fig. 8.** Effect of IL-4 on PDGF-induced mitogenesis in NIH 3T3 fibroblasts. NIH 3T3 cells were grown in serum-free medium for 24 h and then treated with either PDGF BB or PDGF AA at the indicated concentration in the presence (dark gray bars) or absence (light gray bars) of 1 μg/ml IL-4. In some instances, cells were treated with 1 μg/ml IL-4 alone. After 16 h, \[^{3}H\]Thymidine was added, and the cells were harvested after an additional 5-h incubation period as indicated under “Experimental Procedures.” \[^{3}H\]Thymidine uptake was measured using a Beckman 5500 scintillation counter. Each bar represents the average of three determinations from a representative experiment ± S.E.

**PDGF BB**

| Concentration (ng/ml) | Fold Increase in \[^{3}H\]Thymidine Uptake |
|----------------------|------------------------------------------|
| 0                    | 0                                       |
| 1                    | 2                                       |
| 2.5                  | 4                                       |
| 5                    | 6                                       |
| 10                   | 8                                       |
| 20                   | 10                                      |

**PDGF AA**

| Concentration (ng/ml) | Fold Increase in \[^{3}H\]Thymidine Uptake |
|----------------------|------------------------------------------|
| 0                    | 0                                       |
| 1                    | 2                                       |
| 2.5                  | 4                                       |
| 5                    | 6                                       |
| 10                   | 8                                       |
| 20                   | 10                                      |

In the parental NIH 3T3 cells, IL-4 induced Ie binding to approximately 5% of the PDGF BB levels and increased PDGF BB-induced Ie binding by approximately 50%.

To confirm these results we also determined whether NIH 3T3-IL-4R supertransfected with Stat6 demonstrated an analogous effect on PDGF BB-induced Ie binding activity, when stimulated with IL-4, PDGF BB, or both IL-4 and PDGF BB. Like the NIH 3T3-Stat6 transfectant (Fig. 1B), the NIH 3T3-IL-4R-Stat6 supertransfected exhibited dramatically increased Ie binding activity in response to PDGF BB or IL-4 when compared with the NIH 3T3-IL-4R transfectant (Fig. 7A). The Ie binding activity of NIH 3T3-IL-4R-Stat6 after PDGF BB treatment was also increased 5-fold beyond that of IL-4. The treatment of NIH 3T3-IL-4R-Stat6 transfectants with both PDGF BB and IL-4 led to at least a 7-fold increase in the induction of Ie binding activity compared with IL-4 alone. These results strongly suggest that PDGF BB and IL-4 together enhance Ie binding activity.

Our findings prompted us to examine whether IL-4 also affected PDGF BB-induced tyrosine phosphorylation of Jak1. NIH 3T3-IL-4R-Stat6 were stimulated with IL-4, PDGF BB, or both IL-4 and PDGF BB, whole cell extracts prepared, and Jak1 tyrosine phosphorylation assayed. As shown in Fig. 1B, Jak1 tyrosine phosphorylation was slightly greater in PDGF BB-treated than IL-4-treated cells. The combination of both PDGF BB and IL-4 revealed an enhanced tyrosine phosphorylation of Jak1, especially when the levels of recovered Jak1 were compared (Fig. 7B). Thus, PDGF BB and IL-4 appear to signal through Jak1 and Stat6 for activation of downstream effector molecules in NIH 3T3 fibroblasts.

IL-4 Enhances PDGF-induced \[^{3}H\]Thymidine Uptake in NIH 3T3 Fibroblasts—Our results predicted that PDGF and IL-4 share overlapping signal transduction pathways in NIH 3T3 fibroblasts. To further test whether a relationship existed be-
between these two factors and intracellular signaling, we utilized a fibroblast mitogenic assay. While PDGF is a potent mitogen for fibroblasts (1, 2), evidence suggests that IL-4 modulates functional responses of this cell type (13), rather than markedly inducing mitogenesis. Therefore, we examined whether IL-4 had potentiating or inhibitory effects, if any, on PDGF’s induc- tion of DNA synthesis in NIH 3T3 fibroblasts. As shown in Fig. 8, murine IL-4 (1 μg/ml) induced only a 2-fold increase in NIH 3T3 cell DNA synthesis. Concentrations as high as 2 μg/ml resulted in no greater increase in [3H]thymidine uptake (data not shown). PDGF BB (1 ng/ml) induced a 13-fold increase in [3H]thymidine uptake. Strikingly, the addition of IL-4 (1 μg/ml) more than doubled the PDGF BB-mediated mitogenic response. At higher PDGF BB concentrations (5 ng/ml), a 38-fold increase in [3H]thymidine uptake was observed. Addition of IL-4 to PDGF BB at this concentration increased the mitogenic response to 54-fold. Thus, the synergistic effect of IL-4, although observed at every PDGF BB concentration analyzed, was most pronounced at PDGF BB concentrations below those required for maximal induction of DNA synthesis in NIH 3T3 fibroblasts. Similarly, the induction of DNA synthesis by PDGF AA was also increased by IL-4 to levels similar to those observed with PDGF BB (Fig. 8). A dose-dependent effect of PDGF-induced [3H]thymidine uptake was observed using IL-4 in the range of 250 ng/ml to 1 μg/ml, while concentrations of IL-4 from 2 to 5 μg/ml did not show a synergistic effect greater than that observed at 1 μg/ml (data not shown). These results demonstrate that IL-4 enhances PDGF’s induction of DNA synthesis in NIH 3T3 fibroblasts.

The generation of NIH 3T3-Stat6 transfectants enabled us to ask whether IL-4 modulated PDGF BB-induced mitogenesis to a greater extent in cells overexpressing Stat6. As shown in Table 1, the enhancement of PDGF BB-induced [3H]thymidine uptake by stimulation with IL-4 was observed in both wild type NIH 3T3 and NIH 3T3-Stat6 transfectants. However, no significant increase in the synergistic effect of IL-4 on PDGF BB-induced mitogenesis was observed when parental NIH 3T3 were compared with NIH 3T3-Stat6 transfectants.

**DISCUSSION**

The Stats selectively bind to DNA transcriptional elements and activate the transcription of genes that are often associated with differentiation functions and phenotypic changes (33-35). It has recently been demonstrated that IL-4 activates Stat6 which induces transcription of unarranged constant regions for IgE (7) and IgG2 (8) in B cells, leading to immunoglobulin class switching and production of IgG2 and IgG2. Prior to this study, Stat6 activation was thought to be limited to IL-4 and IL-3 (30) and appeared to be more discriminating in its induction of DNA binding than many other Stat molecules which suggested more restricted functions (48).

In the present study, we demonstrated that PDGF is able to activate Stat6 in nonhematopoietic cells. However, we were unable to show a direct association of PDGFRs with Stat6, suggesting that an intermediate tyrosine kinase activated by the PDGFR may induce tyrosine phosphorylation of Stat6 in NIH 3T3 fibroblasts. Moreover, the PDGF Rs do not contain the GYKXXF motif thought to be utilized by the IL-4R for Stat6 binding (28, 32). We were able to demonstrate that the PDGF Rs activate Jak1 which is activated by the IL-4R as well. Additionally, we were able to demonstrate a direct association between Jak1 and PDGF Rs in Jak1 immunoprecipitates. Recent evidence suggests that none of the Jak5 appear to be individually required for PDGFR activation of Stat1 or Stat3 (50) and that Src kinases are also able to constitutively activate the DNA binding activity of Stat3 (51). c-Src has also been identified as a PDGFR substrate (52). Whether Jak1, c-Src, or another intracellular kinase (53) mediate PDGFR activation of Stat6 is currently under investigation.

We demonstrated that induction of Stat6 by binding activity, Jak1 tyrosine phosphorylation, and mitogenesis is enhanced when NIH 3T3 cells are treated with both IL-4 and PDGF. Whether these observed synergies are coincidental or directly related remains to be determined. However, the ability of IL-4 to synergize mitogenically with PDGF AA and BB was comparable, while PDGF BB induced a much more pronounced Stat6 binding activity than PDGF AA. Additionally, IL-4 activates Stat6 in NIH 3T3 fibroblasts but induces only a minor increase in [3H]thymidine uptake. Furthermore, PDGF IL-4 mitogenic synergy was not significantly increased in NIH 3T3-Stat6 transfectants compared with parental NIH 3T3, although these transfectants possessed a higher background as measured by [3H]thymidine incorporation. Whether this result reflects lack of Stat6 involvement in mitogenesis or saturation of Stat6 signaling pathways involved in mitogenesis remains to be resolved.

During the course of these studies, two publications have strongly implicated Stat6 in T lymphocyte proliferation (54, 55) as well as amplification of the proliferative response (54), while another concluded that proliferation was only partly affected (56). Taken together, these observations suggest that the activation of Stat6 may be necessary but not sufficient for mediating optimal mitogenesis (30). Certainly, other signaling molecules (37) are potential candidates for mediating the observed biological synergy. IL-4 has been demonstrated to induce potent tyrosine phosphorylation of IRS-1 and IRS-2 in hematopoietic cells, leading to the activation of phosphatidylinositol 3-kinase, GRB2, and several other SH2-containing molecules (27, 57). Our previous data also demonstrated that expression of either IRS-1 or IRS-2 in the IL-3-dependent hematopoietic 32D cell line was required for eliciting IL-4-induced mitogenesis (27). However, we observed extremely weak IL-4-mediated tyrosine phosphorylation of IRS-1 and no IRS-2 phosphorylation in NIH 3T3 cells, and PDGF did not stimulate any detectable IRS-1 or IRS-2 phosphorylation. Thus, our preliminary data suggest that IRS-1 and IRS-2 tyrosine phosphorylation do not contribute to PDGF-induced DNA synthesis or the observed IL-4-mediated synergy in fibroblasts.

In addition to Stat6, the PDGF-mediated signal transduction pathway has been linked to Stat1 and 3 activation. Stat1 is also activated by a variety of other cytokines and growth factors including interferon, IL-3, IL-5, IL-6, IL-10, growth hormone, epidermal growth factor, and GM-CSF, suggesting that Stat1 binds multiple transcriptional targets and results in a plethora of effects (reviewed in Refs. 34, 57, 58). Stat3 or SIF is activated by IL-2, IL-6, interferon-α, and G-CSF leading to transcription

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4 P. Kriebel and W. J. LaRochelle, unpublished observations.
5 B. K. R. Patel and W. J. LaRochelle, unpublished observations.
6 L. M. Wang and C. C. Lee, unpublished observations.

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**Table I**

| [PDGF] | NIH 3T3 | NIH 3T3-Stat6 |
|--------|---------|--------------|
| ng/ml  |         |              |
| 1      | 1.46 ± 0.03 | 1.63 ± 0.38  |
| 5      | 1.84 ± 0.25 | 1.63 ± 0.41  |
of a set of genes that include the serum response element of the c-fos promoter (57, 58). While Stat1 and -3 also appear to be activated in some breast cancer tissues (59), the role of the Stats in carcinogenesis is actively under investigation.

In summary, our results provide direct evidence that Stat6 and JAK1 activation in fibroblasts remains to be determined, IL-4 and PDGF both elevate the expression of extracellular matrix proteins including collagen (13, 60) and promote chemotaxis (14, 61). Moreover, the levels of IL-4 and PDGF are coincidently increased in the fibrotic disease scleroderma (16, 62). Eludication of the genes that are transcriptionally regulated in this context by Stat6 should reveal potential roles in the proliferation, differentiation, and functional responses in cells of fibroblast origin.

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