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Activation of Phospholipase C-γ1 through Transfected Platelet-Derived Growth Factor Receptor Enhances Interleukin 2 Production upon Antigen Stimulation in a T-Cell Line

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Phospholipase C-γ1 (PLCγ1) plays an important role in the signal transduction pathway by producing second messengers. However, the activation mechanism of PLCγ1 and the role of the phosphatidylinositol pathway for interleukin 2 (IL-2) production in T lymphocytes remain to be determined. To analyze the functional role of this pathway in T cells, we expressed an epidermal growth factor receptor (EGF) or platelet-derived growth factor (PDGF) receptor (EGF-R or PDGF-R), both of which are known to directly activate PLCγ1 in fibroblasts, into a murine T-cell hybridoma. Both receptors were expressed on the cell surface and caused tyrosine phosphorylation of multiple substrates, including the receptor itself, upon ligand binding. While EGF stimulation did not either cause phosphorylation of PLCγ1 or induce Ca^{2+} mobilization in the EGF-R transfectant in this system, PDGF treatment induced tyrosine phosphorylation of PLCγ1 and Ca^{2+} mobilization in the PDGF-R transfectant. Stimulation through PDGF-R enhanced IL-2 production upon antigen stimulation of the transfectants, although PDGF treatment alone did not induce IL-2 production. These results suggest that activation of the phosphatidylinositol pathway affects the downstream pathway to IL-2 production but is not sufficient to produce IL-2 and that cooperation with signals from tyrosine kinase cascades is required for IL-2 production.

Antigen (Ag) recognition by T lymphocytes through the T-cell receptor (TCR) complex initiates signal transduction cascades associated with the rapid activation of phospholipase C-γ (PLCγ). The activation of PLCγ results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield diacylglycerol, which activates protein kinase C, and inositol 1,4,5-triphosphate, which causes the subsequent elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). PLCγ has two different isoforms (γ1 and γ2), and T cells predominantly express the γ1 isoform (4). It has been found that several different inhibitors of tyrosine kinases (PTKs) block early signal transduction events, such as the activation of PLCγ1, inositol phosphate formation, and Ca^{2+} mobilization, as well as late events, including the expression of interleukin 2 (IL-2) receptor and IL-2 secretion and proliferation (7, 11, 25). These data suggest the importance of tyrosine kinases in T-cell activation. Although the TCR complex lacks intrinsic tyrosine kinase activity, it has been shown to associate with non-receptor-type PTKs belonging to the Src family. At present, $p56^{lck}$, $p59^{fyn}$, and $p70^{zap}$ are the most probable candidates to play important roles in TCR signaling (3, 13, 22). $p56^{lck}$ has been demonstrated to associate with CD4/CD8, and the latter two PTKs are considered to be directly associated with the CD3ζ chain. However, the precise mechanism by which signals are transmitted from the TCR complex to the receptor-coupled PTKs and the downstream signaling pathways is not fully understood.

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Moreover, the functional role of the phosphatidylinositol (PI) pathway in IL-2 production is still controversial. Sussman et al. (26) and O'Shea et al. (19) reported the dissociation between PI hydrolysis and IL-2 production, using spontaneous variants or v-*src* transfectants of T-cell hybridomas. In contrast, Desai et al. (6) demonstrated that PLC activation is sufficient to produce IL-2 by using a human muscarinic type 1 (HM1) receptor-transfected T-cell tumor. Since the mechanism of PLCγ1 activation in T cells is not known yet and no specific inhibitor of this enzyme is available, it is not possible to activate or block the PI pathway specifically. In contrast, the mechanism of signal transduction through the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (EGF-R and PDGF-R) has been extensively investigated (1, 8, 15–17, 27). Since these receptors are known to directly activate PLCγ1, it is feasible to activate PLCγ1 independently without TCR stimulation.

We transfected a human EGF-R or PDGF-R gene into a murine T-cell hybridoma and compared the signal transduction pathways through TCR, EGF-R, and PDGF-R. Although each ligand treatment caused tyrosine phosphorylation of multiple substrates, including the receptor itself, only the transfected PDGF-R caused tyrosine phosphorylation of PLCγ1. Moreover, PDGF induced Ca^{2+} mobilization and enhanced IL-2 production upon Ag stimulation. These results indicate that activation of PLCγ1 is important for Ca^{2+} mobilization and affect the downstream pathway to produce IL-2. However, since PDGF alone did not lead to IL-2 production, the activation of PLCγ1 and the PI pathway is not sufficient for IL-2 secretion. Cooperation with signals

from tyrosine kinase cascades is required for IL-2 production.

MATERIALS AND METHODS

Cell lines. 2B4, a cytochrome *c*-specific T-cell hybridoma (10), was maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg of kanamycin per ml, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), and 50 μM 2-mercaptoethanol (complete medium).

Reagents. A monoclonal antibody (MAb) to CD3ε (145-2C11) (14) was provided by J. A. Bluestone (Chicago, Ill.) and purified from ascites fluid on a protein A-Sepharose column (Pharmacia). An MAb to the TCRα chain of 2B4 (A2B4-2) (21) was a generous gift from L. E. Samelson (Bethesda, Md.) and used as the culture supernatant. MAbs to phosphotyrosine (4G10) (United Biotechnology Inc.), to human EGF-R (Takara), and to human PDGF-R β chain (PDGF-R-β) (Oncogene Science), a polyclonal rabbit Ab to PLCγ1 (United Biotechnology), a polyclonal goat anti-hamster Ab (GAH) (Organon Teknika-Cappel), and recombinant human EGF (Genzyme) were from commercial sources. Recombinant human PDGF-BB was kindly provided by C.-H. Heldin (Uppsala, Sweden). Recombinant human [^{125}I]EGF and [^{125}I]PDGF-BB were purchased from Amersham. Moth cytochrome *c* peptide analog DASP, a synthetic peptide (9), was kindly provided by D. Webb (Palo Alto, Calif.).

Flow cytometry. Cells (5×10^5) were stained with various Abs for 30 min at 4°C. After being washed three times with phosphate-buffered saline (PBS), the cells were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin Ab at 4°C for another 30 min. Finally, the cells were washed three times and suspended in 500 μl of PBS. Flow cytometry was performed on a FACScan (Becton Dickinson).

DNA transfection. The human EGF-R expression construct, pNeoSRα h-EGF-R was kindly provided by A. Miyajima (Palo Alto, Calif.). The human PDGF-R expression vector pMKIT-PDGF-R-β was constructed by ligating an *Eco*RI-*Sal*I fragment containing the PDGF-R-β cDNA from pSM1-SK-1 (kindly provided by C.-H. Heldin) into the *Eco*RI-*Xho*I site of pMKITneo (a generous gift from K. Maruyama, Tokyo, Japan). DNA-mediated gene transfer into 2B4 cells was performed by electroporation using a Gene Pulser (Bio-Rad). Briefly, 10^7 T cells in 0.8 ml of K-PBS (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 5 mM $MgCl_2$) were pulsed with 290 mV. Transfectants were selected 1 day after the transfection in the presence of medium containing 1.5 mg of Geneticin (GIBCO) per ml.

Binding assay for EGF or PDGF. Binding of [^{125}I]EGF to EGF-R-transfected cells and of [^{125}I]PDGF-BB to PDGF-R-transfected cells was evaluated by Scatchard plot analysis (23). Briefly, the cells (10^6/200 μl) were incubated on ice for 2 h with the appropriate ^{125}I-labeled growth factor (EGF or PDGF-BB) and unlabeled ligand at different concentrations. The cells were spun through a mixture of 80% silicone oil and 20% olive oil to separate receptor-bound ligand. The value of the radioactivity bound in the presence of an excess of unlabeled ligand was subtracted as nonspecific binding.

Measurement of Ca^{2+} flux. $[Ca^{2+}]_i$ was assessed by fluorimetry with Fura-2/AM (Dojin). Briefly, cells were incubated in complete medium (10^7 cells per ml) at 37°C for 30 min in the presence of 5 μM Fura-2/AM. Dye-loaded cells were washed and resuspended at a concentration of 5×10^6/ml in Ca^{2+} buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mM $NaHPO_4$, 5.5 mM glucose [pH 7.4]). Fluorescence intensity was measured with a fluorescence spectrophotometer (Hitachi model F-2000) with excitation at 340 and 380 nm and emission at 500 nm. After the baselines were stabilized, cells were stimulated by adding either EGF (100 ng/ml), PDGF (50 ng/ml), or 2C11 (100 μg/ml) followed by GAH (333 μg/ml).

Phosphorylation analysis. For TCR stimulation, T cells were incubated with 2C11 (10 μg/ml) for 30 min on ice, washed twice with RPMI 1640, and stimulated by adding prewarmed GAH (100 μg/ml) at 37°C for 2 min. For EGF or PDGF stimulation, cells were stimulated with EGF (100 ng/ml) or PDGF (50 ng/ml) at 37°C for 2 min. For costimulation with 2C11 and PDGF, cells were first incubated with 2C11 as described above and then stimulated with GAH and PDGF simultaneously. The reaction was stopped by adding ice-cold PBS, and cells were lysed for 30 min at 4°C in lysis buffer consisting of 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 4 mM EDTA, 0.15 U of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 10 mM sodium fluoride. The lysates were centrifuged for 15 min at $10^4 \times g$, and the supernatants were incubated with each Ab for 1 h at 4°C and then with protein A-Sepharose for 1 h. After three washes with lysis buffer, the immunoprecipitates were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then separated by SDS-PAGE (8% gel). The protein were transferred onto a polyvinylidene difluoride membrane (Millipore) by using a Sartoblot 2 apparatus (Sartorius). The blots were incubated with 4G10, washed, and further incubated with peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham). The blots were then developed by the enhanced chemiluminescence Western blotting (immunoblotting) detection system (Amersham).

IL-2 assay. IL-2 secretion was assessed as previously described (2). Briefly, 5×10^4 T cells were cultured with various concentrations of DASP and LK cells as Ag-presenting cells in the presence or absence of EGF (100 ng/ml) or PDGF (50 ng/ml) in a 96-well microtiter plate containing 200 μl of complete medium. After 20 h of incubation, IL-2 content in the culture supernatants was determined by measuring the incorporation of [^{3}H]thymidine (ICN Biomedicals Inc.) into an IL-2-dependent T-cell line, CTLL. One unit was defined as the amount of IL-2 providing the half-maximal response. All assays were performed in triplicate.

RESULTS

Expression of EGF-R or PDGF-R in a T-cell hybridoma. The expressible form of a human EGF-R or a human PDGF-R-β cDNA was transfected by electroporation into T-cell hybridoma 2B4, which does not express any endogenous EGF-R or PDGF-R. Geneticin-resistant clones were screened for cell surface expression of transfected receptors by flow cytometry. Several EGF-R- or PDGF-R-expressing transfectants were established and analyzed. One of each clone, BE1.19 for an EGF-R transfectant and BP3.1 for a PDGF-R transfectant, were chosen as the representative clones for detailed description, respectively. The transfectants expressed a high level of EGF-R or PDGF-R and a level of TCR equivalent to that of the parental 2B4 on the cell surface (Fig. 1). To determine the number and affinity of the

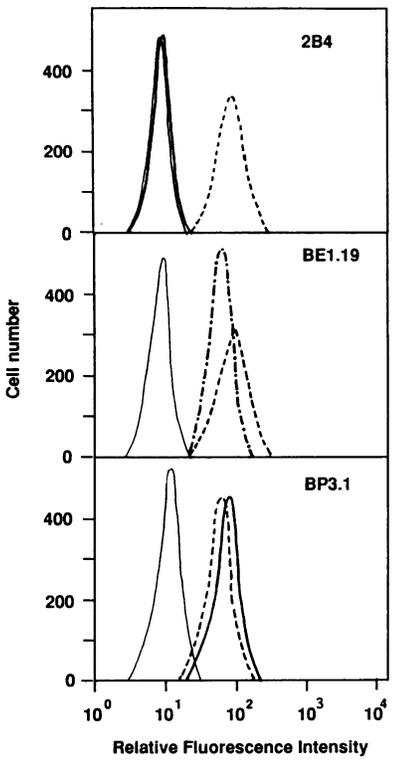

FIG. 1. Expression of the transfected human EGF-R or PDGF-R on the cell surface of transfectants. The parental T-cell hybridoma 2B4, EGF-R transfectant BE1.19, and PDGF-R transfectant BP3.1 were incubated with either control Ab (——), A2B4-2 (– – –), anti-EGF-R (— · — · —), or anti-PDGF-R (▬▬), followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin antibody, and analyzed by FACScan.

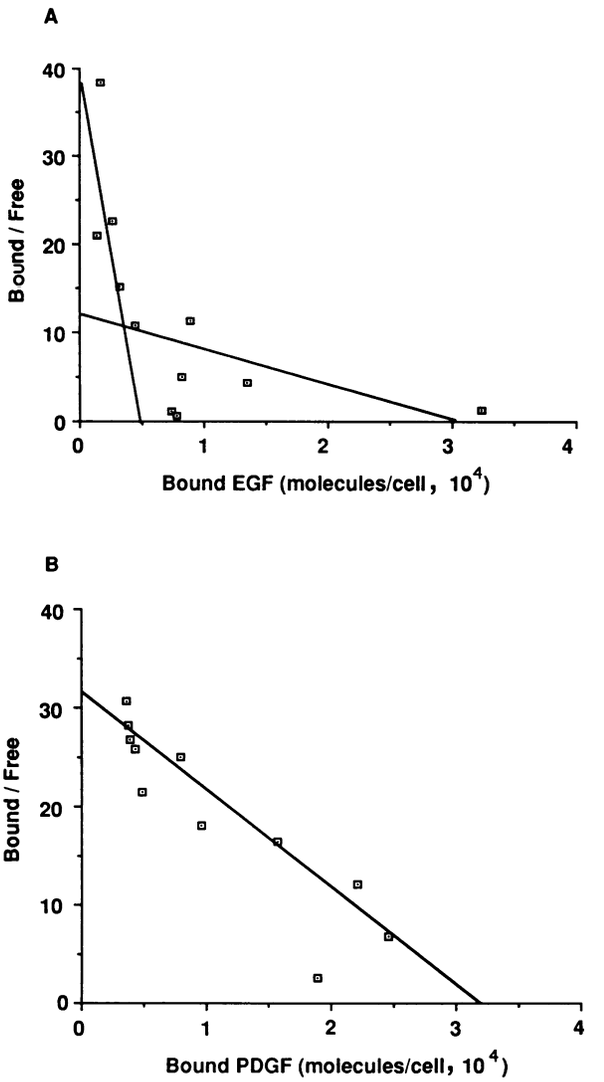

FIG. 2. Scatchard plot analysis of BE1.19 (A) and BP3.1 (B) transfectants. [^{125}I]EGF and [^{125}I]PDGF binding assays were performed on BE1.19 and BP3.1 cells, respectively, as described in Materials and Methods. Binding data were analyzed as described by Scatchard (23).

expressed receptor, Scatchard plot analyses were performed. The analysis of [^{125}I]EGF binding on BE1.19 cells showed that the transfectant expressed the high-affinity (5×10^3 per cell; dissociation constant [K_d], 132 pM) and low-affinity (3×10^4 per cell; K_d, 2.5 nM) receptors (Fig. 2A). The analysis of [^{125}I]PDGF-BB binding on BP3.1 cells demonstrated that PDGF-BB bound to the expressed receptors with a K_d of 0.97 nM and the number of binding sites was approximately 3.1×10^4 per cell (Fig. 2B).

Transfected EGF-R induced tyrosine phosphorylation without affecting TCR signaling. In initial experiments, we analyzed the function of expressed EGF-R on TCR signaling. While EGF stimulation induced tyrosine phosphorylation of multiple substrates, including autophosphorylation of EGF-R, we were not able to detect phosphorylation of PLCγ1 under the conditions used (see Fig. 4A, lane 2 and 3). However, only faint phosphorylation of PLCγ1 was detected after long exposure of the blot (data not shown). Subsequently, EGF stimulation did not either induce Ca^{2+} mobilization (Fig. 3a and b) or enhance IL-2 production in Ag-stimulated cells (see Fig. 5B). These results indicated that the transfected EGF-R was not sufficient to activate the PI pathway in T cells. Since PDGF-R has been shown to associate with PLCγ1 more tightly than EGF-R does, we next analyzed PDGF-R transfectants.

Ca^{2+} signaling in PDGF-R transfectants. To investigate whether the expressed PDGF-R was functional in the transfectants, we first analyzed the function for Ca^{2+} signaling. Whereas the parental 2B4 cells caused a substantial elevation of $[Ca^{2+}]_i$ in response to cross-linkage by 2C11 (Fig. 3c), they showed no increase upon PDGF treatment (Fig. 3d). In contrast, BP3.1 cells showed a significant elevation of $[Ca^{2+}]_i$ upon stimulation with PDGF (Fig. 3f) as well as with 2C11 (Fig. 3e), demonstrating that the transfected PDGF-R is functional for mediating signals for Ca^{2+} mobilization in T cells.

Since BP3.1 cells are also activated by TCR stimulation, we were interested in analyzing the relationship between the signals through PDGF-R and the TCR complex. It has been shown that prolonged exposure of many types of receptors to their cognate agonists can lead to a progressive lack of responsiveness. When this occurs after stimulation by the primary agonists for a given receptor, it is termed homologous desensitization; it is called heterologous desensitization when an agonist binds to a different type of receptor (18, 20). To test whether heterologous desensitization occurs be-

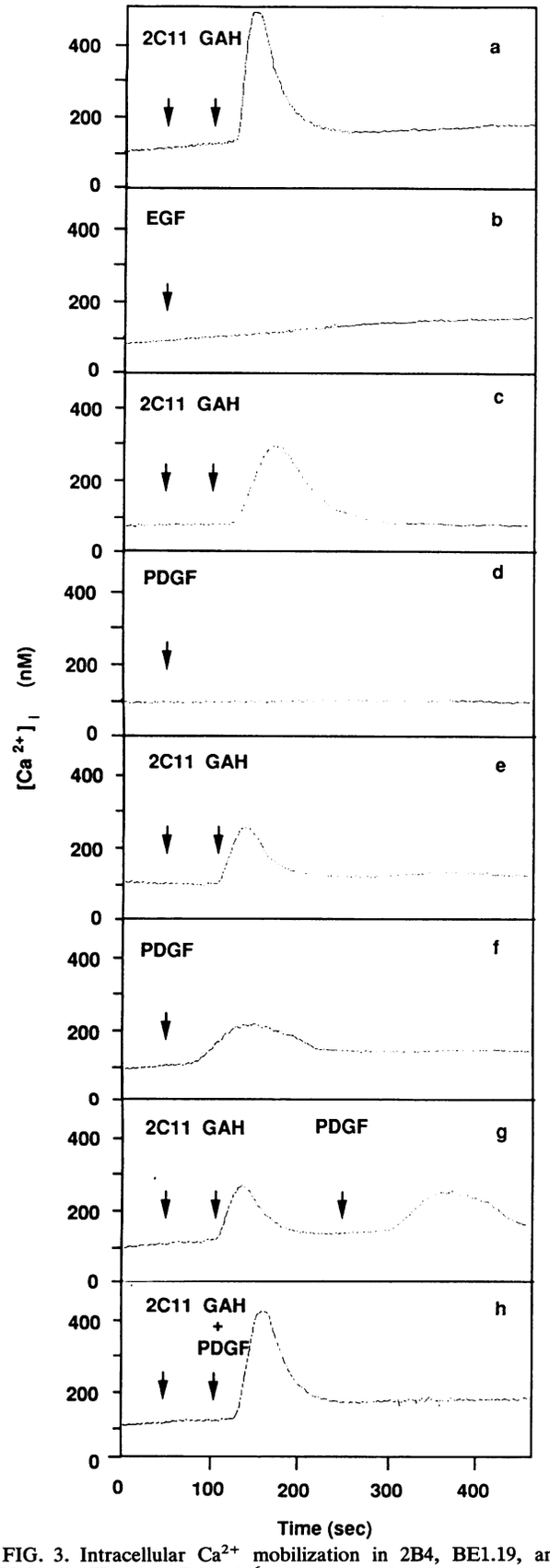

FIG. 3. Intracellular Ca^{2+} mobilization in 2B4, BE1.19, and BP3.1 cells. The cells (7.5×10^6 cells per sample) were loaded with Fura-2/AM (5 μM) and stimulated with either 2C11 (100 μg/ml) followed by GAH (333 μg/ml), EGF (100 ng/ml), or PDGF (50 ng/ml). Addition of the stimulant is indicated by arrows. Changes in Fura-2/AM fluorescence were measured, and $[Ca^{2+}]_i$ was calculated as described in Materials and Methods. (a and b) BE1.19 cells; (c and d) parental 2B4 cells; (e to h) BP3.1 cells.

tween TCR and PDGF-R, cells were stimulated sequentially with 2C11 and PDGF. After the first stimulation with either PDGF or TCR cross-linkage, subsequent activation with the other stimulant (2C11 or PDGF, respectively) independently induced elevation of $[Ca^{2+}]_i$ (Fig. 3g). The first stimulation was not either synergistic or inhibitory for the second stimulation. These results showed that Ca^{2+} signaling upon stimulation through TCR or PDGF-R did not have a mutually desensitizing effect. The independent signaling was confirmed by the observation that the costimulation with 2C11 and PDGF showed a synergistic effect on the Ca^{2+} response (Fig. 3h).

Phosphorylation in the PDGF-R transfectant. Upon ligand binding, PDGF-R is known to induce tyrosine phosphorylation of multiple substrates. We compared the pattern of tyrosine-phosphorylated proteins upon stimulation through either TCR or PDGF-R. PDGF treatment resulted in extensive phosphorylation of a protein of 180 kDa (p180), which was confirmed to be PDGF-R itself by immunoprecipitation with an anti-PDGF-R Ab (Fig. 4B, lane 11). Since the cells were not starved for serum prior to stimulation in this experiment, p180 was slightly phosphorylated even in unstimulated cells (Fig. 4B, lane 5). Comparing the patterns of tyrosine-phosphorylated proteins upon stimulation through TCR and PDGF-R, we found that some phosphoproteins (p180, p150-120, p100, p90, p85, and p80) were shared in TCR- and PDGF-stimulated cells but others were not. Whereas p75 and p38 were unique to PDGF stimulation, p70 and p40 were specifically phosphorylated upon 2C11 stimulation (Fig. 4B, lanes 7 and 9).

PDGF induced tyrosine phosphorylation of PLCγ1 in the PDGF-R transfectant. While several tyrosine-phosphorylated proteins were observed in unstimulated cells, PLCγ1 was phosphorylated only upon stimulation (Fig. 4B, lane 6). In the PDGF-treated cells, the anti-PLCγ1 Ab precipitated strongly phosphorylated proteins (p180 and p145). Judging from their molecular weights, p180 and p145 are likely to be PDGF-R and PLCγ1, respectively. This result indicated that the transfected PDGF-R was functional in terms of the induction of tyrosine phosphorylation of PLCγ1 and that PDGF-R formed a complex with phosphorylated PLCγ1 in T cells, as reported for fibroblasts (17). Comparing the intensity of the phosphorylation of PLCγ1, we found that PDGF treatment induced stronger tyrosine phosphorylation than 2C11 stimulation did. This finding suggests that PLCγ1 is a more susceptible substrate for PDGF-R than the TCR-associated kinases are. Upon stimulation with 2C11, the anti-PLCγ1 Ab precipitated not only PLCγ1 (p145) but also several tyrosine-phosphorylated proteins (p180, p120, and p40) (Fig. 4B, lane 8), suggesting that PLCγ1 associates with several molecules to make complexes in TCR-stimulated cells. These phosphoproteins (p180, p120, and p40) were also coprecipitated with PLCγ1 in the parental 2B4 cells (Fig. 4B, lane 13). Since 2B4 cells do not express PDGF-R, p180 in 2B4 cells is not the phosphorylated form of PDGF-R itself.

Signaling through PDGF-R enhanced Ag-induced IL-2 production. Since PDGF-R transfectants induced early activa-

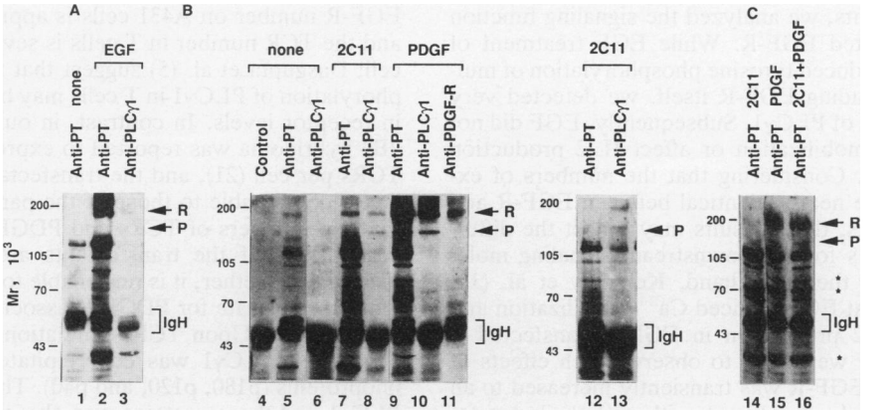

FIG. 4. Tyrosine phosphorylation upon stimulation through TCR, EGF-R, or PDGF-R in BE1.19, BP3.1, and 2B4 cells. A total of 3×10^7 T cells (BE1.19 [lanes 1 to 3], BP3.1 [lanes 4 to 11 and 14 to 16], and 2B4 [lanes 12 and 13]) were stimulated with EGF (100 ng/ml; lanes 2 and 3), with 2C11 (10 μg/ml) followed by GAH (100 μg/ml; lanes 7, 8, and 12 to 14), or with PDGF (50 ng/ml; lanes 9 to 11 and 15) at 37°C for 2 min. For costimulation, cells were stimulated with 2C11 followed by GAH and PDGF (lane 16). The cell lysates were immunoprecipitated with 4G10 (lanes 1, 2, 5, 7, 9, 12, and 14 to 16), anti-PLCγ1 (lanes 3, 6, 8, 10, and 13), anti-PDGF-R (lane 11), or control Ab (lane 4) and then separated by SDS-PAGE (8% polyacrylamide gel), transferred onto polyvinylidene difluoride membranes, and blotted with 4G10. The positions of EGF-R or PDGF-R (R) and PLCγ1 (P) are indicated by arrows. IgH designates the heavy chains of the immunoglobulin used for immunoprecipitation. Summed phosphorylated bands are indicated by the asterisks in the costimulation experiment. Molecular weights of protein standards are indicated at the left.

tion signals such as Ca^{2+} mobilization and tyrosine phosphorylation of multiple substrates, including PLCγ1, upon PDGF stimulation, we next investigated the effect of PDGF stimulation on IL-2 production as one of the representative late events after T-cell stimulation. Whereas PDGF alone did not induce IL-2 production in BP3.1 cells, PDGF significantly enhanced IL-2 production induced by Ag stimulation (Fig. 5A). The augmentation was evident from the shift of the dose-response curve and also the increase of IL-2 production. This augmentation was confirmed by analyses of other PDGF-R transfectants (Fig. 5B), while the effect was not observed in the parental 2B4 cells upon PDGF stimulation (Fig. 5A) or in BE1.19 cells upon EGF stimulation (Fig. 5B). We also confirmed that the augmenting effect of PDGF was not due to the up-regulation of the TCR level on the cell surface of the transfectants (data not shown). We noticed that PDGF-R transfectants were low producers of IL-2 compared with parental 2B4 and EGF-R transfectants. This tendency was observed in independently established transfectants, although the reason is unknown.

To investigate the mechanism of this augmentation, we compared the patterns of phosphorylated proteins upon stimulation through only TCR versus simultaneous stimulation through TCR plus PDGF-R. Costimulation with PDGF and TCR cross-linkage resulted in the sum of both phosphorylated patterns, which were observed with either TCR cross-linkage alone or PDGF treatment alone (Fig. 4C). Such an additive effect may contribute to the augmentation of IL-2 production and also the enhancement of the Ca^{2+} response.

DISCUSSION

We transfected a human EGF-R or PDGF-R into a murine T-cell hybridoma and investigated the functional roles of PLCγ1 and the PI pathway in T-lymphocyte activation. The transfected PDGF-R was shown to be functional in T cells by means of PDGF binding, the induction of phosphorylation of multiple substrates, including PLCγ1, intracellular Ca^{2+} mobilization, and the augmentation of Ag-induced IL-2 production.

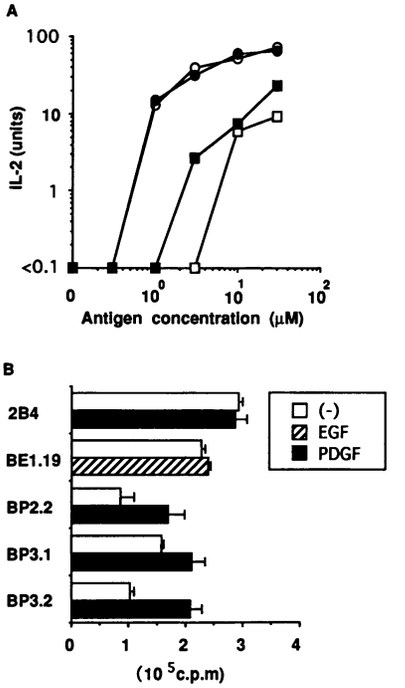

FIG. 5. (A) Augmentation of IL-2 production upon Ag stimulation by PDGF. 2B4 (circles) or BP3.1 (squares) cells were stimulated with various concentrations of Ag peptide (DASP) in the presence (closed symbols) or absence (open symbols) of PDGF (50 ng/ml). After 20 h, supernatants were assayed for IL-2 as described in Materials and Methods. (B) Effect of EGF or PDGF on IL-2 production of various transfectants. IL-2 production is presented in terms of [^{3}H]thymidine uptake as described in Materials and Methods. Experiments were performed with various concentrations of Ag in the presence of EGF or PDGF or the absence of ligands, as indicated, and representative results are shown at 10 μM Ag. Results are presented as means ± standard errors of the means of triplicate determinations.

In initial experiments, we analyzed the signaling function through the transfected EGF-R. While EGF treatment of these transfectants induced tyrosine phosphorylation of multiple substrates, including EGF-R itself, we detected very little phosphorylation of PLCγ1. Subsequently, EGF did not either induce Ca^{2+} mobilization or affect IL-2 production upon Ag stimulation. Considering that the numbers of expressed receptors are nearly identical between EGF-R and PDGF-R transfectants, these results may reflect the difference in their affinities for the downstream signaling molecules in T cells. On the other hand, Kennedy et al. (12) recently observed that EGF induced Ca^{2+} mobilization and enhancement of IL-2 production in EGF-R-transfected T cells. However, they were able to observe such effects of EGF only when the EGF-R was transiently increased to an extremely high level by treatment with sodium butyrate. These data together with our results that the effect was observed only on PDGF-R transfectants, not on EGF-R transfectants, suggest that an extremely high expression of EGF-R is required to induce activation signals in T cells. It is likely that the PDGF-R couples with downstream molecules with higher affinity than EGF-R in T cells as previously shown for fibroblasts (17). Considering that whereas EGF-R neither induced significant phosphorylation of PLCγ1 nor augmented IL-2 production in our system, PDGF-R did both, activation of the PI pathway is important for downstream signaling to IL-2 production.

A previous study by Desai et al. (6) analyzed the role of activation of the PI pathway in T cells by transfecting a G-protein-dependent HM1 receptor into Jurkat cells. They concluded that PLC activation alone is sufficient to produce IL-2. Contrary to that result, our data demonstrated that activation of the PI pathway through tyrosine phosphorylation of PLCγ1 and Ca^{2+} mobilization is not sufficient to result in IL-2 production. It appeared that cooperative signals through both the PI pathway and other downstream pathways from tyrosine kinases are required for IL-2 production under physiological conditions. Indeed, stimulation through the HM1 receptor also induced tyrosine phosphorylation of a 42-kDa molecule (p42). We noticed that p40 was one of the major tyrosine-phosphorylated proteins in our system and was detected only with TCR stimulation, not with PDGF stimulation. Considering the fact that these two molecules are nearly identical in molecular weight, p40 (or p42) could be a critical tyrosine-phosphorylated substrate for mediating signals to produce IL-2.

On the other hand, Sussman et al. (26) reported that IL-2 responses were not impaired despite the lack of PI hydrolysis and Ca^{2+} mobilization in spontaneous variants of the T-cell hybridoma. Similarly, analysis of the v-*src*-transfected T-cell hybridoma showed that elevated tyrosine phosphorylation induced constitutive IL-2 production without affecting the level of PI hydrolysis. It is possible that in these T-cell variants or v-*src*-transfected hybridomas, signaling molecules downstream of the PI pathway are constitutively activated without PI hydrolysis and that the signal from tyrosine kinase pathways is sufficient for IL-2 production. This speculation was supported by the observation that v-*src* expression increased diacylglycerol levels and subsequently activated protein kinase C via phospholipase D without PI hydrolysis (24).

Tyrosine phosphorylation of PLCγ1 was weaker upon TCR stimulation than upon PDGF stimulation. Until recently, the detection of tyrosine phosphorylation of PLCγ1 has been more difficult in T cells than in other tumor cells, such as A431 human epidermoid carcinoma cells. As the EGF-R number on A431 cells is approximately 10^6 per cell and the TCR number in T cells is several ten thousands per cell, Dasgupta et al. (5) suggest that the weakness of phosphorylation of PLCγ1 in T cells may be due to the difference in receptor levels. In contrast, in our system, the parental 2B4 hybridoma was reported to express 2×10^4 to 4×10^4 TCRs per cell (21), and the transfectants expressed TCR at levels comparable to those of the parental cells, suggesting that the numbers of TCRs and PDGF-Rs expressed on the cell surface of the transfectants are comparable. Taking these data together, it is reasonable to assume that PLCγ1 is a better substrate for PDGF-R-associated than TCR-associated kinases. Upon TCR stimulation, we found that phosphorylated PLCγ1 was coprecipitated with several phosphoproteins (p180, p120, and p40). The association between PLCγ1 and these proteins was observed only in stimulated cells. Although we cannot exclude the possibility that these molecules are antigenically related to PLCγ1, identification of these PLCγ1-associated molecules will be important to elucidate the activation mechanism of PLCγ1 in T cells and further downstream signaling for mediating the effector functions of T cells.

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