Evidence for a p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2-independent Pathway in Stimulation of IL-2 Gene Transcription in Human Primary T Lymphocytes*  

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T cell stimulation leads to triggering of signals transmitted from the cell membrane to the nucleus through TCR/CD3 proteins. Characterization of these signals largely results from the use of cell lines stimulated with anti-CD3 monoclonal antibodies. These studies have established that activation caused a rapid increase in the formation of GTP-bound Ras, which stimulates the mitogen-activated protein kinase pathway involving the extracellular-regulated kinase-2 (ERK-2) and activates the nuclear factor of activated T cells (NF-AT) that regulates interleukin-2 (IL-2) gene transcription. In the present study, we used human primary T cells, and we investigated the intracellular signals triggered by two different anti-CD3 monoclonal antibodies (UCHT1 and X-35), which both strongly induce cell proliferation. We found that, in contrast to the commonly used UCHT1, X-35 activated IL-2 gene transcription without stimulation of the Raf-1/mitogen-activated ERK kinase-1 (MEK-1)/ERK-2 phosphorylation cascade; we also showed that X-35 stimulation, which triggers an ERK-2-independent pathway, does not involve activation of p21\textsuperscript{ras}. In addition to demonstrating that activation of p21\textsuperscript{ras} and of its Raf-1/MEK-1/ERK-2 effector pathway is not an event obligatorily triggered upon TCR/CD3 ligation, these results provide the first evidence of the existence of a p21\textsuperscript{ras}/ERK-2-independent pathway for IL-2 gene transcription in human primary T lymphocytes.  

Binding of monoclonal antibodies to the CD3 complex has been used as model system that mimics antigen recognition to characterize the biochemical events leading to interleukin-2 production and T cell proliferation. Several studies have brought evidence that the intracellular signals that mediate activation of transcription factors regulating IL-2 gene transcription in human T cells involve p21\textsuperscript{ras}-mediated signaling pathways (1–5). These studies obtained with T cell lines collectively suggest that the Raf-1/MEK-1/ERK-2 phosphorylation cascade is the necessary (6–8) (if not sufficient (9)) p21\textsuperscript{ras} effector pathway for nuclear factor of activated T cell (NF-AT) induction in human T cells. These conclusions, which mainly result from the expression of dominant negative or constitutively active p21\textsuperscript{ras} (4–6), Raf-1 (10), or MEK-1 (4, 9, 11) mutants in Jurkat cells, have created a paradigm that p21\textsuperscript{ras}/ERK-2 pathway is the major route for activation of IL-2 gene transcription in TCR/CD3 induced activation of T lymphocytes. However, it cannot be excluded that an ERK-2-independent pathway might be used in primary T cells. Indeed a result obtained with splenocytes from transgenic mice expressing an inactive form of MEK-1 (12) suggested the possibility of the existence of a TCR/CD3-induced MEK-1/ERK-2-independent pathway even though one can question whether these cells, which developed in the absence of positive selection, are representative of a normal T lymphocyte population. Therefore, a clear physiological involvement of the MEK/ERK cascade in T cell activation is still a matter of debate, in part due to the fact that molecular genetic approaches are limited to cell lines or transgenic animals. Our aim was to study whether the stimulatory signals from the TCR/CD3 complex that promote IL-2 gene transcription obligatorily involve the p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2 pathway in primary T cells. We used highly purified CD4\textsuperscript{+} human lymphocytes that we stimulated with UCHT1 or X-35, two mitogenic anti-CD3 mAb (13, 14) recognizing the \(\epsilon\) chain of the CD3 complex (15) but presenting a pan thymocyte reactivity and a specific medullary thymocyte reactivity, respectively (14). We analyzed the effect of these antibodies on the Raf-1/MEK-1/ERK-2 phosphorylation cascade. We found that, in contrast to what happens with UCHT1, activation of IL-2 gene transcription triggered upon X-35 ligation occurred without activation of the Raf-1/MEK-1/ERK-2 pathway; moreover, we observed that this ERK-2-independent pathway does not involve activation of p21\textsuperscript{ras}. Altogether, the results we present herein demonstrate that activation of p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an obligatory event triggered upon TCR/CD3 ligation; moreover, they bring evidence that activation of this cascade is not essential for IL-2 gene transcription in human T lymphocytes.  

MATERIALS AND METHODS  
Chemicals and Reagents  
UCHT1 (IgG1) and X-35 (IgG2a) anti-CD3 mAb were from Immunotech (Marseille, France), mouse anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology Inc. (Lake Placid, New York); rabbit anti-ERK-2 Ab, rabbit anti-Raf-1 Ab, and rabbit anti-ZAP-70 Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated rabbit anti-mouse and donkey anti-rabbit were from Amer sham Pharmacia Biotech. Rabbit anti-phosphoserine 473 PKB and...
Peripheral monocyte cells were isolated from peripheral blood of healthy donors. Monocytes were removed by plastic adherence, and CD4+ T cells were purified (>99% pure) by positive immunoselection using magnetic beads coated with anti-CD4 mAb (Dynal International, Oslo, Norway) according to the manufacturer's instructions. Before being used, CD4+ purified T cells were left 15 to 18 h in RPMI 1640 supplemented with 10% fetal calf serum and gentamicin at 37 °C in a 5% CO2-humidified atmosphere. CD4+ cells were stimulated (72 h) either with soluble anti-CD3 mAb or with anti-CD3 coated on anti-IgG-conjugated beads. Proliferation was estimated by a 4-h [3H]thymidine incorporation.

**Analysis of IL-2 mRNA Expression by Reverse Transcription-Polymerase Chain Reaction**

CD4+ T cells were stimulated for 6 h in the presence of 1 μg ionomycin and anti-CD3 mAb (1 μg/ml). The cells were treated with PD098059 (30 μM) as described. Total RNA isolation, reverse transcription reaction, and polymerase chain reaction were performed as already described (16).

**Analysis of MAP Kinase Activation**

**Analysis of MAP Kinase Phosphorylation**—This analysis was performed as described (16). Briefly, CD4+ cells (5 × 10^6/ml) were stimulated with anti-CD3 mAb (10 μg/ml) or phorbol esters (PMA or phorbol dibutyrate (PDBu)). The supernatants were reacted in a 12.5% SDS-PAGE, and the gel was transferred onto a PVDF membrane (polyvinylidene difluoride, NEN Life Science Products). After blocking of nonspecific binding, the membrane was probed with anti-ERK-2 Ab (0.2 μg/ml) and revealed with horseradish peroxidase-conjugated-anti-rabbit Ab and the chemiluminescence detection system. Reprobing of the same blots with the anti-phospho-Thr202/Tyr204 MAPK (ERK) Ab (1:1000) was performed after stripping of bound Ab. The membrane was revealed with 1:10,000 solution of horseradish peroxidase-conjugated anti-mouse Ab and the chemiluminescence detection system (Bio-Rad). The membranes were washed, and the supernatants were resolved in a 12.5% SDS-PAGE gel, and the gel was transferred onto a PVDF membrane. After blocking of nonspecific binding, the membrane was probed with a rabbit anti-phospho-ephrin 473 Ab (1:1000) and revealed with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) followed by enhanced chemiluminescence detection. Reprobing of the same blots with the anti-PKB Ab (1:1000) was performed after stripping of bound Ab. The membrane was revealed with 1:2000 solution of horseradish peroxidase-conjugated anti-mouse Ab and the chemiluminescence detection system. Reprobing of the same blots with the anti-PKB Ab (1:1000) was performed after stripping of bound Ab. The membrane was revealed with 1:2000 solution of horseradish peroxidase-conjugated anti-rabbit Ab and the chemiluminescence detection system.

**Analysis of Tyrosine Phosphorylation**

Cells were stimulated and lysed as described for MAP kinase phosphorylation studies. The proteins were resolved on 10% SDS-PAGE, and the gel was transferred onto a PVDF membrane. After blocking of nonspecific binding, the membrane was probed with the anti-phosphotyrosine 4G10 Ab (1 μg/ml) and revealed with a 1:10,000 solution of horseradish peroxidase-conjugated anti-mouse Ab followed by enhanced chemiluminescence detection system.

**Results**

**Analysis of MAP Kinase Pathway Activation in CD3-stimulated Cells**—We first showed that highly purified CD4+ T cells that do not respond to soluble anti-CD3 mAb are activated by and proliferate in response to both X-35 and UCHT1 when coated on beads (Table I).

- We then analyzed phosphorylation (appearance on electrophoresis) of a slow migrating band (20, 21) and activation of ERK-2. Fig. 1A shows that a shifted band, not present after 1-min stimulation, is clearly detected by anti-ERK-2 Ab after a 5-min

| Stimulation | NS | PHA | Soluble X-35 | Conjugated X-35 | Conjugated UCHT1 |
|-------------|----|-----|--------------|-----------------|-----------------|
| **[3H]Thymidine incorporation** | 160 ± 25 | 59891 ± 3344 | 161 ± 19 | 157 ± 38 | 21810 ± 2080 | 13261 ± 2029 |
activation with UCHT1, then diminishes after 15 min and is no more detectable after 20 min. Conversely, no shifted band can be detected in X-35 stimulation at any time of the analysis. These experiments were performed using 10 μg/ml anti-CD3 mAb. We checked that the difference between the two mAb in term of ERK-2 activation is also observed in a large concentration range (1 to 20 μg/ml) (data not shown).

A parallel study analyzing tyrosine phosphorylation after a 5-min stimulation similarly showed (Fig. 1B) that ERK-2 was tyrosine-phosphorylated in UCHT1 but not in X-35-stimulated cells. We also established that only ERK-2 immunoprecipitated from UCHT1 (or PMA)-treated cells was able to phosphorylate (maximum after a 5-min stimulation) over basal level MBP when used as exogenous substrate (Fig. 1C). Enzymatic activity was confirmed using GST-Elk-1, the GST fusion protein of ERK-2 physiological substrate (22), which was highly phosphorylated by lysates from cells pretreated with PMA or UCHT1 but not from cells treated with X-35 (Fig. 1D). It is noteworthy that we did not detect phosphorylation of ERK1 in neither X-35- nor UCHT1-stimulated cells (not shown).

Analysis of Raf-1, the upstream kinase in the MAP kinase cascade, shows that this MAP kinase kinase is also phosphorylated upon UCHT1 and PMA stimulation but not upon X-35 activation (Fig. 2).

Analysis of Interleukin-2 mRNA Expression in CD3-stimulated Cells—IL-2 gene transcription, a key event in T cell activation and proliferation, is regulated by the coordinate action of multiple nuclear factors including NF-AT. Previous results have brought evidence that NF-AT activation is directly dependent on stimulation of Raf-1/MEK-1/ERK-2 phosphorylation cascade. Since our preceding results suggested that this pathway is not activated in X-35 stimulation, we questioned whether IL-2 gene transcription could occur when the ERK-2 pathway is blocked with PD098059, a specific inhibitor of MEK-1 (23). Fig. 3A confirms that ERK-2 activation, which only occurs in UCHT1 and PMA stimulation (as assessed by the appearance of a slower migrating band and the phosphorylation of GST-Elk-1), is indeed prevented by PD098059. In parallel, Fig. 3B shows that, in the absence of inhibitor, IL-2 mRNA expression is induced by both mAbs, whereas in the presence of inhibitor, IL-2 mRNA expression is blocked in UCHT1-stimulated cells and is not in X-35-activated cells. This result demonstrates that IL-2 gene transcription triggered upon X-35 ligation does not involve activation of Raf-1/MEK-1/ERK-2 pathway.

Effect of X-35 and UCHT1 Treatment on p21ras Activation—Raf-1/MEK-1/ERK-2 has been described as a p21ras effector pathway for NF-AT induction in Jurkat T cells. Rac-1, along with other possible pathways, has also been shown in Jurkat cells to participate in this stimulation as downstream effectors of p21ras (9), confirming that this small G protein played a pivotal role in lymphocyte stimulation. We therefore questioned whether the ERK-2-independent pathway, which is triggered in human primary T lymphocytes upon X-35 stimulation, involves activation of p21ras. Fig. 4 shows that p21ras activation can be detected in UCHT1 (the most intense band appearing at 5 min) as well as in phorbol ester-treated cells (used as a control) but not in X-35-stimulated lymphocytes. This results strongly suggest that X-35 binding to CD3 on purified T cells triggers IL-2 gene transcription through a stimulation pathway independent of p21ras activation.

Effect of UCHT1 and X-35 on Early Signals Linked to TCR Activation—Upstream of the p21ras/MAP kinase phosphorylation cascade, the events that are directly induced upon engagement of the TCR are tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) of ζ and CD3 chains by Src family tyrosine kinases Fyn and Lck (2). These phosphorylated motifs provide docking sites for the protein-tyrosine kinases ZAP-70 and Syk, that are phosphorylated and activated (2, 24). Activation of these protein-tyrosine kinases has been shown to be necessary for propagating downstream signaling. We therefore studied phosphorylation of p56lck, p59fn, and of ZAP-70 in primary T cells stimulated either with UCHT1 or X-35; as shown in Fig. 5A, a shifted band can be observed in p56lck only after a 15-min stimulation with UCHT1 and not with X-35. No activation of p59fn could be detected.
The results we present herein provide evidence that activation of the p21\textsuperscript{ras}/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an event obligatorily triggered upon stimulation of purified T lymphocytes through the TCR/CD3 complex. Moreover, they support the related conclusion that, in primary T cells, IL-2 gene transcription may occur independently of the activation of the MAP kinase pathway. Indeed, we have shown that, in contrast to the commonly used UCHT1, which triggers MAP kinase activation, the anti-CD3 mAb X-35 triggers lymphocyte stimulation leading to IL-2 gene transcription and cell proliferation without activating ERK-2; moreover, using the MEK-1 inhibitor PD098059, we demonstrated that the blockade of ERK-2 phosphorylation has no effect on IL-2 mRNA expression induced by X-35. These results demonstrate that the Ras/Raf-1/MEK-1/ERK-2 phosphorylation cascade is not an exclusive and necessary pathway in TCR/CD3-induced T cell activation. The possibility of the existence of a MAP kinase-independent stimulation of T cells has been suggested using splenocytes from transgenic mice expressing an inactive form of MEK-1 (12); however, as pointed out by others (11), it is unclear whether the splenic T cells in these transgenic mice, which developed in the absence of positive selection, are representative of a normal T lymphocyte population.

It has been demonstrated that Rac-1 participates in the stimulation process in parallel and in addition to ERK-2 pathway but still as an effector of p21\textsuperscript{ras} (9); however, a hypothesis of an involvement of Rac-1 as an effector of Ras seems unlikely since we showed that p21\textsuperscript{ras} is not activated upon X-35 binding. However, the possibility remains that in X-35 stimulation, Rac-1 could act instead of p21\textsuperscript{ras}. Recently Rac-1 and/or CDC-42 were shown to be involved in NF-AT activation through activation of the serine threonine kinase Pak-1 (26); however, evidence has been provided that Pak-1 acts upstream of Ras and participates in a signaling event required for TCR-mediated Ras activation (26). We analyzed Jun-N-terminal kinase (25) stimulation in both UCHT1 and X-35 activation (data not shown); this kinase appeared very faintly but similarly stimulated in both cases and, therefore, is probably not involved in this phenomenon.

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Effect of PD098059.

Fig. 3. Parallel analysis of IL-2 mRNA expression and ERK-2 activation. Effect of PD098059. A, activation of ERK-2 from X-35- or UCHT1-stimulated T cells was evaluated according to its phosphorylation state (using anti-ERK-2 Ab) and kinase activity (using GST-Elk-1 as substrate). PMA stimulation was used as the control. This experiment was performed in the absence or presence of PD098059. This representative set of experiments was performed twice. B, IL-2 mRNA from purified CD4\textsuperscript{+} cells stimulated with X-35 or UCHT1 in the presence of ionomycin was detected by reverse transcription-polymerase chain reaction. These experiments, performed in the absence or in the presence of PD098059, show that inhibition of ERK-2 has no effect on IL-2 mRNA expression induced upon X-35 stimulation. β-2-Microglobulin mRNA expression was used as an internal standard. This is a representative experiment of three. NS, nonstimulated.

Fig. 4. p21\textsuperscript{ras} activation in anti-CD3 stimulation. Purified CD4\textsuperscript{+} T cells were stimulated (5 and 15 min) with X-35 or UCHT1 or with PDBu (5 min) as a control. After cell lysing, the active form of Ras was pulled down using GST-RDB fusion protein. After electrophoresis and electrophoretic mobility shift analysis, the membrane was revealed with anti-Ras mAb. NS, nonstimulated.

either with UCHT1 or X-35 stimulation (not shown). Concerning ZAP-70, the presence of which is detected by anti-ZAP-70 Ab, it appears to be phosphorylated in cells stimulated by both anti-CD3 mAb. NS, nonstimulated.

DISCUSSION

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higher amounts of IL-2 are produced by X-35 than by UCHT1 (not shown). This higher production of the protein correlates with a higher expression of IL-2 mRNA in X-35 stimulation. Therefore it seems that stimulation of the p21ras/ERK-2-independent pathway triggered by X-35 could be more efficient for IL-2 gene transcription and IL-2 production. One could then question whether inhibition of the MAP kinase pathway could be a potentiating factor in activation of primary T cells. This appears unlikely since we showed that MEK/ERK inhibition by PD098059 results in the inhibition of IL-2 mRNA production in UCHT1-stimulated cells. A recent study (39) has also shown on primary T cells stimulated with a mouse mAb to CD3 (IgE isotype) that the blockade of the MEK/ERK pathway inhibited IL-2 production but differentially modulated the production of other cytokines.

It appears, however, that the proximal activation induced by both mAbs after their ligation on CD3 involves phosphorylation of ZAP-70, suggesting that the respective pathways induced by UCHT1 or X-35 diverge downstream in this protein-tyrosine kinase. Concerning p56lck or p59fyn, their autophosphorylation is difficult to detect in primary T cells, and the phosphorylated p56lck band that appears only in UCHT1-activated cells after a relatively long time activation (15 min) is probably not due to its direct autophosphorylation but is likely due to phosphorylation induced by activated ERK-2 as described previously (27, 28). This result is in line with the fact that UCHT1 triggers ERK-2 activation, whereas X-35 does not.

We also considered two other TCR-related signals, i.e. activation of the two protein kinases PKB/Akt and ITK; activation of these kinases is dependent on activation of phosphatidylinositol 3-kinase normally triggered following engagement of the TcR/CD3 complex. We failed to detect phosphorylation of ITK in both cases, but our results show that PKB/Akt is phosphorylated upon UCHT1 treatment and not upon X-35 stimulation, suggesting that the latter anti-CD3 mAb does not induce activation of the TCR-related phosphatidylinositol 3-kinase-dependent pathway.

Studying the overall tyrosine phosphorylation of total lysates from UCHT1- or X-35-stimulated cells, it appears that a single band around 58 kDa is present in X-35 and not in UCHT1 activation. This band unlikely represents phosphorylated p56lck or p59fyn, since we showed that phosphorylation of these protein-tyrosine kinases is difficult to observe in primary T cells even using [γ-32P]ATP. However, this band, which is not
yet characterized, could represent an important signaling molecule involved in the Ras/MEK-ERK-independent pathway triggered by X-35.

Previous results show that the role of MAP kinases in TCR function have looked at regulation of the transcription factor NF-AT in the Jurkat cell line. In these cells, experiments with inhibitory mutants of the MAP kinase pathway have suggested that NF-AT activation is dependent on the Ras/Raf/MEK/ERK signaling cascade. These data now show that in peripheral blood T cells ERK-2 activation is not an obligatory signal for IL-2 gene transcription. This illustrates that Jurkat cells, although a good model for the initial receptor proximal biochemical processes associated with T cell activation, may not be an appropriate model for cytokine gene regulation as it relates to primary human T cells. Interestingly, many of the signaling pathways worked out in Jurkat cells, particularly in the context of TCR/Ras/MEK-ERK-2 pathways, have proven to be important as predicted in TCR function in the thymus.

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