Interleukin-12 Induces Tyrosine Phosphorylation and Activation of 44-kD Mitogen-Activated Protein Kinase in Human T Cells

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Interleukin-12 (IL-12) is a novel cytokine that enhances numerous functional activities of human T cells and natural killer (NK) cells. The present studies were undertaken to characterize some of the early signaling events following IL-12 stimulation of mitogen-activated normal T cells. In these cells, IL-12 induces rapid tyrosine phosphorylation of proteins of 21, 44, and 54 kD. However, IL-12 does not induce tyrosine phosphorylation in normal resting T cells. In conjunction with increased tyrosine phosphorylation of several substrates, IL-12 stimulation resulted in increased in vitro kinase activity of immunoprecipitated tyrosine phosphorylated proteins. The 44-kD protein has been characterized as one isoform of the mitogen-activated protein (MAP) kinase family. Increased tyrosine phosphorylation of MAP kinase following IL-12 stimulation was also associated with enhanced enzymatic activity of this protein in vitro as measured by myelin basic protein phosphotransferase assay. These studies identify MAP kinase as one of the intracellular elements of the IL-12 signaling pathway in human T cells.

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MATERIALS AND METHODS

Reagents. Recombinant IL-12 (rIL-12) was kindly provided by Dr Steven Herrmann (Genetics Institute, Cambridge, MA). Conjugated anti-CD3 fluorescein isothiocyanate (FITC) was obtained from Coulter Immunology (Hialeah, FL). Mouse antiphosphotyrosine (Ptyr) antibody (4G10) was kindly provided by Dr Brian Drucker (Dana-Farber Cancer Institute, Boston, MA). Affinity-purified rabbit polyclonal antibodies anti-erkl-III (R1) against protein kinase subdomain III region and anti-erkl-CT (R2) against C-terminus rat brain erk-1 were generated as previously described and are commercially available from Upstate Biotechnology (Lake Placid, NY). Alkaline phosphatase (AP)-conjugated anti-mouse or anti-rabbit immunoglobulin affinity-purified antibodies were purchased, along with developing reagents nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), from Promega, Madison, WI. Phytohemagglutinin (PHA) was purchased from Wellcome (Dartford, England). Myelin basic protein (MBP) and other reagents not specified here were obtained from Sigma Chemical (St Louis, MO).

Cells. Peripheral blood mononuclear cells (PBMC) were obtained from normal donors following Ficoll-Hypaque density-gradient centrifugation. Activated T cells were obtained by culturing
Fig 1. IL-12-induced tyrosine phosphorylation in resting PBMC and PHA-activated T cells. (A) Resting PBMC were stimulated for the times indicated by CD3 cross-linking (CD3 X-L) (lanes 2-5), 100 U/mL rIL-12 (lanes 6-9), or CD3 X-L plus rIL-12 (lanes 10-13). (B) Mitogen-activated T cells were stimulated for the times indicated with 100 U/mL rIL-12. Cell preparations contained > 90% CD3⁺ cells. Phosphotyrosyl proteins were separated under reducing conditions by 5% to 16% gradient SDS-PAGE and detected by immunoblotting with anti-Ptyr monoclonal antibody. The molecular sizes (in kilodaltons) of major phosphorylated substrates are indicated.
T cells were stimulated for the times indicated by CD3 X-L (lanes 2-5). 100 U/mL rIL-12 (lanes collected and assayed for MBP phosphotransferase activity as pre-

A MonoQ column attached to a Pharmacia (Piscataway, NJ) fast-

44-kD protein was apparent within 5 minutes following IL-

pH 7.2, 25 mmol/L β-glycerophosphate, 2 mmol/L EDTA, 5 mmol/L EGTA, 2 mmol/L Na2VO4, and 1 mmol/L DTT) and loaded onto a MonoQ column attached to a Pharmacia (Piscataway, NJ) fast-phase liquid chromatography (FPLC) system. After washing with 2 mL of buffer A, the column was developed with a 10-mL linear 0-

to 0.8-mol/L NaCl gradient in buffer A. Fractions (250 μL) were collected and assayed for MBP phosphotransferase activity as previously described.19

RESULTS

IL-12–induced tyrosine phosphorylation in mitogen-activated T cells. As shown in Fig 1A, IL-12 stimulation of resting PBMC did not result in increased tyrosine phosphorylation detectable by immunoblotting experiments. Resting PBMC responded to CD3 cross-linking (CD3 X-L) with tyrosine phosphorylation of several substrates, but simultaneous incubation with IL-12 did not modify the phosphorylation pattern induced by CD3 perturbation. In contrast, tyrosine phosphorylation of several proteins was observed (Fig 1B) when PHA-activated PBMC, containing more than 90% T cells, were stimulated with IL-12. The molecular weights of the principal tyrosyl phosphorylated proteins were 21, 34, 44, and 54 kD. As shown in Fig 1B, the phosphorylation events observed following IL-12 stimulation were time-dependent and different proteins appeared to be phosphorylated in a sequential order. The phosphorylated 44-kD protein was apparent within 5 minutes following IL-12 stimulation. At this time, the phosphorylation of the 21-kD protein became appreciable, reaching a maximum 10 minutes after stimulation and disappearing by 30 minutes. The phosphorylation of a 54-kD protein was also evident in this experiment at 10 minutes. A 34-kD protein is only phosphorylated approximately 30 minutes following IL-12 activation. These findings indicate the induction of an ordered sequence of tyrosine phosphorylation events following IL-12 stimulation of mitogen-activated T cells. IL-12 was active on PHA-activated T cells at concentrations of 0.1 to 100 U/mL, with maximal activity noted between 10 and 100 U/mL (data not shown).

As noted previously, one major tyrosine phosphorylated protein following IL-12 stimulation of activated T cells was approximately 21 kD. This appeared to represent the ζ component of the CD3 complex, which is known to undergo tyrosine phosphorylation following CD3 X-L. To investigate this possibility further, the phosphorylation of the 21-kD protein was evaluated in mitogen-activated T cells following stimulation with either IL-12, CD3 X-L, or the addition of both reagents simultaneously. As shown in Fig 2, both stimuli induced tyrosine phosphorylation of 21-kD

![Image](https://via.placeholder.com/150)

**Fig 2.** Tyrosine phosphorylation of a 21-kD protein following CD3 X-L and IL-12 stimulation of mitogen-activated T cells. PHA-activated T cells were stimulated for the times indicated with 100 U/mL rIL-12 (lanes 6-9), or CD3 X-L plus rIL-12 (lanes 10-13). Phosphotyrosyl proteins, separated by 5% to 16% gradient SDS-PAGE, were detected by immunoblotting with anti-Ptyr monoclonal antibody. The 21-kD phosphoprotein is indicated.

![Image](https://via.placeholder.com/150)

**Fig 3.** Immunoprecipitate kinase assay following IL-12 stimulation of activated T cells. PHA-activated T cells were stimulated for the times indicated with 100 U/mL rIL-12. Phosphotyrosyl proteins were immunoprecipitated using anti-Ptyr monoclonal antibody and subjected to in vitro kinase reaction consisting of a 15-minute incubation in kinase buffer containing 20 μCi [γ-32P]ATP. Isotype-matched irrelevant mouse antibody was used as immunoprecipitation control. Proteins were resolved under reducing conditions in 10% SDS-PAGE; 110-, 70-, 56-, and 42-kD proteins are indicated. Exposure time was 30 minutes.
proteins, and this effect appeared to be slightly enhanced by the combination of both stimulations. In additional experiments not shown, IL-12 stimulation of mitogen-activated T cells for periods of 10 minutes to 12 hours did not alter surface expression of CD3.

In vitro kinase activity of anti-Ptyr immunoprecipitates following IL-12 stimulation. To examine further the effects of IL-12 on protein tyrosine kinases, an in vitro immunocomplex kinase assay was performed with anti-Ptyr immunoprecipitates obtained following IL-12 stimulation of PHA-activated T cells. Results were compared to PHA-activated T cells without further stimulation. Figure 3 shows that several tyrosine phosphorylated proteins were increased within 5 minutes following IL-12 stimulation and phosphorylation was decreased by 30 minutes. The main substrates phosphorylated in vitro were 44, 56, 85, and 110 kD.

Characterization of the p44 protein as MAP kinase. In the immunoblot experiments shown in Fig 1B, one of the major substrates phosphorylated on tyrosine following IL-12 stimulation was a 44-kD protein. The 44-kD isoforms of tyrosine phosphorylated MAPK migrate in this region of the gel. To determine whether IL-12 stimulation leads to tyrosine phosphorylation of MAPK, anti-Ptyr immunoprecipitates from lysates of activated T cells were blotted with anti-erk-CT, which is immunoreactive with both 42- and 44-kD isoforms. Mitogen-activated T cells were stimulated with 100 U/mL IL-12 for various times and compared with T cells without further stimulation. Figure 4 shows a time-course experiment confirming that the 44-kD MAP kinase isoform was tyrosine-phosphorylated within 5 minutes following IL-12 stimulation. Phosphorylation of MAPK was clearly decreased by 10 minutes following IL-12 stimulation. As in previous experiments, this effect was transient and had decreased by 10 minutes.

Increased MAPK activity following IL-12 stimulation. To evaluate whether IL-12-induced tyrosine phosphorylation of MAPK also resulted in increased enzymatic activity of this kinase, an in vitro phosphotransferase assay was performed on extracts from mitogen-activated T cells following stimulation with 100 U/mL of IL-12. Results were compared with those obtained with activated T cells without further stimulation. MAPK activity was assayed on fractions obtained following anion exchange chromatography and detected using the MAPK substrate MBP, as described in the Methods. Figure 5A shows that a major peak of enzymatic activity was observed in fractions 29 and 30. This peak coincided with the maximal immunoreactivity of MAPK, as detected by immunoblotting of the various chromatographic fractions (Fig 5B and C). IL-12 stimulation of activated T cells caused an approximate threefold increase of MBP phosphotransferase activity as compared with activated T cells not further stimulated (Fig 5A). Increase in MBP phosphorylation was not associated with an increase in the amount of MAPK themselves, as shown in Fig 5B and C. This finding indicates that increased enzymatic activity was due to a posttranslational modification of MAPK.
Figure 6 summarizes results of experiments evaluating the increase of MBP phosphorylation by MAPK at various times after IL-12 stimulation. Fractions with MAPK enzymatic activity were obtained by anion exchange chromatography from T-cell extracts as previously described. MBP phosphotransferase activity was maximal 5 minutes following IL-12 stimulation and returned to basal level by 10 minutes. Taken together, these data confirmed the results shown in Fig 1B and Fig 4 indicating that IL-12-induced MAPK activation represents a rapid and transient event in the cell activation process, with a peak of activity at approximately 5 minutes following stimulation.

DISCUSSION

Although various studies have recently identified several of the functional effects of IL-12 on T cells and NK cells, little is known about postreceptor events that mediate and propagate the IL-12-induced signal. Our current results, which indicate that IL-12 does not induce tyrosine phosphorylation events in resting T cells, are consistent with the previous demonstration that resting T cells express few, if any, receptors for IL-12. After mitogen activation, T cells are known to express IL-12 receptors, and our current studies demonstrate that IL-12 stimulation of PHA-activated T cells involves tyrosine phosphorylation signaling pathways. Proteins of 44 kD, 21 kD, and 54 kD represent the major substrates phosphorylated on tyrosine residues. Immunoblotting experiments showed that the 44-kD proteins represent two isoforms of the MAPK family of serine-threonine kinases. Significantly, tyrosine phosphorylation of MAPK paralleled the increase of in vitro MAPK activity in MonoQ chromatographic fractions, as measured by phosphorylation of MBP substrate. Furthermore, tyrosine phosphorylation of a 21-kD protein, possibly the p component of the CD3/T-cell receptor complex, followed tyrosine phosphorylation of MAPK.

MAPK represents a heterogeneous family of structurally and functionally related serine-threonine protein kinases that migrate in polyacrylamide gels in the 40- to 46-kD region.20-22 Most members of this family require both tyrosine and threonine phosphorylation to express full enzymatic activity,23 thus representing a well-characterized link between the tyrosine and serine-threonine kinase systems. Several lines of evidence suggest that tyrosine phosphorylation represents a positive regulatory signal for MAPK. Various growth factors, including nerve growth factor, platelet-derived growth factor, epidermal growth factor, IL-3, and granulocyte-macrophage colony-stimulating factor, activate MAPK through a tyrosine phosphorylation pathway.17,24-27 Furthermore, a tight correlation between tyrosine phosphorylation and increased enzymatic activity has been clearly demonstrated in both human neutrophils and T cells, indicating that the two events are temporally related.27,28

Since the IL-12 receptor has not been completely characterized, the biochemical mechanism by which IL-12 stimulation activates MAPK remains to be elucidated.7,8 However, several lines of evidence indicate that MAPK represents an intermediate element in a signaling cascade that involves at least two other molecules: the dual specificity MAPK kinase, able to induce both tyrosine and threonine phosphorylation of MAPK, and Raf1 serine-threonine protein kinase.21,29,31 Furthermore, protein kinase C (PKC) appears to phosphorylate and activate Raf1 directly.32 The complexity of the upstream regulatory events in MAPK activation suggests that the IL-12 receptor may activate multiple signaling systems, as already shown for other growth factor receptors.27 Future studies will be necessary to establish whether IL-12 signaling is mediated, at least in part, via a PKC → Raf1 → MAPK kinase cascade.

To date, extensive analysis of early events of cell activation following cytokine stimulation has led to the demonstration that tyrosine phosphorylation events represent a general phenomenon and are a prerequisite for cell activation. Nevertheless, the function-related specificity that results from tyrosine phosphorylation of individual substrates remains poorly defined. Of note, IL-12 is a potent mitogen for activated T cells, while it has a relative inhibitory effect on IL-2-induced proliferation of activated NK cells. This demonstration that the same cytokine has opposite effects on different cell populations suggests that distinct signaling pathways may be activated in these two cell types.

The observation that the same receptor can recruit different pathways, which under certain conditions can be dissociated, is already known. Specifically, it has been reported that CD3 ligation in Jurkat cells results in Ca2+ flux, PKC activation, and MAPK activation, whereas CD3 ligation in CD3+ PKC-deficient HPB-ALL cells induces tyro-
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A possible explanation for the different behavior of T and NK cells in response to IL-12 could be that IL-12 signaling involves both tyrosine and threonine phosphorylation pathways in activated T cells, but that tyrosine phosphorylation predominates in NK cells. Nonetheless, a definitive interpretation is not yet possible since it has been shown that MAPK can be also activated independently of serine-threonine pathways.35,36

IL-12-activated human T cells represent an excellent experimental model to explore further the roles of MAPK and other signal transducing proteins in intracellular communication. A better understanding of intracellular pathways activated by this cytokine may lead to more rational approaches in the use of this biological agent in the immunotherapy of cancer patients, as well as in patients with immunodeficiency syndromes.

REFERENCES


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