Biochemical and Biophysical Research Communications **259,** 172–177 (1999)
Article ID bbrc.1999.0746, available online at http://www.idealibrary.com on IDEAL®

Interleukin-17 Induces Rapid Tyrosine Phosphorylation and Activation of Raf-1 Kinase in Human Monocytic Progenitor Cell Line U937

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Received April 12, 1999

Interleukin-17 is a T cell derived pro-inflammatory cytokine exhibiting multiple biological activities in a variety of cells and believed to fine tune all general phases of hematopoietic response. However, the signaling mechanism of this novel cytokine remains unknown. The purpose of this study was to determine whether Interleukin-17 induces tyrosine phosphorylation of proteins and to find out whether the raf-1 kinase signaling pathway is involved in mediating its signaling. Using immunoblotting and immunocomplex kinase assays, we report that the early signaling events triggered by rhIL-17 involves rapid tyrosine phosphorylation of several cellular proteins including raf-1 within 0.5 to 30 min. Optimal stimulation of tyrosine phosphorylation was observed with 0.5 to 1.0 ng/ml of Interleukin-17. Further, Interleukin-17 stimulates rapid activation of raf-1 kinase. These findings provide the first evidence that the mechanism of IL-17 signaling involves rapid tyrosine phosphorylation and activation of raf-1 serine/threonine kinase. © 1999 Academic Press

Interleukin-17 (IL-17), initially known as CTLA8, is a novel cytokine that appears to be part of a unique ligand-receptor system that regulates the production of various cytokines (1-3). IL-17 was originally cloned from a T cell hybridoma produced by fusion of a mouse cytotoxic T cell clone and a rat T cell lymphoma (4, 5). The human IL-17 was cloned based on its homology to the rodent sequence and to an Open reading frame (ORF) of Herpes virus saimiri (HVS) (1, 5, 6). Besides the rat and human factors, mouse IL-17 has also been cloned (7, 8).

Human IL-17 is a variably glycosylated, 20-30 kDa homodimeric polypeptide secreted by $CD4^+$ activated memory T cells (1, 6). The human IL-17 consists of a 136 amino acid residue mature segment and it shows a sequence identity of 62.5% and 58% to the mouse and rat sequences respectively (8). IL-17 shares homology to the thirteenth ORF of HVS. Out of the 151 amino acid residues constituting the HVS-ORF 13 gene, there is a 57% amino acid residue identity to the rat IL-17 (4, 5, 8) and 72% identity to the human IL-17 (1, 5, 8). The receptor for IL-17 also has been recently isolated from mouse EL4 thymoma cells (7, 9) and it is approximately 120 kDa in size.

Though the biological functions of IL-17 have not been fully elucidated, the limited literature available on IL-17 so far suggest that it could be a major vehicle which fine tunes all phases of hematopoiesis (2). Fibroblasts, when cultured in the presence of IL-17, are able to sustain $CD34^+$ hematopoietic progenitor cells and direct their maturation towards neutrophils (5, 6). IL-17 has been found to induce the release of IL-6, IL-8, and G-CSF, cytokines which regulate hematopoiesis (10) and also to have synergistic or antagonistic effects with other cytokines (11). However, the signaling pathway(s) utilized by IL-17 in various cells still remain unknown.

Cytokine-mediated tyrosine phosphorylation of cellular proteins is known to regulate the involvement of key signal transduction molecules including the JAK-STATs and the members of the Ras-Raf-MAP Kinase pathway in multiple signaling cascades responsible for regulation of growth and differentiation. Because of the relevance of tyrosine phosphorylation in the biological functions of several cytokines and hematopoietic growth factors, we asked whether tyrosine phosphorylation plays a role in the signaling pathway(s) utilized by IL-17.

In this study, we report that in human monocytic leukemia cells (U937), and human NK cells, IL-17 induces rapid dose and time dependent stimulation of tyrosine phosphorylation of several cellular proteins. Immunoprecipitation, Western blot analysis and kinase assay results revealed that in U937 cells, IL-17

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0006-291X/99 $30.00

mediates rapid tyrosine phosphorylation of raf-1 suggesting that the mechanism of IL-17 involves initial activation of tyrosine kinases coupled to downstream activation of raf-1 kinase.

In conclusion, we have provided evidence that tyrosine as well as serine/threonine kinases may play important role in transducing the IL-17 signal to the nucleus.

MATERIALS AND METHODS

U937 cells. Human leukemia U937 cells obtained from ATCC were maintained at 37°C under 5% CO_2 tension in RPMI 1640 medium containing 10% FBS and 50 units/ml each of penicillin and streptomycin. Viability of cells was estimated to be greater than 95% by Trypan blue dye exclusion. Human recombinant IL-17 was purchased from R & D systems, Minneapolis, MN and diluted into tissue culture medium in the absence of fetal bovine serum (FBS). FBS was from Atlanta Biologicals, Atlanta, GA, & Pepstatin A, leupeptin, aprotinin, phenyl methyl sulfonyl fluoride (PMSF), microcystin and EDTA were products of Sigma Chemicals, St. Louis, MO. The anti-phosphotyrosine antibody coupled to horse-radish peroxidase (RC20) and monoclonal antibody to raf-1 was from Transduction Labs, Lexington, KY. Coomassie protein assay reagent and Supersignal CL-HRP detection reagent were purchased from Pierce, Rockford, IL; protein A-agarose from Boehringer-Mannheim and anti-mouse Ig peroxidase linked antibody from Amersham, Arlington Heights, IL.

NK cell isolation. Highly purified human NK cells were obtained by immunomagnetic isolation technique (12). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (American Red Cross, Portland, Oregon) by Ficoll-Hypaque gradient centrifugation. To select for peripheral blood lymphocytes (PBL), monocytes were separated by plastic adherence by incubating the PBMC suspension in petri dishes for 45 min at 37°C in 5% CO_2 environment. The enrichment of human NK cells from the non-adherent cells was achieved by negative selection using anti CD19, CD3, and CD14 mAb and magnetic Dynabeads M-450 coated with goat anti mouse IgG. Mononuclear cells (2×10^8) were incubated in the presence of the mAbs at a 1:20 dilution for 30 min at 4°C. After the incubation, a magnet (Dynal MP-6) was used to separate beads with attached $CD3^+$ T, $CD14^+$ monocytes and $CD19^+$ B cells from NK cells. The purity of the NK cells was assessed to be about 96% $CD56^+$ cells as determined by immunohistochemical staining (DAKO). Then the cells were washed twice and resuspended in RPMI 1640 for assaying.

Western blotting and detection of phosphotyrosine bands. U937 cells (5×10^6) were pretreated with orthovanadate (5 mM) for 30 min at 37°C to inhibit endogenous phosphatases. Thereafter, aliquots of vanadate pretreated cells were exposed to various doses of IL-17 for varying periods of time at 37°C. Following treatment, cells were rapidly packed by microcentrifugation at 13,000 × g for 20 sec, washed twice in PBS and were lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM Na Vanadate, 1% Brij-35, 0.5% Triton X-100, 10 ug/ml each of aprotinin and Leupeptin, 1 mM PMSF and 1 μM of Microcystin) by extensive vortexing. Samples were spun down to remove cell debris and the total protein content was determined using Coomassie protein assay reagent. For all immunoprecipitation and kinase assay experiments, known quantity of the protein samples were used. For dose and time course experiments, pellets of untreated (control) and treated cells were resuspended in SDS-gel sample buffer containing 0.1% SDS and vortexed extensively to completely lyse the cells. Protein samples were analyzed on 10% or 12% polyacrylamide SDS gels run at 35 mA for 4 hrs. Protein bands were transferred to nitrocellulose filters by electrotransfer under a constant current of 140 mA for 3 hrs at 4°C. The efficiency of transfer was verified by rapid ponceau (0.4% in 5% TCA) staining and destaining of the filters. The destained membrane filters were incubated in a blocking buffer, TBST (TBS containing 0.1% Tween 20 and 1% BSA) at 37°C for 20 min. Next, the filters were immunoblotted in a blocking solution containing anti-phosphotyrosine antibody RC20 (1:2500 dilution) for 30 min at 37°C. The filters were washed in a wash buffer (blocking buffer minus BSA) for 30 min with three changes of buffer. Finally, the filters were incubated in ECL detection reagent for one minute followed by exposure to X-ray films for autoradiography (13). Similar protocol was followed for the untreated and treated NK cells.

Immunoprecipitation of raf-1 and immunocomplex kinase assay. To determine whether stimulation of cells cause tyrosine phosphorylation of raf-1, we used specific monoclonal anti-raf-1 antibody in immunoprecipitation reaction to detect raf-1 protein from the cell extract. Briefly, cells were lysed in lysis buffer (TBS containing 0.2% NP-40, 2 mM EDTA, 0.5 mM PMSF and 1 μg/ml each of protease inhibitors, leupeptin, aprotinin and pepstatin-A) by extensive vortexing. Next, unbroken cell debris and unsolubilized membranous materials were removed by microcentrifugation at 13,000 × g for 5 min. The protein content of the supernatant was determined by using Coomassie protein assay reagent. 500 mg of protein was was incubated with 5 μl of monoclonal antibody to raf-1 for 3 hrs with gentle shaking at 4°C. Protein A agarose was then added to capture the immunocomplex during a 1 hr incubation at 4°C. The protein A agarose-immunocomplex mixture was pelleted and washed for 30 min in lysing buffer (with three changes of buffer). The final protein A agarose-immunocomplex pellet was resuspended in SDS-gel sample buffer, vortexed extensively and boiled for 3 min to solubilize the bound protein for SDS gel analysis. In order to determine whether the immunoprecipitated protein was phosphorylated on tyrosine residue, Western blot analysis using RC20 was performed after immunoprecipitation.

To determine whether treatment of cells with IL-17 causes activation of raf-1 kinase activity, following immunoprecipitation, the protein A agarose-immunocomplex pellet was dissolved in a kinase reaction buffer (250 mM HEPES, pH 7.4, 1% NP-40, 20 mM $MnCl_2$, 5 mM vanadate, 50 μM Na_2ATP containing histone H1 (10 ug) and 0.2 mCi of ^{32}P-ATP and incubated on ice initially for 5 min. The tubes were then vortexed and incubated at 30°C for 10 min. The kinase reaction was terminated by transferring the tubes to ice incubating for 15 min. 70 μl of SDS-gel sample buffer (3×) was added to each sample and the samples were extensively vortexed and boiled for 3 min to completely solubilize the proteins for PAGE. The samples were resolved on 12% polyacrylamide gels and the proteins were transferred to nitrocellulose membrane. The membrane was air dried, and exposed to X-ray films for autoradiography (13).

RESULTS

The role of tyrosine phosphorylation in cytokine-receptor signaling cascades in regulation of cellular functions is very well established as evidenced by the fact that the initial signaling events triggered by several cytokines including IL-2, IL-3, IL-4, IL-6, IL-13 and IL-15 involve rapid induction of phosphorylation of proteins on tyrosine residues (14-18). Also, it is well established that cytokine-mediated phosphorylation of key signaling protein molecules occurs on tyrosine residues (19). Earlier studies in our lab have shown that in U937 cells, the optimum effect of IL-17 on tyrosine phosphorylation was attained between 0.5 ng/ml and 1.0 ng/ml of this cytokine. As the concentration increased beyond 2.5 ng/ml, a gradual decline in the effect of this cytokine on tyrosine phosphorylation was

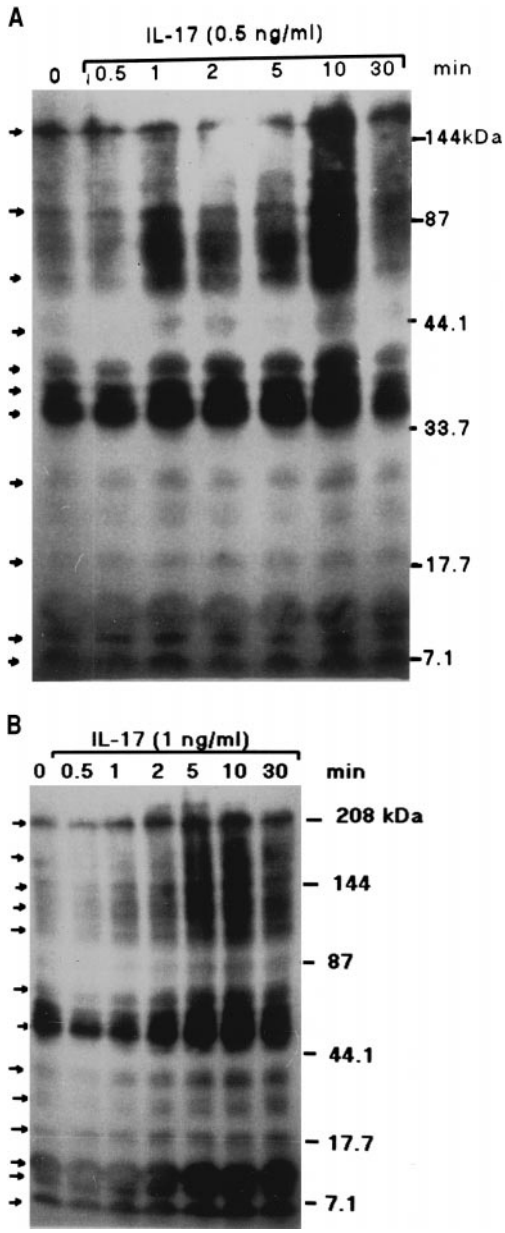

FIG. 1. (A and B) Time course stimulation of tyrosine phosphorylation in U937 cells by IL-17. Human U937 cells (5×10^6) were untreated or treated with either 0.5 ng/ml (A) or 1.0 ng/ml (B) of IL-17 for various periods of time. Total cell lysate proteins were analyzed for tyrosine phosphorylation using RC20. Control (CTL.) = Untreated.

noticed. Therefore, we decided to use either 0.5 or 1.0 ng/ml of IL-17 for all the experiments described in this report.

To further establish the role of tyrosine phosphorylation in the mechanism of action of IL-17, the U937 cells were treated with recombinant human IL-17 (0.5ng/ml and 1.0 ng/ml) for various periods of time. At the indicated time points, samples were analysed for phosphotyrosyl bands. The results in Figures 1A & 1B indicate that IL-17 has the ability to elicit rapid stimulation of phosphorylation of a number of proteins (indicated by arrows) on tyrosine residues. As could be seen, IL-17 elicited a maximum effect on tyrosine phosphorylation of bands with relative molecular weights ranging from of 6.9 to 167 kDa. The effect of IL-17 on tyrosine phosphorylation could be noticed from as early as one minute, with a gradual increase through 10 minutes, beyond which there was a decline. Similar pattern of time-dependent alteration of tyrosine phosphorylation was detected with either 0.5 or 1.0 ng/ml of IL-17 (Figures 1A & 1B).

In order to demonstrate that activation of tyrosine phosphorylation by IL-17 occurs also in normal human untransformed cells, we examined the effects of this cytokine on phosphotyrosine levels in natural killer (NK) cells extracted from normal peripheral blood mononuclear cells (PBMC). The data in Figure 2 confirms that IL-17 is capable of inducing tyrosine phosphorylation in normal blood NK cells as well, although the amount of IL-17 required to stimulate a response was higher than in the leukemic cells. Increased phosphorylation was detected in three major bands corresponding to 42, 28 and 14.3 kDa. However, after a prolonged exposure of the membrane to film, a 97 kDa phosphotyrosine band was detected in extracts from IL-17 treated cells. From these experiments, it can be inferred that the initial signaling events evoked by IL-17 include rapid stimulation of tyrosine phosphory-

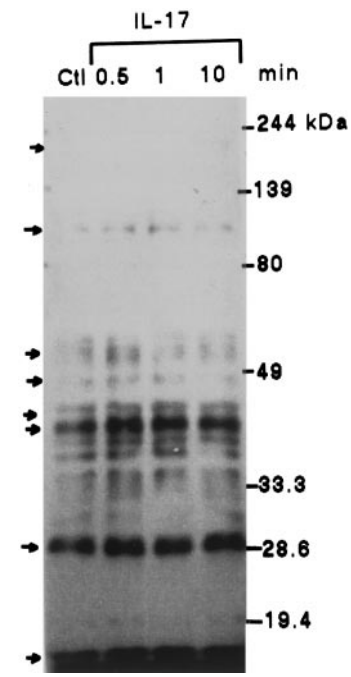

FIG. 2. Time course stimulation of tyrosine phosphorylation in NK cells by IL-17. Human natural killer (NK) cells were untreated or treated with 10ng/ml of IL-17 for various periods of time. Total cell lysate proteins were analyzed for tyrosine phosphorylation. Control (CTL.) = Untreated.

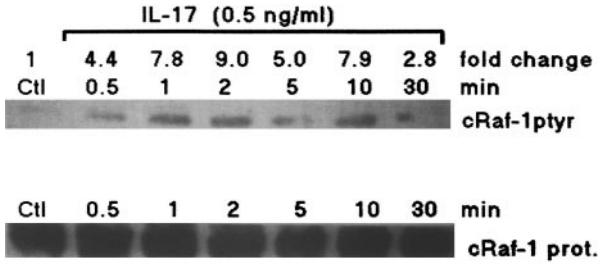

FIG. 3. Effects of IL-17 on raf-1 tyrosine phosphorylation. Vanadate pretreated human U937 cells (5×10^6) were either untreated or treated with 0.5 ng/ml of IL-17 for various periods of time. Total cell extract (500 μg protein) was immunoprecipitated with anti-raf-1 monoclonal antibody. Immunocomplexes were captured on Protein-A agarose and samples were subjected to Western blot analysis. Raf-1 phosphotyrosyl protein was detected by Western blot hybridization with RC20 as described in Materials and Methods.

lation suggesting that tyrosine phosphorylation may be relevant to the mechanisms of action of this cytokine.

IL-17 has been considered to play a role in fine tunning all the phases of hematopoiesis including differentiation (2, 20). There are many reports implicating raf-1 kinase as a major signal transduction molecule involved in serine/threonine phosphorylation of major cellular proteins including histone H1 during cell activation by mitogens, growth harmones and cytokines (13). To determine whether raf-1 kinase plays a role in the signaling events evoked by IL-17, the status of tyrosine phosphorylation of raf-1 in untreated and IL-17 treated cells was compared. Cells were exposed to IL-17 (0.5 ng/ml) for varying times, and the raf-1 protein was immunoprecipitated with anti-raf-1 monoclonal antibody followed by Western blotting with antiphosphotyrosine antibody (RC20). Figure 3 shows that in comparison to the untreated control, there was an immediate increase in raf-1 phosphotyrosine level in IL-17 stimulated cells noticeable by 0.5 min (4.4 fold increase). Increased phosphorylation could be noticed upto 2 min (9 fold), after which the level decreased (5 fold). A second increase in the phosphorylation level was seen at the 10 min time point (7.9 fold) after which the level dropped to 2.8 fold at 30 min. The changes in raf-1 phosphotyrosine levels were not due to variation in protein levels as could be seen from Fig. 3.

Since the level of tyrosine phosphorylation of raf-1 changed in response to IL-17, we decided to investigate whether modulation of tyrosine phosphorylation of raf-1 by IL-17, results in alteration of raf-1 kinase activity. Immunocomplex kinase assays were performed on an immunoprecipitated raf-1 protein by following the extent of incorporation of ^{32}P into histone H1 in extracts from untreated and IL-17 treated cells. The results in Fig. 4 indicate that IL-17 stimulates rapid activation of raf-1 kinase activity as demonstrated by the time-dependent increase in ^{32}P incorporation into histone H1 in IL-17 treated cells up to 2 min. Compared to the untreated cells, the increase of ^{32}P incorporation in treated cells was 2.6, 15.6 and 25 folds at 0.5 min, 1 min and 2 min respectively. After the peak at 2 min, a gradual decrease was noticed at 10 min (7.7 fold) and 30 min (2.3 fold). The pattern of changes noticed in the tyrosine phosphorylation levels in the kinase assay results correlated well with that of raf-1 protein (Fig. 3) indicating that IL-17 induced modulation of tyrosine phosphorylation of raf-1 is associated with activation of the phosphotransferase activity of raf-1 kinase.

DISCUSSION

Though limited in number, the reports available on the biological activity of IL-17 suggest that it can be considered the newest T cell derived hematopoietic cytokine, which might ultimately have the ability to fine tune or impact all general phases of a hematopoietic response (2, 21). To understand how this cytokine may have an overall control on hematopoiesis, it is absolutely essential to elucidate its signal transduction pathway(s) and to identify the molecules which participate in the cellular sequence of events involved in the signaling pathway(s) of this cytokine. As a major step in this direction, we are providing the first evidence that the mechanism of action of IL-17 involves rapid activation of tyrosine phosphorylation of several key signaling molecules including phosphorylation and activation of raf-1 serine/threonine kinase. Our results indicate that IL-17 induced stimulation of tyrosine phosphorylation could be detected on several proteins ranging in relative molecular weight of 6.9 to 167 kDa. This effect was found to be both dose and time dependent in U937 cells. Exposure of cells to varying doses of

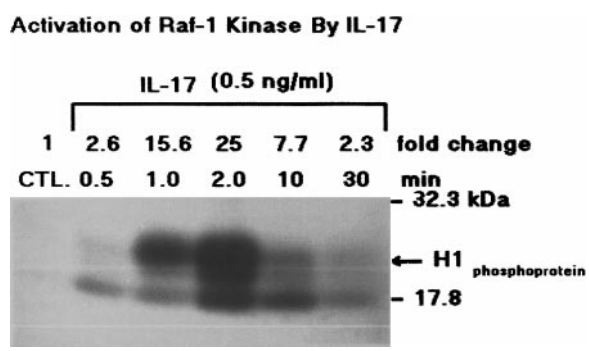

FIG. 4. Stimulation of activation of raf-1 kinase activity by rhIL-17. Vanadate pretreated U937 cells (5×10^6) were either untreated or treated with 0.5 ng/ml of rhIL-17. Total cell extract (500 μg protein) was immunoprecipitated with anti-raf-1 monoclonal antibody. Immunocomplexes were captured on Protein-A agarose, dissolved in raf-1 kinase reaction mixture containing 10 μg histone H1 and raf-1 kinase induced ^{32}P incorporation into histone H1 was assessed as described in Materials and Methods.

IL-17 revealed that the maximum tyrosine phosphorylation could be attained with between 0.5 ng/ml and 1.0 ng/ml of IL-17. IL-17-dependent stimulation of tyrosine phosphorylation was also detected in human NK cells, although the amount of IL-17 required to stimulate a response was higher than in the leukemic cells (10 ng/ml). Increased phosphorylation was detected in four major bands corresponding to 97, 42, 28 and 14.3 kDa. From these experiments, it can be inferred that the initial signaling events evoked by IL-17 include rapid stimulation of tyrosine phosphorylation suggesting that tyrosine phosphorylation may be relevant to the mechanism of action of this cytokine and the effect is not restricted to transformed cells alone.

Signal transduction pathways enable extracellular signals to activate latent transcription factors in the cytoplasm of the cells. Dimerization, nuclear localization and binding to specific DNA sequences result in the induction of gene transcriptions by these proteins (19, 22). Early tyrosine phosphorylation events are necessary for the functioning of either the JAK/STAT or the Ras/Raf/MAPK pathways. Raf-1 is a 74 kDa serine/threonine kinase located in the cell cytoplasm that has been identified as a component of growth-factor-activated signal transduction pathways, which is activated by phosphorylation in cells stimulated with a variety of mitogens and growth factors including hematopoietic growth factors (23). It is well documented that raf-1 is required for growth factor induced proliferation of leukemic progenitor cell lines and normal murine and human bone marrow derived progenitor cells regardless of the growth factors used to stimulate growth whose receptors may be members of several different structural classes like the a) hematopoietin receptor family (IL-2, IL-3, IL-4. GM-CSF & EPO), b) the tyrosine kinase receptor class (Steel factor & CSF-1) and c) receptors that include gp130 subunit (IL-6, leukemia inhibitory factor & oncostatin-M) (24). The ability of IL-17 to stimulate tyrosine phosphorylation and activation of raf-1 kinase reported here strengthens the notion that perhaps the Ras-Raf-MAPK pathway is utilised by this cytokine in transducing its signals to the nucleus. We have found that one of the proteins which experiences changes in tyrosine phosphorylation and activation in response to IL-17 is raf-1 kinase. This is demonstrated by a 9 fold increase in tyrosine phosphorylation and a 25 fold increase in raf-1 kinase mediated incorporation of ^{32}P into histone H1 in IL-17 treated cells. Cellular activation by cytokines, mitogens and growth hormones involve stimulation of tyrosine phosphorylation and activation of key signal transduction molecules including c-fes, raf-1, MAP kinase kinase and MAP kinase (13). Activation of Raf-1/MAP kinase pathway has been shown to occur in case of several other cytokines like IL-1 beta and TNF-alpha (25), IL-2 (26), IL-5 (27), IL-6 (28), IL-8 (23) and IL-13 (13). Thus, the mechanism of IL-17 may be similar to that of other cytokines.

As suggested by previous workers, it is likely that IL-17 functions as a true pleiotropic cytokine which controls several functions like inflammation, immunity and hematopoiesis. It is a fact that stimulation of the Raf/MAP kinase pathway activates transcription factors that control the expression of inflammatory cytokines (29) and IL-17 is known to induce secretion of IL-8. IL-17 also induces secretion of IL-6, G-CSF and PGE2, molecules which produce different activities. Furthermore, murine IL-17 is known to stimulate activation of the transcription factor NFκB, which is known to regulate a number of gene products involved in cell activation and growth control (7). Thus, by stimulating the release of a number of cytokines, IL-17 may have a broad effect on the overall process of hematopoiesis. Whether or not regulation of all these cellular functions by IL-17 requires initial tyrosine phosphorylation and activation of multiple signaling pathways remains to be established.

Clearly, this report provides the first evidence suggesting that tyrosine phosphorylation of numerous proteins including activation of raf-1 kinase may be relevant to the mechanism by which IL-17 regulates cellular functions. Further studies are in progress to elucidate the roles of other protein kinases in mediating IL-17 signal to the nucleus.

ACKNOWLEDGMENTS

We acknowledge the research funding from the NIH/NCI and from the CTR Inc., to Dr. S. E. Adunyah. In addition, we are grateful to the National Heart Lung Blood Institute for research support through Meharry's Comprehensive Sickle Cell Center. Finally, we are grateful to Dr. Harold L. Moses of Vanderbilt Cancer Center for his valuable discussions.

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