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Interleukin-18 Activates the IRAK-TRAF6 Pathway in Mouse EL-4 Cells

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The pleiotropic biological activities of interleukin-18 (IL-18) are mediated by IL-18 receptor (IL-18R). When the ligand binds to the IL-18R, IL-18R initiates a signaling cascade that results in the activation of nuclear factor kappa B (NF-κB). When mouse EL-4 cells were exposed to IL-18, IL-1 receptor-associated kinase (IRAK) was recruited to IL-18R and was phosphorylated. In addition, tumor necrosis factor receptor-associated factor-6 (TRAF6) was associated with IRAK. Therefore, we concluded that IL-18/IL-18R-mediated signaling may share the IRAK/TRAF6 pathway through NF-κB activation with the IL-1/IL-1 receptor system. © 1998 Academic Press

Interleukin-18 (IL-18), previously known as interferon-γ-inducing factor (IGIF), is a multifunctional cytokine playing various regulatory roles in the immune system (1, 2). Thus far, induction of cytokine production such as interleukin-2 (IL-2), interferon-γ and granulocyte/macrophage-colony stimulating factor by T cells (2, 3), enhancement of natural killer cell activity (2, 3), inhibition of IgE production by activated B cells (4), activation of Fas ligand-mediated cytotoxicity of T helper type 1 (Th1) cell clones (5) and natural killer cells (6), and anti-tumor effects have been reported (7). However, the intracellular pathway by which IL-18 exerts such multiple functions through its receptor remain largely unknown. To investigate the basis of IL-18 receptor (IL-18R)-mediated signals, it is important to elucidate the molecular mechanisms of IL-18 signal transduction pathways leading to expression of a specific phenotype. We have been focusing on IL-18-induced early events in both the cytoplasm and the nucleus, which result in transcriptional activation of IL-18-inducible cytokine genes (e.g., IL-2). We have demonstrated that IL-18 rapidly induced activation of NF-κB in a Th1 clone (8). Recently, we identified IL-18R and that was previously known as an orphan receptor, interleukin-1 receptor related protein (IL-1Rrp) (9, 10). Transfection of IL-18R cDNA into COS cells resulted in functional receptor expression capable of initiating responses that lead to NF-κB activation (10). IL-18R shares some structural similarities with IL-1 receptor type I (IL-1RI). In particular, the cytoplasmic domain of IL-18R has significant sequence identity (35%) with that of IL-1RI. Interestingly, several amino acids in the IL-1RI cytoplasmic domain that are essential for activation of NF-κB are highly conserved between IL-1RI and IL-18R (10, 11). Furthermore, deletion or mutation of these amino acids resulted in loss of the coprecipitation of the serine-threonine kinase IRAK (12). Thus, IL-18 might share the signaling pathway with IL-1, at least as regards NF-κB activation. A recent study on the IL-1 activated NF-κB pathway has shown that IL-1 mediated association of the IL-1 receptor type I (IL-1RI) and the IL-1 receptor accessory protein (IL-1RAcp) leads to activation of the serine-threonine kinase IRAK (12-14). IL-1 stimulation induces the formation of a complex between IRAK and TRAF6 (15), further, the latter was found to associate with NF-κB-inducing kinase (NIK) (16), which activates a signaling cascade involving IκBα kinase (IKKα/CHUK). IKKα specifically modulates IκBα that sequesters the NF-κB in the cytoplasm (17, 18).

In this report, we show that IL-18 induces recruitment of IRAK to the IL-18R and the formation of a complex between IRAK and TRAF6.

MATERIALS AND METHODS

Cells and cell culture. The mouse cell line, EL-4, was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Dai-

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Abbreviations used: IL-18, interleukin-18; IL-18R, IL-18 receptor; IL-1, interleukin-1; IL-1RI, IL-1 receptor type I, NF-κB; nuclear factor-κB; IRAK, IL-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor-6.

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nippon Pharmaceutical Co., Ltd., Osaka, Japan), 60 μg/ml penicillin and 50 μg/ml streptomycin.

Cytokine and antibodies. Recombinant murine IL-18 was prepared from cultures of murine IL-18 cDNA-expressing *Escherichia coli* as described previously (1). Recombinant IL-1β was purchased from R&D Systems., Inc. (Minneapolis, MN). Anti-IRAK antibody was raised against a fused protein containing glutathione S-transferase and human IRAK (amino acids 546-712). Anti-TRAF6 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IL-18R-antibody was produced in rabbits immunized with a fusion protein a 6-amino-acid histidine fragment bound to the C terminal of the extracellular domain (residues 1-326) of mouse IL-18R (unpublished data).

Immunoprecipitation and in vitro kinase assays. Cells (2×10^7) were harvested after stimulation and suspended in 1 ml of lysis buffer (0.2%NP-40, 20mM Hepes-KOH [pH 7.8], 150mM NaCl, 1mM sodium vanadate, 5μg/ml aprotinin and 5μg/ml leupeptin). Lysates were cleared by centrifugation and mixed with 20 μl of protein-G-Sepharose (Pharmacia, Uppsala, Sweden) and 3 μl of anti-IRAK or anti-TRAF6 antibodies, followed by a 2 hr incubation at 4 °C. The beads were washed four times with 1 ml of the lysis buffer in the absence of protease inhibitors. The immunoprecipitates were eluted with Laemmuli's sample loading buffer, separated on a 7.5% or 12.5% SDS-polyacrylamide gel, and transferred to a PVDF membrane (Immobilon P, Millipore, Bradford, MA). The membranes were incubated with either 1:1000 diluted anti-IRAK antisera, 1:1000 diluted anti-IL-18R antisera or 1μg/ml anti-TRAF6 antibody for 1 hr at room temperature, washed three times with TBST (20mM Tris-HCl [pH 7.4], 150mM NaCl, 0.1% Tween 20), and then incubated with 1:5000 diluted horseradish peroxidase-conjugated swine anti-rabbit or anti-goat antibodies (DAKO, Glostrup, Denmark). After washing, immunocomplexes were visualized by a chemiluminescence system (ECL, Amersham, Buckinghamshire, England). IRAK activation was measure as described elsewhere (12). The IRAK immunocomplex was incubated in 20 μl kinase buffer with 10 μCi of [γ-^{32}P]ATP for 20 min at 30 °C. The phosphorylated proteins were separated by SDS-PAGE (7.5%) and visualized by autoradiography.

Electrophoretic mobility shift assays (EMSA). EMSA was performed as described previously (8), using a radiolabeled double-stranded oligonucleotide containing a murine IL-2 promoter NF-κB binding site (GGGATTTCACC).

RESULTS AND DISCUSSION

To investigate further the molecular events from IL-18R to NF-κB activation, it is important to clarify whether IL-18 activates signaling molecules common to the initial steps in IL-1-mediated NF-κB activation.

We used a murine Th0 -like cell line EL-4 to compare signalling by IL-18 and IL-1β in a cell type that expresses both IL-18R and IL-1R. It has been reported that IL-1 activates EL-4 and induces the production of IL-2 and interleukin-4 when costimulated with phorbol 12 -myristate-13-acetate (19). IL-18 also activates EL-4 cells and induces the production of IL-2 in the presence of anti-CD3ϵ antibody (Arai, N. *et al;* unpublished data).

Initially, we tested whether NF-κB was activated by IL-18. The cells were treated for 30 min with IL-18 or IL-1β, nuclear extracts were prepared, and binding activity to an oligonucleotide containing the NF-κB binding site was assessed by EMSA. As shown in Fig.1, IL-18 induced DNA binding activity of NF-κB to the same as IL-1β stimulation. Supershift assay revealed that the upper main band is composed of the p65/p50 heterodimer and the lower band of the p50/p50 homodimer of the NF-κB subunit (data not shown).

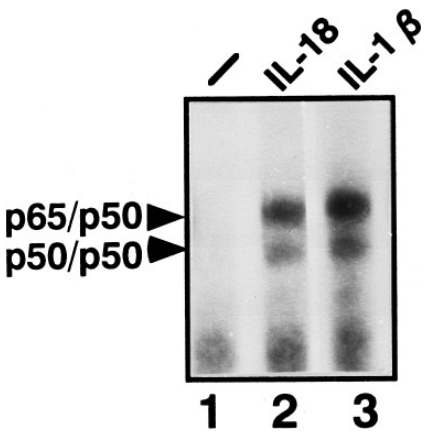

FIG. 1. IL-18-dependent NF-κB activation in EL-4 cells. After 1 h stimulation with 50 ng/ml of IL-18 or IL-1β, nuclear extracts were prepared from EL-4 cells. Lane 1: untreated, Lane 2: treated with IL-18, Lane 3 : treated with IL-1β, Arrowhead: NF-κB band.

As mentioned in the Introduction, recent studies have shown that the first step in the IL-1-induced NF-κB activation pathway is initiated by IRAK that is recruited to the IL-1RI in a ligand-dependent manner (13). To investigate whether the IL-18 induced association of IRAK with IL-18R was IL-18 dependent, we used protein immunoblot analysis to detect IL-18R in the IRAK immunoprecipitate before and after IL-18 treatment. Anti-IL-18R antisera recognized a protein of 90−105 kD in the IRAK immunocomplexes prepared from EL-4 cells treated with IL-18 (Fig.2, lane 4). No such protein was detected in immunocomplexes with untreated cells or immunocomplexes with normal rabbit serum (Fig.2, lane 1−3). In addition, specificity of the anti-IL-18R antibody used was confirmed by western blotting analysis. This 90-105 kD protein was also detected in extracts of chinese hamster ovary cells transfected with an expression vector containing the IL-18R cDNA, but not from mock-transfected cells, and antibodies raised against IL-1RI did not cross-react with the 90-105 kD protein (data not shown).

Furthermore, activation of IRAK was also examined by *in vitro* kinase assay. After cell stimulation for 10 min, IRAK was immunoprecipitated and kinase activity was examined. Both IL-18 and IL-1β treated cell extracts immunoprecipitated with anti-IRAK antisera contained kinase activity and resulted in the phosphorylation of a protein that co-migrated with IRAK (Fig.3, lane 5, 6). A faster migrating band was detected after immunoprecipitation with normal serum or with anti IRAK antiserum, and the density of this band seems

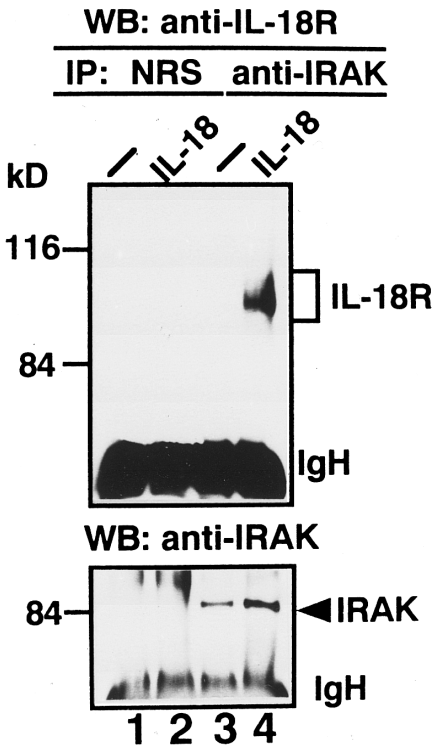

FIG. 2. Association of IRAK with IL-18R. Proteins were immunoprecipitated (IP) with antibody to IRAK or normal rabbit serum (NRS) from extracts of EL-4 cells, untreated (−) or treated with 50 ng/ml of IL-18 for 5 min. The immunoprecipitated proteins were separated by SDS-PAGE (7.5% gel) and then immunoblotted (WB) with rabbit antisera to IL-18R (upper panel). The filter was stripped and reprobed with anti-IRAK antisera (lower panel). NRS, normal rabbit serum; IgH, Immunoglobulin heavy chain.

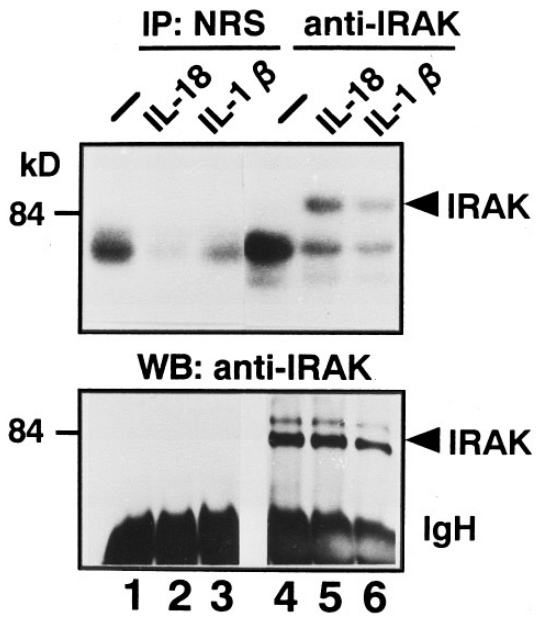

FIG. 3. IL-18-dependent activation of IRAK. Proteins were immunoprecipitated with antibody to IRAK from extracts of EL-4 cells untreated (−) or treated with 50 ng/ml IL-18 or IL-1β for 10 min. The immunoprecipitated proteins were divided into two aliquots. One aliquot was subjected to *in vitro* kinase assay (upper panel); the other was used in immunoblot analysis to detect IRAK (lower panel).

to decrease following IL-18 or IL-1stimulation. This band is now under investigation. IRAK activity was also detected in immunoprecipitates of extracts of an IL-18-stimulated human T cell line, MOLT-16, demonstrating that these phemonomena are not unique to EL-4 cells (data not shown). Taken together, when IL-18 associates with IL-18R, IRAK appears to be recruited to IL-18R and becomes phosphorylated.

It has been reported that a downstream molecule of IRAK is TRAF6 (15). In order to delineate the ligand-dependent association of IRAK and TRAF6, co-immunoprecipitation analysis was performed. Immunoprecipitation of TRAF6 demonstrated that IRAK associates with TRAF6 in both IL-18 and IL-1β treated cells (Fig.4, lane 2, 3), but not in untreated cells (Fig.4, lane 1).

In conclusion, IL-18 mediates the recruitment of IRAK to IL18R, and the formation of a complex between IRAK and TRAF6. This pathway may involve further downstream pathways to NF-κB activation. While this manuscript was in preparation; Robinson *et al.* reported that IRAK was also activated in mouse Th1 cells after IL-18 stimulation (20) and their results would support our conclusions.

There is a region of considerable sequence homology region which is reported to be critical for generating signals for NF-κB activation in IL-1RI and other members of the IL-1 receptor family such as ST2L *etc.* (21, 22). It is possible that these members may also activate

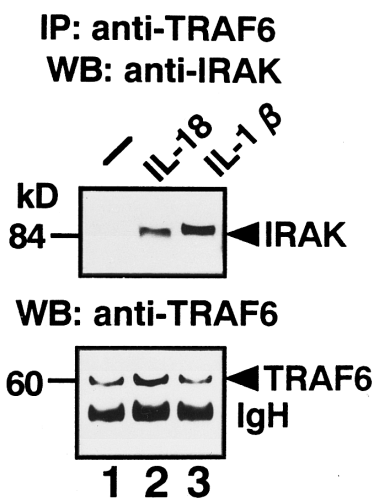

FIG. 4. IL-18-dependent association of TRAF6 with IRAK. Cells were stimulated as described in the legend of FIG.3. Proteins were immunoprecipitated with goat antibody to TRAF6. The immunoprecipitated proteins were separated by SDS-PAGE (12.5%) and then immunoblotted (WB) with rabbit antisera to IRAK (upper panel). The filter was stripped and reprobed with anti-TRAF6 antibody (lower panel).

the IRAK/TRAF6 pathway. If so, then the determinant for ligand-specificity is not clear at this point. We observed different IL-18R/IL-1RI expression patterns in different subsets of murine helper T cell clones. Th0-like mouse EL-4 cells possess both IL-18R and IL-1RI. Th1 clones express only IL-18R. In contrast, Th2 clones express only IL-1RI (unpublished observation). Differential expressions of these receptors may be one of the mechanisms for ligand specificity and this point should be clarified.

It has been proposed that recruitment of IL-1RAcp is a critical early step in the signaling cascade mediated by the IL-1RI activation complex (14). Whether IL-18R requires additional subunits to form a heteromeric receptor, IL-1RAcp or similar molecules, is a question that remains to be answered.

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