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JAK3 Janus kinase is involved in interleukin 7 signal pathway

Yi-Xin Zeng[a], Hiroshi Takahashi[b], Masao Shibata[c], Katsuiku Hirokawa[d,*]

[a]*Department of Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan*
[b]*Laboratory of Histochemistry, Mitsubishi Kasei Institute of Life Science, Kasei, Japan*
[c]*MBL Co., Nagoya, Japan*
[d]*Department of Pathology, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan*

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Abstract Interleukin (IL) 7 is an important cytokine regulating both T and B cell development and inducing the formation of lymphokine-activated killer cells and cytolytic T lymphocytes. This study reports the role of JAK family kinases in the IL-7 signalling pathway in a T cell clone. The results have shown that out of 4 members of JAK family tyrosine kinases (JAK1, JAK2, JAK3 and Tyk2), only JAK3 was tyrosine-phosphorylated and activated in cells of a T cell clone by stimulation with IL-7. Furthermore, STAT1α (STAT, the signal transducers and activators of transcription) and p44 of MAPK (mitogen-activated protein kinases) were tyrosine phosphorylated by IL-7 stimulation, indicating that the two signal pathways might be involved in IL-7 signal transduction.

Key words: Interleukin 7; JAK3 tyrosine kinase; Signal transduction; T cell; STAT; MAP kinase

1. Introduction

Interleukin 7 (IL-7) was originally discovered as a pre-B cell growth factor in long-term bone marrow culture system [1]. But subsequent studies showed that this factor elicited a variety of biological activities and affected T cells as well as pre-B cells [2,3]. More intriguingly, IL-7 is capable of induce the formation of lymphokine-activated killer (LAK) cells and cytolytic T lymphocytes (CTL) from both murine and human T cells [4,5]. Thus, IL-7 is an important factor for the regulation of both B and T cell development and may have therapeutic applications in cancer immunotherapy and treatment of immunodeficiency diseases. However, the mechanism by which IL-7 receptor transmit signals after IL-7 binding remains elusive, although the receptor for IL-7 was cloned several years ago [6] and protein tyrosine kinases might be involved in it [7].

A number of growth factor receptors, such as PDGF-R, FGF-R, NGF-R and IGF-R, have tyrosine kinase domains in their cytoplasmic regions and are capable of mediating biological response by activating their intrinsic kinase activity, while most of the cytokine receptors lack such kind of kinase activity and additional cytoplasmic tyrosine kinases are required to transmit signals from the receptors to downstream molecules [8]. In the last two years, a large number of studies identified the important role of JAK family tyrosine kinases (JAKs) in signal transduction of cytokines. Namely, JAKs are physically associated with the membrane-proximal region of specific receptors for cytokines and are phosphorylated and activated by the binding of ligand with corresponding receptors, resulting in the activation of downstream transcription factors called the signal transducers and activators of transcription (STATs) [9]. Recently, the fourth member of this family, JAK3, was cloned and found to be related with the signal pathway of interleukin 2 (IL-2) and interleukin 4 (IL-4) [10–12]. Since the third molecule of IL-2R, γ chain, also composes a part of receptors for IL-4 and IL-7 [13] and is physically associated with JAK1 and JAK2 tyrosine kinases [14], it is likely that JAK3 also plays a role in IL-7 signal pathway. Accordingly, in the present study, we examined whether the stimulation with IL-7 can induce the tyrosine phosphorylation and activation of JAK3 in a T cell clone and the result clearly showed tyrosine phosphorylation of JAK3 in dose- and time-dependent manners, when the cells were stimulated with IL-7. Moreover, in association with activation of JAK3, we found that STAT1 and mitogen-activated protein kinases (MAP Kinases) are also activated in the cell line by IL-7 stimulation.

2. Materials and methods

2.1. Cell culture

A murine T helper cell clone (YT-5) was kindly provided by Dr. Nariuchi (Inst. Med. Sci. Univ. of Tokyo). This cell line was maintained in RPMI-1640 media in the presence of 10% of fetal bovine serum (FBS) and 10% of culture supernatant of Con A-stimulated rat spleen cells, and receiving repeated stimulation by 40 Gy-irradiated C3H splenocytes every three weeks.

2.2. Generation of anti-JAK3 polyclonal antisera

By RT-PCR and cDNA cloning, we obtained a partial cDNA clone which was first identified as a novel member of JAK kinase family and later revealed to be identical to JAK3 [15]. Based on the cDNA sequence, a peptide corresponding to the hinge region between the two kinase domains of JAK3 (amino acid 924–938, SDPTPGIPSPRDELC) was synthesized and was coupled to keyhole limpet hemocyanin by glutaraldehyde and used for immunization of rabbits.

2.3. Immunoprecipitation, SDS-PAGE and Western blotting

Cells were harvested and starved in RPMI-1640 with 0.5% (w/v) of dialyzed bovine serum albumin (BSA) (Sigma) for 2–3 h before stimulation. Recombinant mouse IL-7 (UBI) was added and incubated for indicated time and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 100 μM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride) at a concentration of 2×10^7 cells/ml. Supernatant were separated from debris by centrifugation for 10 min at 4°C and were either directly used for SDS-PAGE as cell lysate or processed for immunoprecipitation as previously described by Harlow and Lane [16]. The cell lysates or immunoprecipitates were

*Corresponding author. Fax: (81) (3) 3813 1790.

Abbreviations: IL, interleukin; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, chemiluminescence.

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subjected to 10% or 6% SDS-PAGE, respectively. Gels were transferred electrophoretically to nitrocellulose membrane (Schleicher and Schuell). The membranes were blocked in 5% (w/v) of BSA in Tris-buffered saline solution (TBS), incubated in relevant primary antibody, and after washing were incubated in horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (DAKOPATTS, Denmark). Primary antibodies employed were anti-phosphotyrosine monoclonal antibody (4G10), polyclonal antisera against MAP kinase R1 (erk1-III), and MAP kinase R2 (erk1-CT), polyclonal rabbit antisera against JAK1 and JAK2. All these were purchased from UBI. Anti-TYK2 antibody and anti-STAT1 antibody (polyclonal E-23 and monoclonal C-136) were purchased from Santa Cruz Biotechnology. Bands in the washed membrane were detected by the enhanced chemiluminescence (ECL) system (Amersham Life Science). The used membranes were stripped for re-probing by washing once in 2% SDS (w/v), 100 mM 2-mercaptoethanol and 62.5 mM Tris-HCl, pH 6.8, at 50°C for 30 min and twice in PBS containing 0.1% (v/v) Tween-20 at room temperature for 10 min. Competition study was done by pre-incubation of the anti-JAK3 antisera with the JAK3 specific peptide (100 μg/ml of antisera) for 1 h at 4°C prior to addition of the mixture to cell lysate for immunoprecipitation.

2.4. *In vitro kinase assay*

The washed immunoprecipitates on protein A-Sepharose (Pharmacia) were further washed once with kinase buffer (50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 10 mM HEPES, pH 7.0) and incubated for 5 min at room temperature in equal volume of kinase buffer containing 0.25 mCi/ml [γ-^{32}P]ATP (Amersham Life Science). The reaction was stopped by adding equal volume of 2 × Laemmli's sample buffer (100 mM Tris-HCl, pH 7.5, 20% (v/v) glycerol, 2 mM dithiothreitol, 2% (w/v) SDS and 0.004% (w/v) bromophenol blue) and boiling for 3 min. The proteins were separated by 6% SDS-PAGE and transferred onto nitrocellulose membrane and detected by exposing to X-ray film. The filter was then probed by anti-JAK3 antisera.

3. Results and discussion

3.1. *Tyrosine phosphorylation of JAK family kinases induced by IL-7*

Studies carried out in a series of mutants which were selected for their inability to respond to interferon (IFN)-α/β or IFN-γ or both evidently demonstrated that JAK1 and TYK2 were essential for an IFN-α/β response and JAK1 and JAK2 were essential for an IFN-γ response [9]. In addition, the homodimerization of IL-6 signal transducer gp130 was shown to activate JAK2, JAK1 and to a lesser extent, TYK2 [9]. Recently, both JAK3 and JAK1 were shown to be involved in IL-2 signalling [11]. These data suggested the requirement for two or more kinases in cooperation in the cytokine signalling process, though in the cases of IL-3 and IL-5, only JAK2 was supposed to be involved. Thereby we chose to examine the phosphorylation pattern of all the four identified JAK kinases upon stimulation by IL-7. As shown in Fig. 1, only the tyrosine phosphorylation of JAK3 was up-regulated by IL-7 stimulation, although both JAK1 and JAK3 had a high expression level at both protein and mRNA levels (as detected by Northern blot analysis, data not shown). Probably in the case of IL-7, only one kinase is required presumably for signalling, at least in the cell line checked, or alternatively the phosphorylation of other JAKs was too weak to be detected in our system. On the other hand, we can not exclude the possible existence of novel members of JAK family involving in the process. Interestingly, in previous studies using human mature T cells [7,17], a protein

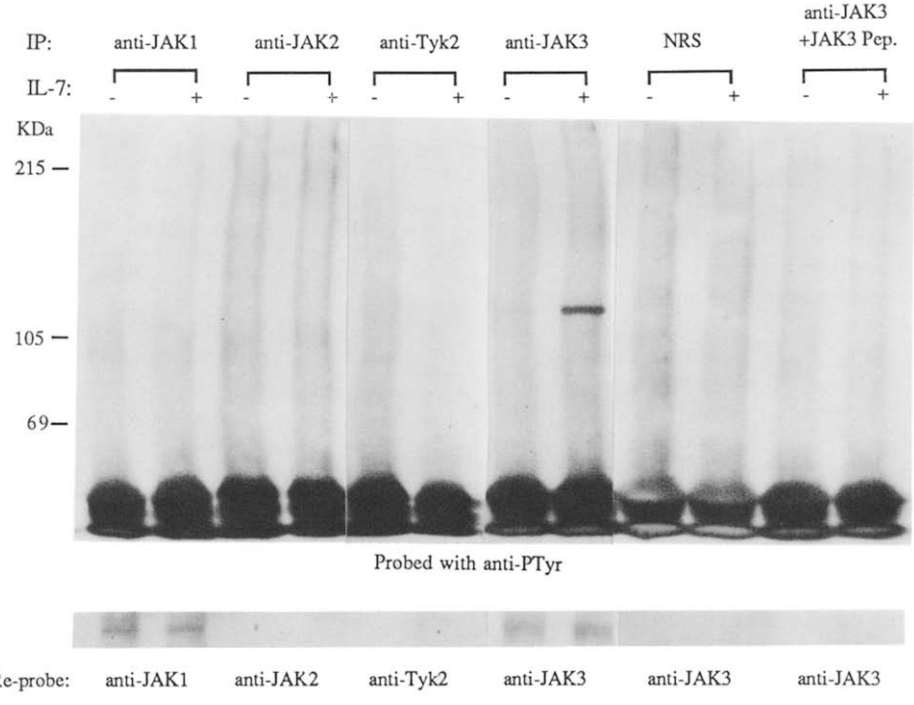

Fig. 1. Tyrosine phosphorylation of JAK family kinases in a helper T cell line (YT-5) by IL-7 stimulation. Cells (routinely, 2×10^7/lane was used) of YT-5 T cell clone were not stimulated (−) or stimulated (+) by IL-7 (20 ng/ml) for 5 min. The cells were lysed and immunoprecipitated (IP) by the indicated antibodies or normal rabbit serum (NRS) or JAK3 peptide-treated anti-JAK3 antisera (100 μg of peptide/ml of antisera) as described in section 2. The immunoprecipitates were separated by 6% of SDS-PAGE and transferred to nitrocellulose membrane. The filter was first probed with anti-phosphotyrosine monoclonal antibody 4G10 (anti-PTyr) and detected by ECL system. The used filter was then stripped and re-probed with the corresponding antibodies (bottom line) which were used for immunoprecipitation. Comparable amount of proteins were present in lanes of JAK1 and JAK3. However, tyrosine phosphorylation was observed only in the lane of anti-JAK3 with IL-7 stimulation.

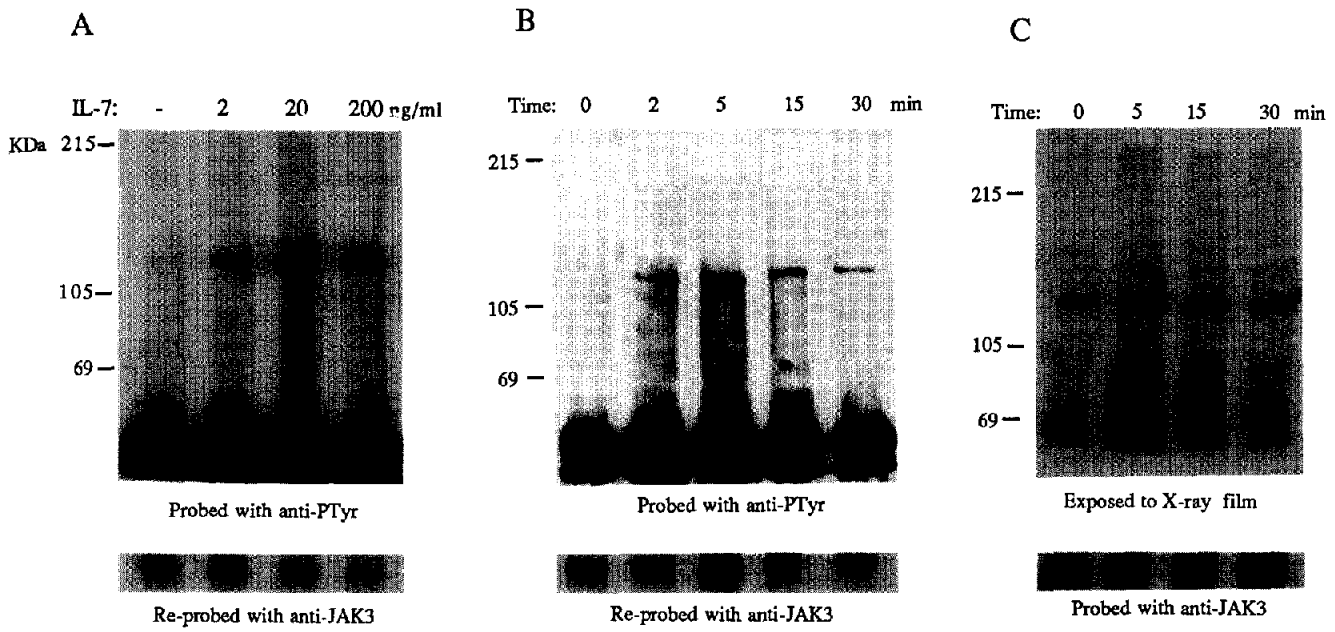

Fig. 2. Tyrosine phosphorylation and in vitro activation of JAK3 in a helper T cell line (YT-5) by IL-7 stimulation. (A) Dose-dependent tyrosine phosphorylation of JAK3. The cells were unstimulated (−) or stimulated by 2, 20, or 200 ng/ml of IL-7 for 5 min before cell lysis and then immunoprecipitated by anti-JAK3 antisera. The precipitated materials were electrophoresed in 6% SDS-PAGE, transferred to nitrocellulose membranes and analyzed by immuno-blotting with anti-phosphotyrosine antibody 4G10 (anti-PTyr). The same membrane was re-probed with anti-JAK3 antisera. The detection was done using ECL system. JAK protein was present in all lanes (bottom lines), but tyrosine phosphorylation was detected after the stimulation and peak response was seen at dose of 20 ng/ml. (B) Time course of tyrosine phosphorylation of JAK3. The cells were not stimulated (0) or stimulated with 20 ng/ml of IL-7 for the indicated time and analyzed as described in Fig. 2A. Peak response was seen at 5 min after the stimulation. Bottom line shows a comparison of JAK3 protein level. (C) In vitro autophosphorylation of JAK3. Unstimulated (0) or stimulated cells by 20 ng/ml of IL-7 for the indicated time were immunoprecipitated by anti-JAK3 antisera. The 6% SDS-PAGE-separated proteins were transferred onto nitrocellulose membrane which was then exposed to a X-ray film. The same membrane was again probed with anti-JAK3 antisera. The peak of in vitro autophosphorylation was seen at 5 min after the stimulation. The bottom line indicate JAK3 protein level.

of about 130 kDa was rapidly tyrosine phosphorylated when the cells were treated with IL-7. It is highly possible for that protein to be the human homolog of the murine JAK3 detected in our experiment. In order to confirm that the IL-7-regulated protein is JAK3, a competition experiment was done by using the JAK3 specific peptide which the anti-JAK3 antisera was raised against. The result showed a complete blockage by the peptide (Fig. 1).

3.2. *Dose- and time-dependent tyrosine phosphorylation and in vitro activation of kinase activity of JAK3 by IL-7*

Fig. 2A shows a dose-dependent tyrosine phosphorylation pattern of JAK3 induced by IL-7. Coincident with previous findings showing that at 1 ng/ml [7], IL-7 stimulated the tyrosine phosphorylation of four proteins including the 130 kDa one, we found that 2 ng/ml of IL-7 clearly up-regulated the magnitude of tyrosine phosphorylation of JAK3 and the increased dosage (20 ng/ml) enhanced the extent of phosphorylation greatly, but when further increased to 200 ng/ml, no obvious enhancing effect was observed. The protein of JAK3 detected with anti-JAK3 antisera by immuno-blotting was shown at the bottom of Fig. 2A,B,C. Thus we considered the concentration of 20 ng/ml as optimal and was used in all other experiments in this study.

To see the time course of tyrosine phosphorylation of JAK3, we stimulated the cells with IL-7 for various time before preparing cell lysate. Fig. 2B indicates that JAK3 was clearly phosphorylated 2 min after stimulation, peaked at 5 min and then diminished. To link the tyrosine phosphorylation with enzymatic activity, in vitro autophosphorylation was performed. The results in Fig. 2C show a similar time course of the kinase activity as seen in tyrosine phosphorylation (Fig. 2B); i.e. the peak was again observed at 5 min after stimulation. The kinase activity was not precipitated in the presence of competing JAK3 peptide (data not shown). These results strongly indicated that JAK3 was regulated by IL-7 for its tyrosine phosphorylation and kinase activity in vitro as well.

3.3. *Tyrosine phosphorylation of STAT1 and MAP kinase by IL-7*

STATs is a family of transcription factors composed of at least four members, and recent studies suggested its response to JAKs-mediated IFNs and IL-6 stimuli and its role in linking JAK kinases with cytokine-responsive gene expression [18,19]. In this study, when checking the phosphorylation pattern of whole cell lysate, we found that a protein with the same size as STAT1α (p91) was up-regulated for its tyrosine phosphorylation by IL-7 (Fig. 3, middle arrow). By re-probing with anti-STAT1 antisera which recognize both STAT1α and β (p91 and p84), the antibody identified a band at 90 kDa which comigrated with the above mentioned tyrosine phosphorylated protein (Fig. 3, right and upper panel). Another protein showing elevated tyrosine phosphorylation by IL-7 stimulation (Fig. 3, lower arrow) had a size of about 44 kDa and was revealed

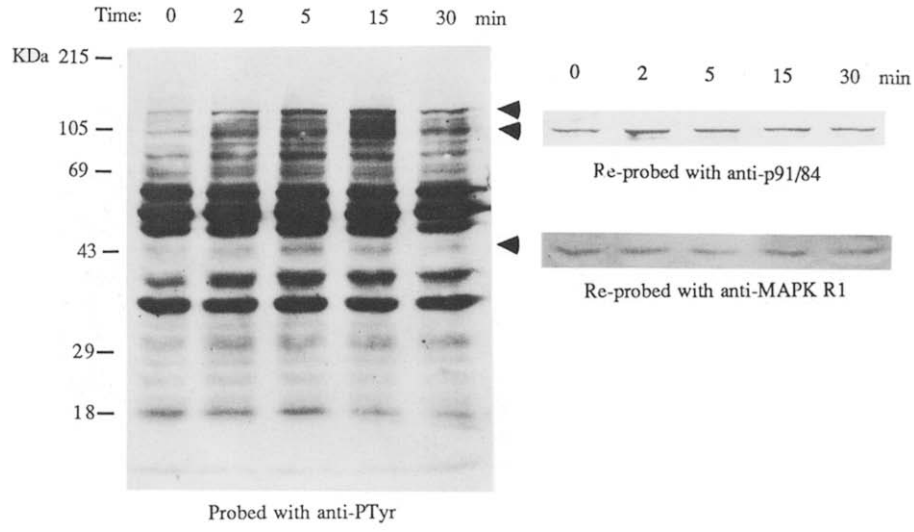

Fig. 3. Tyrosine phosphorylation pattern of the whole cell lysate of a helper T cell line (YT-5) stimulated by IL-7. The cells were not stimulated (0) or stimulated with 20 ng/ml of IL-7 by the indicated time and the cell lysate was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was first probed with anti-phosphotyrosine antibody 4G10 (anti-PTyr: the left figure) and then re-probed with anti-p91/84 (STAT1α: the right upper), anti-MAPK R1 (erk I-III: the right lower) and anti-JAK3 antibodies (data not shown), respectively. The upper arrow indicates the band detected by anti-JAK3, the middle arrow indicates the band detected by anti-p91/84 and the lower arrow indicates the band detected by anti-MAPK R1. Both STAT1α and p44 of MAPK were significantly tyrosine phosphorylated after IL-7 stimulation and the response peaked between 5 and 15 min.

presumably to be the p44 isoform of MAP kinases by re-probing with anti-MAP kinase R1 (anti-erk1-III) (Fig. 3, right and lower panel). These two proteins also showed a similar time-dependent kinetics of phosphorylation as observed in JAK3 (Fig. 3, upper arrow) which was detected by re-probing with anti-JAK3 (data not shown). To further prove these findings, we carried out immunoprecipitation experiments using two additional antibodies against MAP kinases (anti-erk1-CT) and STAT1 (monoclonal antibody C-136) which can be applied for immunoprecipitation. As expected and shown in Fig. 4A, the p44 isoform of MAP kinases showed a significant change in tyrosine phosphorylation when the cells were treated with IL-7; i.e. tyrosine phosphorylation first decreased 2 min after stimulation, increased thereafter and peaked 15 min after the treatment. STAT1α (the p91 isoform) also displayed a significant increase in tyrosine phosphorylation 15 min after the cells were

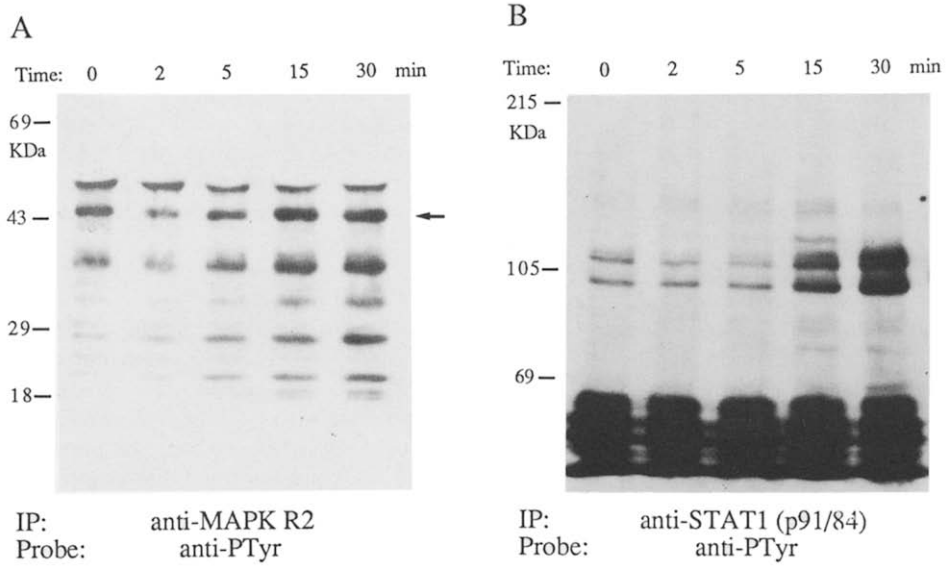

Fig. 4. Tyrosine phosphorylation of p44 of MAP kinases and STAT1α (p91) induced by IL-7 stimulation. The cells were not stimulated (0) or stimulated with 20 ng/ml of IL-7 for the indicated time. Cell lysates were prepared and immunoprecipitated (IP) either with: (A) polyclonal antiserum against MAP kinases (erk1-CT), or (B) monoclonal antibody against STAT1 (ISGF-3 p84/91). The immunoprecipitates were separated by 10% (A) or 6% (B) SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with anti-phosphotyrosine monoclonal antibody (4G10, anti-PTyr) and detected by ECL system. The arrows indicate the bands with appropriate size of p44 of MAP kinase (A) and p91 of STAT1 (B), respectively. Both of these proteins showed a significant level of tyrosine phosphorylation after IL-7 stimulation.

stimulated with IL-7 (Fig. 4B). Therefore, these data strongly support that two different signal pathways may be involved in IL-7 signalling.

In this respect, one report [7] demonstrated that two proteins of about 95 kDa and 45 kDa showed a significant level of tyrosine phosphorylation in human T cells after stimulation with IL-7, although they were not characterized by immunochemical method. In previous studies, a direct association between JAK1 and STAT1α (p91) was suggested in the IFNs and IL-6 signalling pathways [18,19], therefore a similar association between JAK3 and STAT1α might also exist in IL-7 singalling. Moreover, MAP kinases were tyrosine phosphorylated and activated by a number of cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M [20] and IL-12 [21], and tyrosine kinases were suggested to be required for the activation of MAP kinases [20]. On the other hand, phospholipase C-γ1, a key molecule in the phospholipid signal pathway after T cell antigen receptor (TCR) engagement, was not activated by IL-7 [16]. Taken together, these results may suggest that both the STAT1α and p44 of MAP kinases might be phosphorylated and activated by IL-7 induction and this IL-7-induced response may use different signalling pathways from the TCR.

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