Interleukin 2 induces rapid phosphorylation of cellular proteins in murine T-lymphocytes

Michiaki Kohno*, Shigeki Kuwata, Yuziro Namba and Masao Hanaoka

*Departments of *Biophysics* and *Pathology, Institute for Virus Research, Kyoto University, Shogoin-kawahara-machi, Sakyo-ku, Kyoto 606, Japan*

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When quiescent murine T-lymphocyte cells were stimulated by the addition of interleukin 2 (IL-2), they reinitiated DNA synthesis after a lag period of 5 h. Under these conditions, rapid but transient phosphorylation of two cellular proteins with M_r values of 27000 and 26000 was detected; maximal phosphorylation occurred within 10–15 min after the addition of IL-2. The protein of M_r 27000 contained phosphoserine, while the protein of M_r 26000 contained phosphothreonine.

Interleukin 2 *Protein phosphorylation* *T-lymphocyte* *DNA synthesis* *Cell proliferation*

1. INTRODUCTION

Interleukin 2 (IL-2) maintains the continuous proliferation of normal T-lymphocytes [1]. The ultimate effects of IL-2, induction of DNA synthesis and mitosis, occur after a sequence of events initiated by binding of IL-2 to a specific cellular receptor; the IL-2 receptor is expressed only on antigen- or lectin-activated lymphocytes but not on quiescent cells [2]. We have previously shown that IL-2 acts on the restricted process of a cell cycle which occurs 3–5 h before the beginning of DNA synthesis [3], although the molecular mechanism by which IL-2 produces its effect on cell multiplication has not been identified.

Receptors for some peptide growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin, contain an inherent, growth factor-dependent protein kinase activity specific for tyrosine [4]. In addition, calcium- and phospholipid-dependent, serine/threonine-specific protein kinase, kinase C, has recently been reported as a receptor for tumor promotors such as 12-*O*-tetradecanoyl phorbol-12-acetate (TPA) [5,6]. These observations suggest that phosphorylation of some cellular proteins by receptor kinases constitutes one of the first mitogenic responses of cells to growth factors. In fact, we and others have recently demonstrated that several growth factors can stimulate rapid phosphorylation of certain cellular proteins in a number of cell types [7–11].

In this study, we investigated whether IL-2 also induced phosphorylation of cellular proteins in murine T-lymphocytes.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The T-lymphocyte cell line (TN-9) whose proliferation depends on the presence of IL-2 was established from C3H mouse spleen cells, and had been maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 30% culture supernatant of Con A-stimulated rat spleen cells for more than 6 months.

2.2. *IL-2*

2.2.1. Source of IL-2

Male Wistar rat spleen cells (5×10^6 cells/ml) in RPMI-1640 medium supplemented with 2% FCS and 2 μg/ml Con A were incubated at 37°C for

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18 h. The culture supernatants harvested by low speed centrifugation (Con A sup) were used for the culture of the cells and for the purification of IL-2.

2.2.2. Assay of IL-2 activity

IL-2 activity was determined by its ability to induce DNA synthesis of TN-9 cells, measured by the incorporation of [^{3}H]thymidine into acid-insoluble material. The unit of IL-2 activity was determined as the dilution of a given solution which exhibits half-maximum stimulation of DNA synthesis on TN-9 cells.

2.2.3. Purification of IL-2

Con A sup (2.5 l) was concentrated, dialyzed against 20 mM Hepes, pH 7.0, and applied to a DEAE-cellulose column equilibrated with the same buffer. Elution from the column was carried out with a linear gradient of NaCl (0–0.2 M) and the pooled active fractions were further purified by passing through a Sephadex G-75 column equilibrated with 20 mM Hepes, 150 mM NaCl, pH 7.2. Partially purified IL-2 thus obtained had a specific activity of 1×10^5 units/mg protein, approx. 2000-fold purification was achieved.

2.3. *Radiolabeling of cells and cell lysis*

TN-9 cell suspension in RPMI-1640 medium supplemented with 30% Con A sup and 10% FCS (2×10^5 cells in 2 ml) was incubated at 37°C for 24 h. Culture fluid was replaced with 2 ml RPMI-1640 containing 10% FCS and incubation was continued for 12 h. After washing with phosphate-free Eagle's minimum essential medium containing 20 mM Hepes, pH 7.4, and 2% dialyzed FCS, cells suspended in the same medium (1×10^6 cells/ml) were incubated at 37°C for 20 min. The cells were labeled by the addition of 1 mCi/ml of [^{32}P]orthophosphate (carrier-free, JAERI, Japan) to the medium for 60 min. Purified IL-2 (10 units/ml) was then added to the medium and the cells were incubated for up to 30 min. Labeled cells were chilled, washed with ice-cold phosphate-buffered saline, then lysed and treated with nuclease as described in [7].

2.4. *Two-dimensional gel electrophoresis*

Cell lysates were subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) in 9.2 M urea for 2000 V·h followed by SDS-polyacrylamide gel electrophoresis on 10% or 12.5% slab gels [12]. After electrophoresis, proteins were visualized by silver staining, while ^{32}P-labeled polypeptides were located on dried gels by autoradiography using a fluorescent screen. For the detection of alkali-resistant phosphoproteins, gels were incubated in 1 M NaOH at 45°C for 1.5 h, then neutralized and dried [13].

2.5. *Phosphoamino acid analysis*

Gel portions from non-alkali-treated gels were incubated in 5.7 M HCl for 1 h at 110°C and released phosphoamino acids were recovered from the hydrolysate, separated by electrophoresis at pH 3.5 (pyridine-acetic acid H_2O, 1:10:189) on thin layer cellulose plates as described [7].

3. RESULTS

3.1. *Induction of DNA synthesis in TN-9 cells with IL-2*

TN-9 cells absolutely require IL-2 for their continuous proliferation. The cells maintained in the

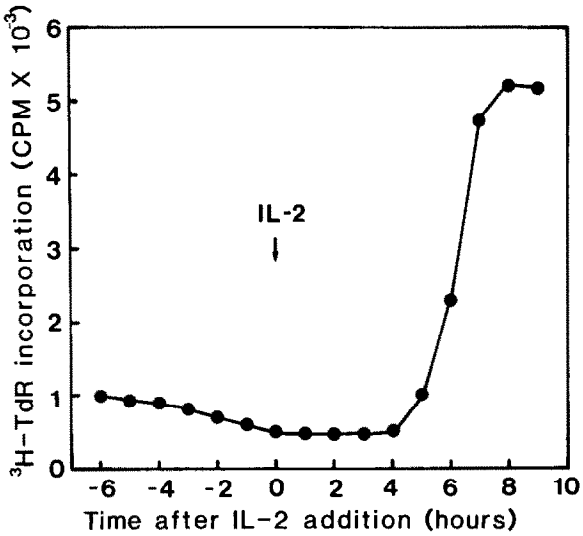

Fig.1. IL-2 induced DNA synthesis in IL-2 arrested TN-9 cells. TN-9 cells in RPMI-1640 medium containing 30% Con A sup and 10% FCS was incubated at 37°C for 24 h. Culture fluid was replaced with RPMI-1640 containing only 10% FCS, and incubation was continued for 12 h. DNA synthesis was reinitiated by the addition of 0.2 μCi [^{3}H]thymidine to 10^5 cells in 0.2 ml, and pulsing for 30 min at 37°C. Acid-insoluble radioactivity was determined as described [3]. Each value is the average of triplicate cultures.

presence of sufficient IL-2 ceased DNA synthesis rapidly upon transfer to IL-2-free medium, and began to die after 16 h. TN-9 cells precultured for 12 h without IL-2 reinitiated DNA synthesis after a lag period of 5 h when purified IL-2 (10 units/ml) was added (fig.1); cell number increased to 1.7-fold by 30 h after the addition of IL-2. These results suggest that the majority of the resting cells were stimulated synchronously by IL-2 to enter the DNA synthesizing stage leading to cell proliferation. Under such conditions, protein phosphorylation stimulated by IL-2 in TN-9 cells was analyzed.

3.2. *Protein phosphorylation stimulated by IL-2*

TN-9 cells cultured for 12 h without IL-2 were prelabeled for 60 min with $^{32}P_i$, exposed to 10 units/ml of IL-2 for 30 min, lysed and analyzed by two-dimensional gel electrophoresis. By comparing the autoradiographs of gels from IL-2-treated and control cultures, two proteins whose phosphorylation had reproducibly increased after

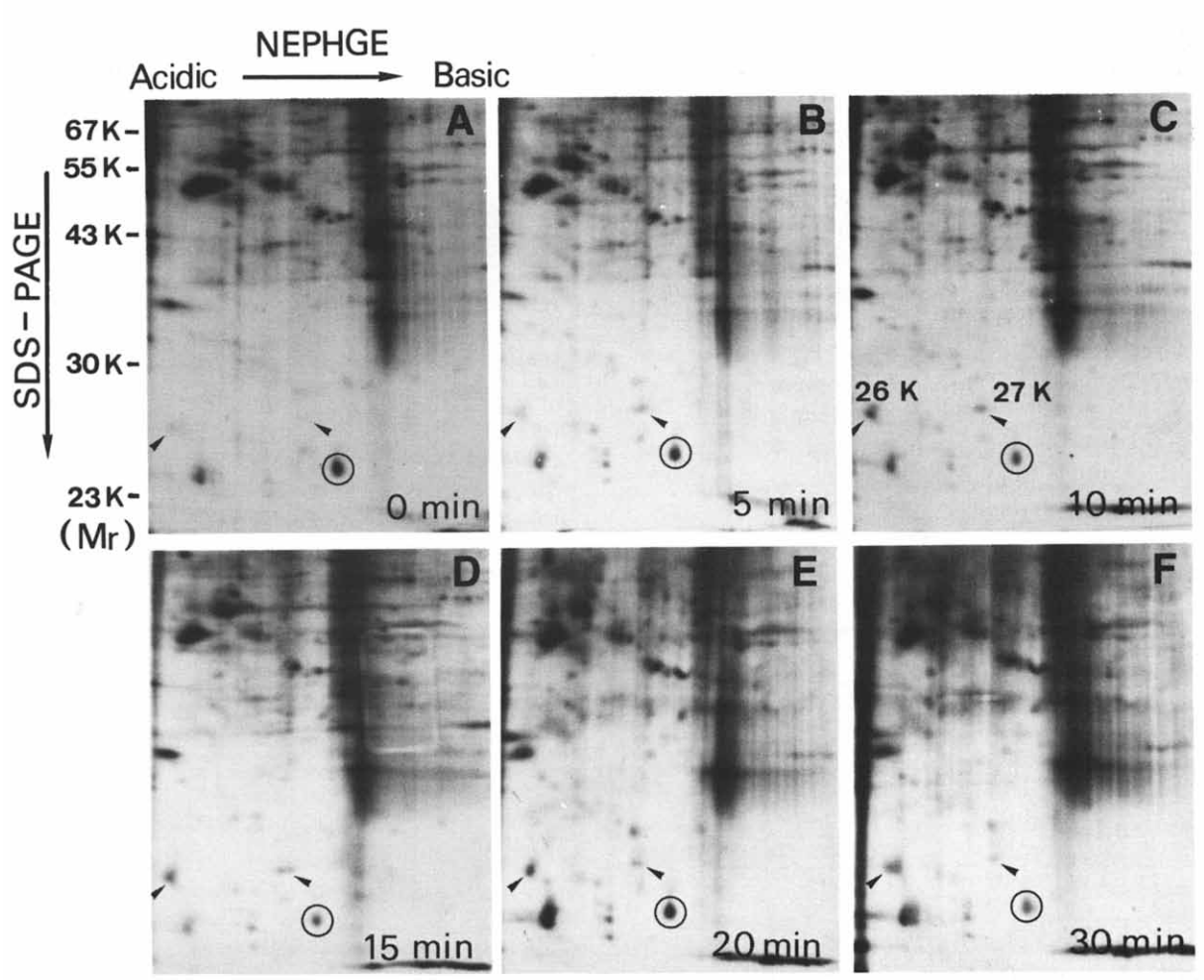

Fig.2. Phosphoproteins of TN-9 cells. IL-2 arrested TN-9 cells were labeled with ^{32}P, treated with 10 units/ml of IL-2 for 0 min (A), 5 min (B), 10 min (C), 15 min (D), 20 min (E), and 30 min (F), lysed and analyzed by two-dimensional gel electrophoresis as described in section 2.4. Each gel contained the lysates of about 1×10^5 cells. The gels were fixed, stained, dried and exposed to X-ray film for 24 h at −70°C. Arrowheads indicate the positions of the proteins with M_r values of 27000 (27 K) and 26000 (26 K) phosphoproteins, while the circle indicates the position of the phosphoprotein (M_r 24000).

IL-2 stimulation were found (fig.2); these proteins had apparent M_r values and apparent isoelectric points of about 27000 (p*I* 6.2) and 26000 (p*I* 4.7). These two phosphoproteins appeared to be minor species and were not identified by the silver staining method on two-dimensional gels. Cycloheximide did not prevent the increases in phosphorylation of the above mentioned proteins, when added at a concentration of 100 μg/ml 15 min prior to IL-2, suggesting that phosphorylation of pre-existing apoproteins of these phosphoproteins had occurred.

Figs 2 and 3 show the kinetics of phosphorylation of the proteins with M_r values 27000 and 26000 induced by IL-2. The extent of phosphorylation of the two proteins was quantitated by measuring the peak intensities of spots in autoradiographs and normalized by comparing them with that of the phosphoprotein, M_r 24000 (fig.2, encircled), in each film. The phosphoprotein (M_r 24000) was chosen as a reference because it showed little difference in phosphorylation with or without IL-2. Phosphorylation appeared rapidly after IL-2 treatment; maximal phosphorylation of the protein with M_r 27000 occurred within 10 min (12-fold) and that of the protein with M_r 26000 within 15 min (8-fold), then the level of phosphorylation of both proteins decreased gradually.

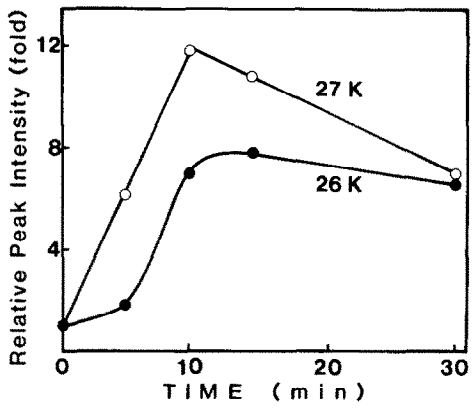

Fig.3. Kinetics of phosphorylation of the proteins (M_r 27000 and 26000) induced by IL-2. The relative peak intensities of phosphoproteins [M_r 27000 (27 K) and 26000 (26 K)], determined by densitometry of the autoradiographs and normalized by comparing them with that of phosphoprotein (M_r 24000) in each film (fig.2), are shown as the duration of time for IL-2 treatment.

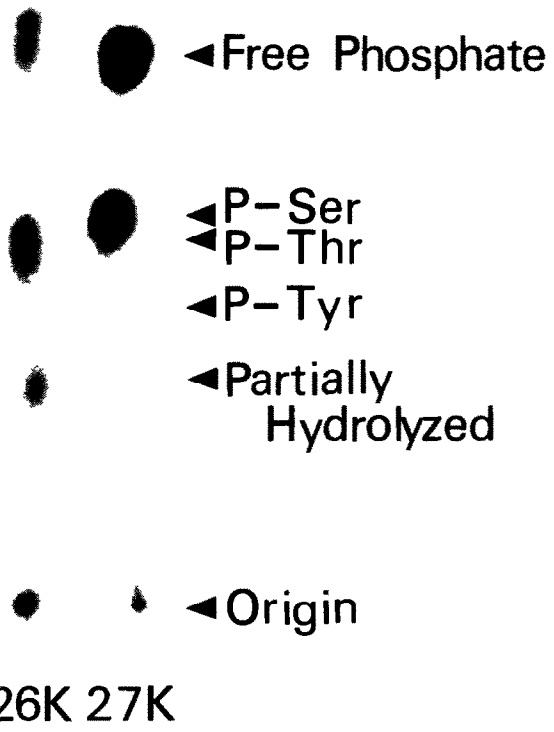

Fig.4. Phosphoamino acid analysis of the proteins (M_r 27000 and 26000). Portions containing the proteins [M_r 27000 (27 K) and 26000 (26 K)] were cut from two-dimensional gels of TN-9 cells labeled with ^{32}P and treated for 15 min with 10 units/ml of IL-2. Partial acid hydrolysis was performed and phosphoamino acids were separated on a cellulose plate by electrophoresis (1000 V, 60 min) at pH 3.5 as described [7]. Marker phosphoamino acids, phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), were identified by ninhydrin staining.

To know whether IL-2 induced the phosphorylation of these proteins on the tyrosine residue as various mitogens do in fibroblasts [7], the gels were treated with alkali [13] and re-exposed. Among the two IL-2 induced phosphoproteins shown in fig.2, only the protein of M_r 26000 was alkali-resistant (not shown), which suggests that it contains phosphotyrosine or phosphothreonine. To determine the phosphoamino acid content of the proteins (M_r 27000 and 26000), these phosphoproteins from IL-2-treated cells were excised from non-alkali-treated gels and subjected to

partial acid hydrolysis. As shown in fig.4, the protein with an M_r of 27000 contained phosphoserine, while the other protein contained phosphothreonine but not phosphotyrosine.

4. DISCUSSION

Treatment of IL-2 arrested T-lymphocytes with IL-2 induced the phosphorylation of two cellular proteins (M_r 27000 and 26000). The phosphorylation of these proteins appeared to be a rapid but transient response of cells to IL-2; it could be an early event in the transmission of the intracellular growth signal caused by IL-2. IL-2 stimulated the phosphorylation of the proteins mentioned above at serine or threonine but not tyrosine, suggesting that IL-2 activated some serine/threonine-specific protein kinase. Although some growth factor receptors have tyrosine kinase activity [4], the IL-2 receptor does not seem to have such an activity. Recently, the entire sequence of the human IL-2 receptor was determined [14,15]; its cytoplasmic domain has only 13 amino acid residues and it seems too small to harbor any kinase activity. The IL-2/IL-2 receptor interaction has been reported to produce rapid redistribution of kinase C from the cytosol to the plasma membrane, where it is activated [16]. In addition, TPA induced the phosphorylation of the two cellular proteins (unpublished). Thus, the protein kinase that directly participates in the phosphorylation of the proteins with M_r values of 27000 and 26000 seems to be kinase C. Studies are in progress on the cellular localization of these proteins and their biological significance in signaling cell growth.

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