Characterization of Interleukin 2 Stimulated 65-Kilodalton Phosphoprotein in Human T Cells†

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ABSTRACT: We have characterized the cellular proteins which are rapidly phosphorylated by interleukin 2 (IL 2) in a human IL 2 dependent cell line. When treated with IL 2, the phosphorylation of five proteins, 65, 50, 37, 24, and 21 kDa, was found in IL 2 dependent cell lines by two-dimensional gel electrophoretic analysis. After cell conversion from an IL 2 dependent state to an IL 2 independent state, one of the five phosphoproteins, the 65-kDa protein, became constitutively phosphorylated even without addition of IL 2. Also, in other IL 2 independent cell lines, such as KUT-2 and HUT-102, constitutive phosphorylation of the 65-kDa protein occurred without IL 2-stimulation. So our researchers were focused on biochemical characterization of the 65-kDa protein. It was found that the 65-kDa protein was one of the major cellular proteins by comparing the results of two-dimensional gel electrophoretic analysis of [^{32}P]P_i-labeled and [^{3}H]leucine-labeled cellular proteins and peptide mapping analysis. Subcellular fractionation studies indicated that the 65-kDa protein is a cytosol protein. The 65-kDa protein was purified from cytosol of a human T cell line, and its amino acid composition and amino acid sequences of its three oligopeptides were determined. It was found that the 65-kDa protein is identical with l-plastin.

Interleukin 2 (IL 2),[1] a T-cell-derived growth factor, has been shown to support the in vitro growth of T, B, and other lymphocytes [for a review, see Smith (1980)]. These cells are known to express receptor for IL 2 on their surface (Robb et al., 1981; Cantrell & Smith, 1980; Leonard et al., 1984), with recent studies demonstrating two classes, high- and low-affinity IL 2 receptors (Robb et al., 1984; Hatakeyama et al., 1989). Although the cell growth signal is believed to be transduced from the high-affinity IL 2 receptor (Fujii et al., 1986), little is known about the exact molecular mechanism of the intracellular signaling pathway leading to reinitiation of DNA synthesis induced by IL 2.

Among the variety of changes induced upon addition of growth factors to quiescent cells are changes in the phosphorylation of cellular proteins, which occurs extremely rapidly. Thus, particular attention has been focused recently on the possible role of protein kinases in the reinitiation of DNA synthesis. In fact, many growth factor receptors, such as those for epidermal growth factor (Ushiro & Cohen, 1980), insulin (Kasuga et al., 1981), platelet-derived growth factor (Ek et al., 1982; Nishimura et al., 1982), insulin-like growth factors (Jacobs et al., 1983; Rubin et al., 1983), and colony stimulating factor 1 (Sherr et al., 1985), have an intracellular tyrosine kinase domain that has been implicated in transduction of the mitogenic signal [for a review, see Hunter and Cooper (1985)]. Other serine/threonine kinases can also be activated by the interaction of growth factors and their receptors. A likely candidate is the Ca^{2+}/phospholipid-dependent enzyme protein kinase C, which is known to be a receptor for the tumor promoters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which stimulate the kinase activity (Castagna et al., 1982; Niedel et al., 1983). Protein kinase C activation has been proposed to mediate the responses of many extracellular ligands in a variety of cell signaling systems [for a review, see Nishizuka (1984)]. Taken together, these observations suggest that the phosphorylation of certain cellular proteins by receptor kinases or protein kinase C may constitute one of the earliest mitogenic responses of cells to growth factors. In fact, some reports have demonstrated that several growth factors can stimulate rapid phosphorylation of cellular proteins at tyrosine, serine, and/or threonine in a number of cell types (Cooper et al., 1982; Nakamura et al., 1983; Kohno, 1985).

In IL 2 and its receptor system, IL 2 dependent cell growth has recently been suggested to be closely related to the activation of protein kinase C (Farrar & Anderson, 1985; William & Francis, 1986), although the precise mechanism of its activation remains to be elucidated. In this context, we and others have found that IL 2 rapidly induces phosphorylation of some cellular proteins in murine (Kohno et al., 1986) and human IL 2 dependent T cells (Farrar et al., 1986; Ishii et al., 1987; Saltzman et al., 1988).

In the present study, we have further analyzed the IL 2 stimulated serine phosphorylation of the 65-kDa protein in IL 2 dependent and independent human T lymphocytes. We have also purified the 65-kDa protein from the cytosol fraction of a human T cell line and characterized its biochemical nature.

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[1] Abbreviations: ATL, adult T cell leukemia; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); IL 2, interleukin 2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; protein kinase C, Ca^{2+}/phospholipid-dependent enzyme; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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Materials and Methods

Chemicals. Recombinant human IL 2 (rIL 2) was kindly supplied by Takeda Chemical Industries, Osaka, Japan. The sources of other chemicals and culture reagents purchased for use in this work were as follows: *Staphylococcus aureus* nuclease (code NFCP), deoxyribonuclease (code DPFF), and ribonuclease A (code RASE), Worthington Biochemicals; *Staphylococcus aureus* V_8 protease, Miles Laboratories; sodium orthovanadate, Sigma Chemical Co.; urea, Schwarz/Mann; ampholyte, LKB–Produkter AB; poly(ethylene glycol) 20000 and lysyl endopeptidase from *Achromobacter lyticus*, Wako Pure Chemicals; DE-52, Whatman Chemical Separation; RPMI 1640 medium, Nissui Laboratories; fetal calf serum, Microbiological Associates; [^{32}P]P_i, New England Nuclear; [^{3}H]leucine, ICN Radiochemicals; [^{35}S]methionine, Amersham International; Enhance, New England Nuclear.

Cells. UW-4B is an IL 2 dependent cell line derived from PBL of an adult T-cell leukemia (ATL) patient. This cell line originated from UW-4. Although UW-4 is unstable in the absence of IL 2, UW-4B is a variant which can be cultured without any cell death for 48 h in the absence of IL 2. The protein content of UW-4B does not decrease for 24 h in the absence of IL 2. UW-4X is the IL 2 independent spontaneous variant of UW-4B cells selected by the long-term culture of UW-4B in IL 2 free culture medium. KUT-2 and HUT-102, the ATL-derived IL 2 independent cell lines, were also used. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. This medium is referred to as RPMI complete medium. For the culture of UW-4B cells, this medium was supplemented with human recombinant IL 2 (10 ng/mL).

Radiolabeling of Cells. IL 2 independent cells were labeled during exponential growth, while IL 2 dependent cells (UW-4B) were preincubated in medium without IL 2 for 20 h before labeling. The radiolabeling procedure for cells was as follows: 5×10^6 cells were incubated in 1 mL of phosphate-free medium containing 1 mCi of [^{32}P]P_i at 37 °C for 60 min in plastic tubes. Cells were then harvested and washed twice with chilled PBS in microtubes. For IL 2 treatment, 20 ng/mL human rIL 2 was added to each culture 15 min before the cells were harvested. The cells were also labeled with [^{3}H]leucine (50 μCi/mL) or [^{35}S]methionine (50 μCi/mL) under the conditions described above for 2 h at 37 °C in the medium lacking each amino acid.

Preparation of Cell Lysates. The pelleted cells were resuspended in 150 μL of 20 mM Tris-HCl, pH 8.8, containing 2 mM $CaCl_2$, *Staphylococcus aureus* nuclease (50 μg/mL), and 100 μM sodium orthovanadate and were lysed in 50 mM Tris-HCl, pH 7.0, containing 0.25% SDS, 1% 2-mercaptoethanol, 1 mM PMSF, deoxyribonuclease (100 μg/mL), ribonuclease A (50 μg/mL), and 5 mM $MgCl_2$. The lysates were kept in ice for 1 min and then mixed with (final concentrations) 9.5 M urea, 4% NP-40, 5% 2-mercaptoethanol, and 2% ampholytes, pH 5–8. The final volume of the sample was 600 μL/5×10^6 cells. All samples were rapidly frozen and stored at −70 °C until use.

Two-Dimensional Gel Electrophoresis. Cell lysates were subjected to isoelectric focusing in 9.2 M urea at 7000 V·h with pH 5 through pH 8 ampholytes followed by SDS–polyacrylamide gel electrophoresis on 9% slab gels. After electrophoresis, the gels were fixed with 10% trichloroacetic acid, 10% acetic acid, and 30% methanol and dried. [^{32}P]P_i-labeled polypeptides were located by autoradiography, a fluorescent screen being used. Gels containing samples which were to be subjected to peptide analysis were dried without fixation, after being washed with 20% methanol. [^{3}H]Leucine- and [^{35}S]-methionine-labeled polypeptides were located by fluorography after treatment with Enhance.

Peptide Mapping. One-dimensional peptide mapping was carried out as described by Cleveland et al. (1977) using *S. aureus* V_8 protease.

Subcellular Fractionation. Subcellular fractionation was carried out according to the method of Farrar and Anderson (1985) with some modifications. Briefly, the culture cells, UW-4B, were washed first with PBS and then with 0.25 M sucrose containing 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine, and 1 mM PMSF, pH 7.4 (SEAT buffer). Cells were then resuspended in SEAT buffer (1×10^7 cells/mL) and were lysed by pipetting the suspension 30 times with a disposable 26-gauge needle/syringe on ice. Nuclei and any unbroken cells were removed by centrifugation at 200*g* for 5 min. The supernatant was centrifuged at 100000*g* for 60 min to separate the membrane fraction of the resulting pellet from the cytosol fraction of the supernatant. Finally, for electrophoresis, urea, NP-40, 2-mercaptoethanol, and ampholytes were added to both subcellular fractions, and the samples were stored at −70 °C until use.

Phosphoamino Acid Analysis. Dried polyacrylamide gel pieces containing phosphoprotein as identified by autoradiography were washed for 2 h at 37 °C with 20 mL of 10% methanol. The gel pieces were then dried at 70 °C for 60 min and extracted with 2 mL of 50 mM NH_4HCO_3 containing 100 μg of TPCK-trypsin for 24 h at 37 °C. After centrifugation, extracts were lyophilized and hydrolyzed in 0.3 mL of 6 N HCl for 2 h at 110 °C. Each sample was diluted with 2 mL of water, lyophilized, dissolved in 0.5 mL of water, and lyophilized again. The lyophilized materials were finally dissolved in 10 μL of water containing 1 mg/mL each of phosphotyrosine, phosphoserine, and phospothreonine, and applied onto cellulose thin-layer plates. Electrophoresis was performed at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by volume) at 1000 V for 60 min. Standard phosphoamino acids were detected by reaction with ninhydrin, while radioactive amino acids were detected by autoradiography.

Purification of the 65-kDa Protein. From a 5-L culture of a human T-cell line (Jurkat cell), approximately 5×10^9 cells were harvested and washed with Dulbecco's PBS. Packed cells (8 g) were suspended in 10 volumes of a solution containing 25 mM Tris-HCl, 25 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM PMSF, pH 7.8, and broken at 4 °C by 40–60 strokes with a tight-fitting pestle in a Dounce homogenizer. The homogenates were centrifuged at 10000*g* for 30 min, and the supernatant was dialyzed against 65% saturated ammonium sulfate solution (pH 6.2 adjusted with ammonia water) at 4 °C to a final concentration of 55% saturation at equilibrium. The final turbid fluid was centrifuged at 6000*g* for 30 min, after which the supernatant was brought to 75% saturation by adding saturated ammonium sulfate solution (pH 6.2). The precipitate, collected by centrifugation, was dissolved in 30 mL of sodium phosphate buffer (20 mM, pH 7.5), dialyzed against the same buffer, and applied to a DEAE-cellulose column (DE-52). After extensive washing of the column with 20 mM sodium phosphate buffer, pH 7.5, adsorbed material was eluted with a salt and pH gradient (20 mM sodium phosphate buffer, pH 7.5, 100 mM sodium phosphate buffer, pH 5.5, and 150 mM NaCl). Each fraction from the DEAE-cellulose column was analyzed by two-dimensional gel electrophoresis, and the fractions containing the 65-kDa protein were pooled, concentrated with poly(ethylene glycol) 20000, and then gel filtrated through Sephadex G-100

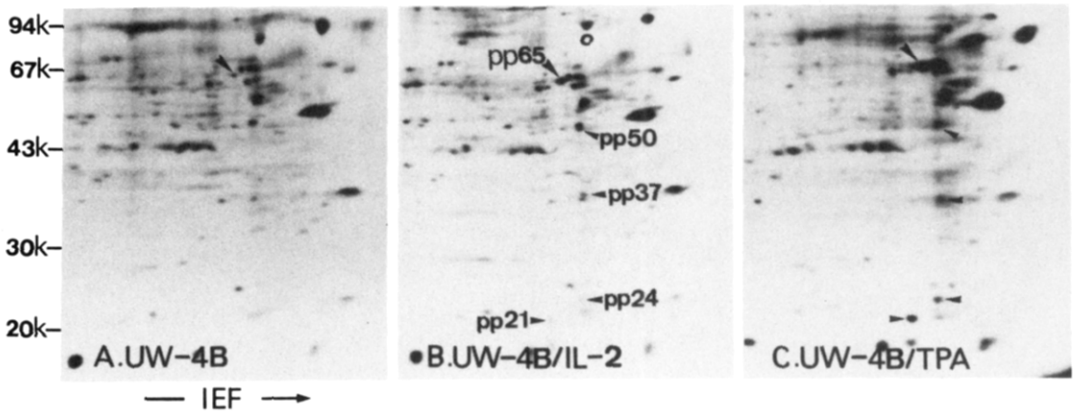

FIGURE 1: Analysis of phosphoproteins of UW-4B cells by two-dimensional gel electrophoresis. UW-4B cells precultured in IL 2 free medium for 20 h were labeled with 1 mCi/mL [^{32}P]P_i for 60 min and then treated with 20 ng/mL rIL 2 (B) or 10 ng/mL TPA (C) for 15 min at 37 °C. (A) Control without any stimulation. Cells were lysed and analyzed by two-dimensional gel electrophoresis as described under Materials and Methods. Each gel contained the lysates of about 2.5×10^5 cells. The gels were fixed, stained, dried, and exposed to film. The acidic end of the gel is to the right and the basic end to the left. The arrowheads indicate the positions of phosphorproteins of M_r 65K, 50K, 37K, 24K, and 21K.

equilibrated with sodium phosphate buffer (50 mM, pH 6.8). The 65-kDa protein-containing fractions were then applied to a hydroxylapatite column (5 cm^3 bed volume), and linear gradient elution was carried out with sodium phosphate buffer (50–100 mM). The 65-kDa protein, eluted with 80–85 mM sodium phosphate buffer, was stored at −20 °C.

Preparation and Sequencing of Cleaved Peptide Fragments. The purified 65-kDa protein was reduced with 20 mM dithiothreitol in 0.2% SDS and 200 mM Tris-HCl buffer, pH 8.3, and alkylated with iodoacetate (50 mM final concentration). After dialysis against 50 mM Tris-HCl buffer (pH 7.5), urea was added to the final concentration of 4 M and incubated at 30 °C for 1 h. The sample was then diluted 2-fold with 50 mM Tris-HCl buffer, and lysyl endopeptidase was added to a final concentration of 4 μg/mL. After the incubation at 32 °C for 4 h, the cleaved peptides were separated by reverse-phase HPLC on a 250 × 4.6 mm reverse-phase column (TSKgel ODS-120T, Tosoh, LTD). The starting solvent was water with 0.05% trifluoroacetic acid, and the limiting solvent was 70% isopropyl alcohol with 0.05% trifluoroacetic acid. The purified peptides were subjected to gas-phase automated Edman degradation in a Applied Biosystems sequenator, Model 470A, as described by Henderson et al. (1980).

Results

IL 2 Stimulated Protein Phosphorylation. Protein phosphorylation in the IL 2 dependent human T-lymphocyte cell line, UW-4B, was analyzed by two-dimensional gel electrophoresis. By comparison of autoradiographs of gels of IL 2 treated and control cultures, at least five proteins whose phosphorylation had reproducibly increased after IL 2 stimulation were found; these proteins had apparent molecular weights of 65 000, 50 000, 37 000, 24 000, and 21 000, respectively (Figure 1A,B). Phosphorylation of the five proteins appeared rapidly and reached a maximum after 15 min after IL 2 treatment (data not shown). Increased phosphorylation of the five proteins described above was also observed when the cells were exposed to 10 ng/mL TPA; the extent of phosphorylation induced by TPA was higher than that induced by IL 2 (Figure 1C).

Protein Phosphorylation in IL 2 Independent Variants. We isolated a variant, UW-4X, that showed continuous growth in the absence of IL 2 (Namba et al., unpublished results). UW-4X cells were labeled with [^{32}P]orthophosphate without IL 2 stimulation, and cellular phosphoproteins were analyzed

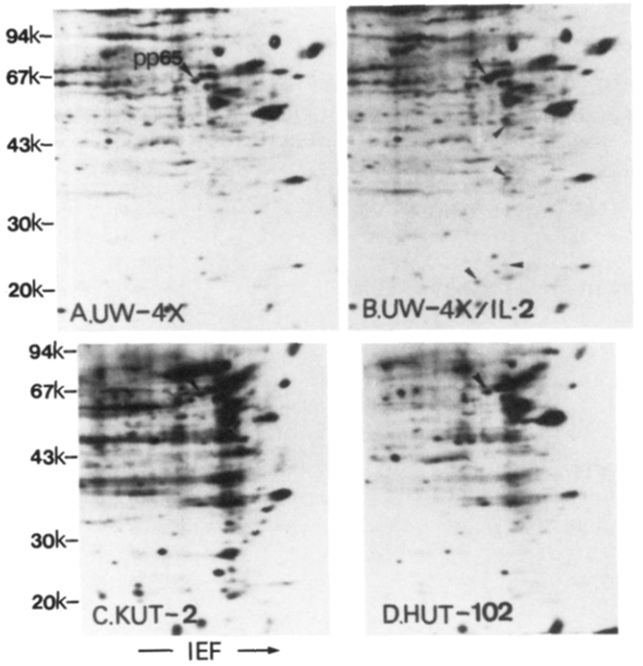

FIGURE 2: Analysis of phosphoproteins of IL 2 independent cells. UW-4X, KUT-2, and HUT-102 cells were labeled with [^{32}P]P_i and mock-treated (A, C, D) or treated with 20 ng/mL rIL 2 for 15 min (B). Labeled phosphoproteins were analyzed by two-dimensional gel electrophoresis as described in the Figure 1 legend. Gels shown in (A) and (B) contained the lysates of about 2.5×10^5 UW-4X cells; (C) 3×10^5 KUT-2 cells; and (D) 2×10^5 HUT-102 cells.

(Figure 2A). In these cells, the 65-kDa protein was constitutively phosphorylated to a higher extent when compared with that in IL 2 dependent cells without IL 2 stimulation (see Figure 1A). As with UW-4B (see Figure 1B), addition of IL 2 to UW-4X cells enhanced phosphorylation of the 65-kDa protein as well as four other proteins (Figure 2B).

High degrees of constitutive phosphorylation of the 65-kDa protein were also observed in other IL 2 independent cell lines such as KUT 2 and HUT 102 without the addition of IL 2 (Figure 2C,D). Thus, the phosphorylation of the 65-kDa protein seemed particularly interesting, and it was further characterized in detail.

The phosphorylated residues in the 65-kDa phosphoprotein were examined by cellulose electrophoresis of hydrolyzed phosphoamino acids. As shown in Figure 3, a spot corresponding to phosphoserine, but not to phosphothreonine and

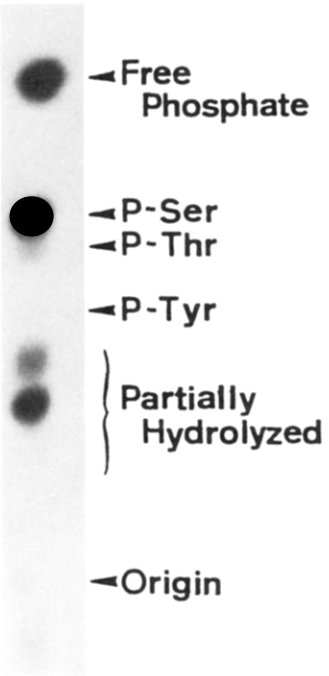

FIGURE 3: Phosphoamino acid analysis of the 65-kDa phosphoprotein. A portion containing the 65-kDa protein was cut from a two-dimensional gel of UW-4B cells labeled with [^{32}P]orthophosphate and treated for 15 min with 20 ng/mL human rIL 2. Partial acid hydrolysis was performed, and phosphoamino acids were separated on a cellulose plate by electrophoresis (1000 V, 60 min) at pH 3.5 (pyridine/acetic acid/H_2O, 1:10:189, by volume) as described under Materials and Methods. Marker phosphoamino acids are phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr), identified by ninhydrin staining.

phosphotyrosine, was demonstrated in the 65-kDa phosphoprotein.

Identification of the 65-kDa Apoprotein. The 65-kDa phosphoprotein (pp65) was also detected on two-dimensional gel electrophoresis of UW-4B cells prelabeled with [^{3}H]leucine and then treated with 20 ng/mL IL 2 for 15 min (Figure 4B); in contrast, it was only slightly observed in IL 2 free control cultures (Figure 4A). In addition, the [^{3}H]leucine-labeled pp65 spot on gels of UW-4X cells without IL 2 treatment was apparently increased in intensity (Figure 4C) when compared to that in UW-4B IL 2 free control cultures. This result parallels the constitutively high phosphorylation of this protein in UW-4X cells.

A 65-kDa protein (p65) spot was also detected in close vicinity to the position of the pp65 at its slightly basic side upon two-dimensional gel electrophoresis of cells labeled with [^{3}H]leucine (Figure 4). The ratio of pp65 to p65, determined by measuring the intensity of autoradiogram spots with a densitometer using each actin protein spot as an internal reference, was less than 1/10 in IL 2 stimulated UW-4B or in UW-4X cells. In order to determine whether p65 is the unphosphorylated form of pp65, we compared the peptide maps of the [^{35}S]methionine-labeled p65 and pp65 by partial proteolysis with *S. aureus* V_8 protease. The proteolytic cleavage patterns for these two proteins were almost identical with each other (Figure 5, lanes 1–4). The peptide maps of [^{35}S]methionine-labeled pp65 and [^{32}P]P_i-labeled pp65 were also compared; all [^{32}P]P_i-labeled peptide fragment bands well, coinciding with those of some [^{35}S]methionine-labeled fragments (Figure 5, lanes 5–8). All these results strongly indicate that [^{32}P]P_i-labeled pp65 and [^{35}S]methionine-labeled pp65 analyzed are the same molecule and that the p65 represents the unphosphorylated form, or apoprotein, of pp65.

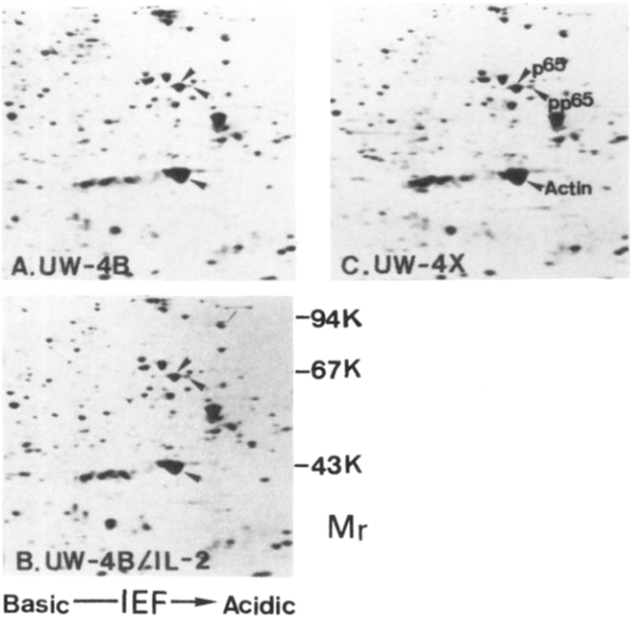

FIGURE 4: Analysis of cellular proteins labeled with [^{3}H]leucine by two-dimensional gel electrophoresis. UW-4B (A, B) precultured in IL 2 free medium for 20 h and UW-4X cells (C) were labeled with 50 μCi/mL [^{3}H]leucine for 2 h. UW-4B cells were treated with 20 ng/mL rIL 2 for 15 min before the cells were harvested (B). Labeled proteins were analyzed by two-dimensional gel electrophoresis as described under Materials and Methods. Each gel contained lysates of about 2×10^5 cells. The arrowheads indicate the positions of the 65-kDa protein (p65, left) and the 65-kDa phosphoprotein (pp65, right), respectively. Actin was used for position control.

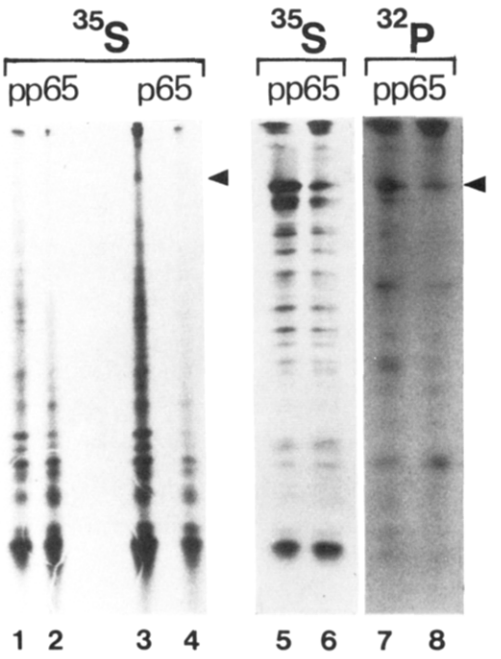

FIGURE 5: Partial proteolytic peptide mapping of p65 and pp65. The spots corresponding to [^{35}S]methionine-labeled p65 (lanes 3 and 4) and pp65 (lanes 1, 2, 5, and 6) and the spots corresponding to [^{32}P]P_i-labeled pp65 (lanes 7 and 8) from IL 2 treated UW-4B cells were excised, inserted in the wells of SDS–PAGE (15% acrylamide concentration), and overlaid with buffer containing 100 ng (lanes 1–4) or 25 ng (lanes 5–8) of *S. aureus* V_8 protease as described by Cleveland et al. (1977). [^{35}S]Methionine-labeled polypeptides were located by fluorography after treatment with Enhance, while [^{32}P]P_i-labeled polypeptides were located by autoradiography. Arrowheads indicate the position of the unhydrolyzed p65.

Subcellular Localization of the 65-kDa Protein. The 65-kDa protein was a rather abundant cellular protein in T and B lymphocyte and monocyte cell lines, regardless of whether

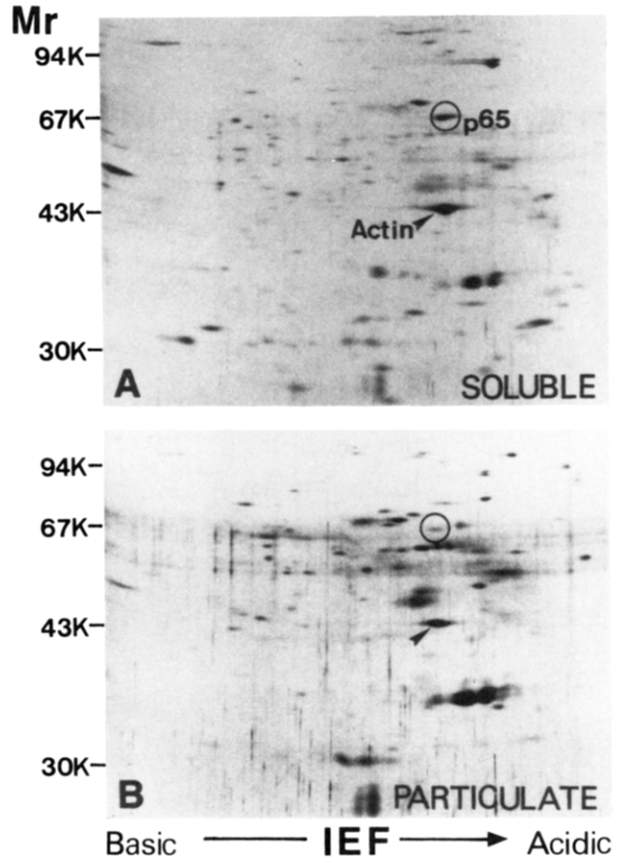

FIGURE 6: Subcellular location of the 65-kDa protein. Cytosol fractions (A) and particulate (B) were prepared from UW-4B cells as described under Materials and Methods. Each fraction was subjected to two-dimensional gel electrophoresis, and proteins were visualized by staining with silver nitrite. A circle surrounds the position of p65, while an arrowhead indicates the position of actin.

of not those cells were bearing HTLV-I. Although a lower amount of p65 was also found in a myeloid cell line, K562, it was not detectable in normal fibroblasts when whole cell lysates were analyzed by two-dimensional gel electrophoresis (Table I). Next, particulate and cytosol fractions were prepared from UW-4B cells, and subcellular localization of the p65 was examined. As shown in Figure 6, the majority of the p65 was found in the cytosol fraction.

Purification of the 65-kDa Protein. Using fractionation by ammonium sulfate precipitation, we recovered more than 80%

Table I: Semiquantitative Analysis of the 65-kDa Protein of Various Human Cells[a]

cells	65-kDa protein
normal lymphocyte	
PBL	+++
ATL-derived T cell lines	
HUT-102	+++
KUT-2	+++
MT-1	+++
MT-2	+++
TL-Mor	+++
TL-Oml	+++
UW-4B	+++
UW-4X	+++
ATL-derived B cell line	
KUT-3	+++
non-ATL T cell lines	
CCRF-CEM	+++
ILT-Mat	+++
ILT-Mor	+++
Jurkat	+++
non-ATL B cell line	
Raji	+++
monocyte cell line	
U937	+++
myeloid cell line	
K562 (CML)	+
fibroblasts	
skin fibroblast	–
FS-4	–

[a] Cell lysates of various human cells (about 3×10^5 cells) were analyzed by two-dimensional gel electrophoresis, and proteins were visualized by staining with silver nitrite. Relative peak intensity of the 65-kDa protein spot as compared with that of actin in each gel was determined by densitometry.

of the p65 in the 55–75% saturation fraction. Most of the p65 thus recovered was eluted in a NaCl concentration range of 90–100 mM from a DEAE-cellulose column (Figure 7A,B). After gel filtration through Sephadex G-100, fractions containing p65 were charged on a hydroxylapatite column. The p65 was eluted at a 80–85 mM concentration of sodium phosphate buffer (Figure 8A,B). p65 was purified to almost a single band (Figure 8B) and single spot (Figure 8C) using these four purification steps.

Amino Acid Sequence Analysis of the 65-kDa Protein. In repeated attempts, we were unable to obtain an amino-terminal sequence of the purified p65 by the Edman degradation method, suggesting that its amino-terminal end was blocked. We then attempted to obtain internal peptide sequence information of p65. Thus, purified p65 was digested with lysyl endo-

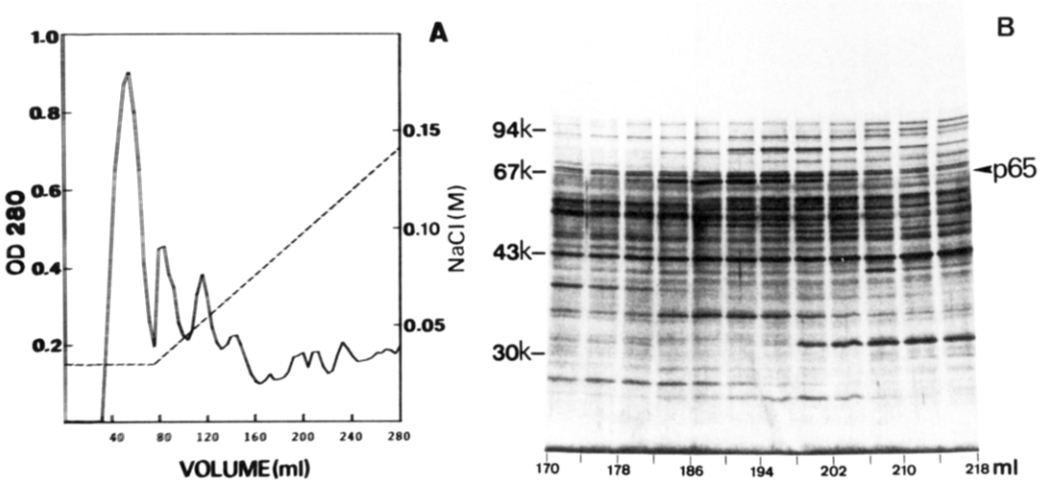

FIGURE 7: Purification of the 65-kDa protein. (A) Ion-exchange chromatography of cellular proteins on DEAE-cellulose column performed as described under Materials and Methods. (B) Silver nitrite stained SDS–PAGE of column fractions in (A) from the elution volume of 170–218 mL (left to right, 4 mL/fraction). The arrowhead indicates the position of p65.

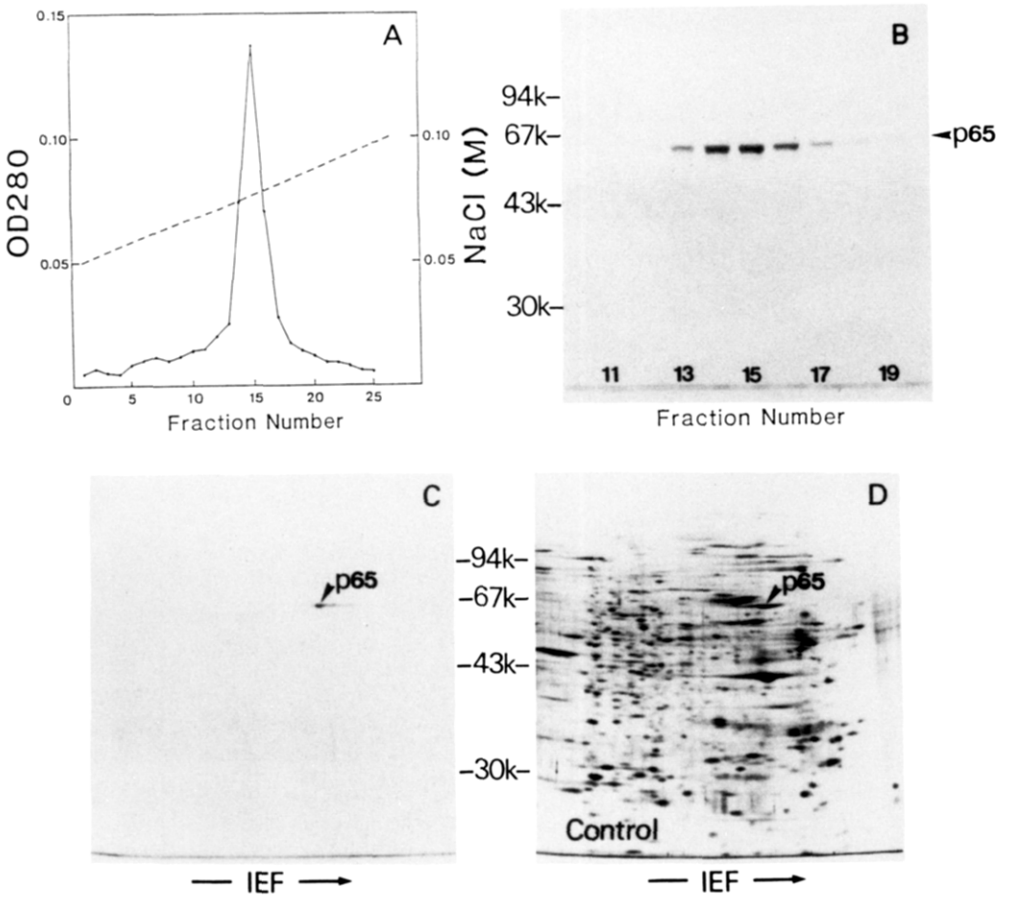

FIGURE 8: Purification of the 65-kDa protein. After gel filtration through Sephadex G-100, the proteins were separated by using a hydroxylapatite column as described under Materials and Methods (A), and each fraction was analyzed by SDS–PAGE stained with silver nitrite (B). The proteins in column fraction 15 were analyzed by two-dimensional gel electrophoresis and stained with silver nitrite (C). (D) An all-cellular protein (Jurkat cells) control for (C). Arrowheads indicate the position of p65.

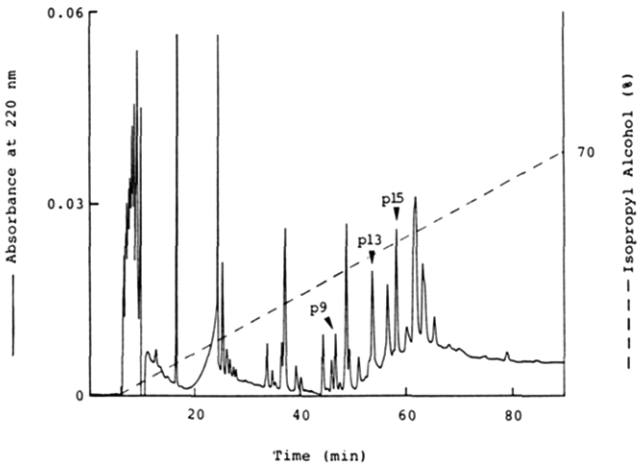

FIGURE 9: Reverse-phase HPLC of cleaved peptides derived from the purified 65-kDa protein. Purified 65-kDa protein was digested with lysyl endopeptidase, and the cleaved peptide fragments were separated by reverse-phase HPLC on a column of TSKgel ODS-120T, as described under Materials and Methods. The arrows indicate the fragments which were used for amino acid sequence analysis.

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l-plastin 1'  MARGDLDQDGRISFDEFIKIFHGLKSTDVAKTFRKAINKKEGICAIGGTSEQSSVGTQHS
          61'  YSEEEKYAFVNWINKALENDPDCRHVIPMNPNTNDLFNAVGDGIVLCKMINLSVPDTIDE
         121'  RTINKKKLTPFTIQENLNLALNSASAIGCHVVNIGAEDLKEGKPYLVLGLLWQVIKIGLF
         181'  ADIELSRNEALIALLREGESLEDLMKLSPEELLLRWANYHLENAGCNKIGNFSTDIKDSK
                                    ********************
                          peptide #13 ► LSPEELLLRWANYHLENAG
         241'  AYYHLLEQVAPKGDEEGVPAVVIDMSGLREKDDIQRAECMLQQAERLGCRQFVTATDVVR
         301'  GNPKLNLAFIANLFNRYPALHKPENQDIDWGALEGETREERTFRNWMNSLGVNPRVNHLY
         361'  SDLSDALVIFQLYEKIKVPVDWNRVNKPPYPKLGGNMKKLENCNYAVELGKNQAKFSLVG
         421'  IGGQDLNEGNRTLTLALIWQLMRRYTLNILEEIGGGQKVNDDIIVNWVNETLREAEKSSS
                                                ********************
                                      peptide #15 ► VNDDIIVNWVNETLREAE
         481'  ISSFKDPKISTSLPVLDLIDAIQPGSINYDLLKTENLNDDEKLNNAKYAISMARKIGARV
                                                                ******
                                                      peptide #9 ► IGARV
         541'  YALPEDLVEVNPKMVMTVFACLMGKGMKRVX
               ************
               YALPEDLVEVNP
```

FIGURE 10: Amino acid sequences of the lysyl endopeptidase cleaved peptide fragments of the 65-kDa protein. Three oligopeptide sequences determined by microsequencing in a gas-phase sequenator are indicated, in comparison with the amino acid sequence of l-plastin (Lin et al., 1988).

peptidase, and the resultant peptides were separated by reverse-phase HPLC (Figure 9). We determined the amino acid sequences of three (9, 13, and 15) peptide fragments. As shown in Figure 10, a computer-assisted search for protein sequence homology of these peptide sequences with reported protein sequences revealed that these peptides exist in l-plastin (Lin et al., 1988). The molecular weight of l-plastin calculated from its amino acid sequence is 64000. Furthermore, p65 and l-plastin are some of the most abundant proteins constitutively expressed in human leukemia cell lines, and both of them migrate to the isoelectric point almost identical with β-actin in the two-dimensional gel electrophoresis, suggesting that p65 is identical with l-plastin.

Discussion

IL 2 rapidly induced the phosphorylation of 65-, 50-, 37-, 24-, and 21-kDa proteins in the IL 2 dependent human T cell line UW-4B. IL 2 also induced the phosphorylation of the same set of cellular proteins in an IL 2 independent variant, UW-4X. As UW-4X cells express the same level of high- and low-affinity IL 2 receptors as UW-4B cells do, it seems likely

that the increased phosphorylation of these five cellular proteins is a common response to IL 2 in the cells expressing the IL 2 receptor. Increased phosphorylation of the same set of proteins was also induced by TPA. In this context, IL 2/IL 2 receptor interaction has recently been reported to induce rapid and transient activation of protein kinase C (Farrar & Anderson, 1985). Thus, direct participation of protein kinase C in the phosphorylation of the five cellular proteins described above seems likely. The precise mechanism of how IL 2/IL 2 receptor interaction activates protein kinase C, however, remains to be clarified.

Among the IL 2 induced phosphorylation of the five cellular proteins described above, that of the 65-kDa protein seemed particularly interesting, which was revealed from the analysis of phosphoproteins in some IL 2 independent cell lines, UW-4X, KUT-2, and HUT-102 cells. The culture supernatants of these variants contained no detectable IL 2 activity as determined by stimulation assay of IL 2 dependent cells (Namba et al., unpublished results). An autocrine stimulation of cells has recently been shown to occur with a minute amount of growth factor present even within the cell (Nienhuis, 1988); thus, at present, we cannot rule out the possible autocrine growth of those variant cells. Nevertheless, the important point is that only the 65-kDa protein, but not the other four proteins, was constitutively phosphorylated to a higher extent in UW-4X, KUT-2, and HUT-102 cells without exogenous IL 2 stimulation. It might be that constitutive serine phosphorylation of the 65-kDa protein above a threshold level is causally related to acquisition of the ability of UW-4X, KUT-2, and HUT-102 cells to grow in an IL 2 independent manner. IL 2 induced serine phosphorylation of 68-kDa protein (Farrar et al., 1986) and 67-kDa protein (Ishii et al., 1987) in human T lymphocytes has recently been reported. Their cytosol location as well as their mobilities on two-dimensional gel electrophoresis suggests that all those proteins are identical with the 65-kDa protein described here.

By comparing the peptide maps of the [^{35}S]methionine- and the [^{32}P]P_i-labeled 65-kDa proteins by partial proteolysis with *S. aureus* V_8 protease, we identified the unphosphorylated form, apoprotein, of the 65-kDa phosphoprotein, which was one of the major cytosol proteins. We succeeded in purifying the 65-kDa protein to homogeneity from the soluble fraction of Jurkat cells. We determined the amino acid sequences of its lysyl endopeptidase cleaved, three internal oligopeptides. A computer-assisted search for the sequence homology of these peptides with reported proteins strongly suggested that the 65-kDa protein is the leukocyte isoform of plastin (l-plastin), which was originally identified as an abundant, transformation-induced protein in chemically transformed human fibroblasts (Lin et al., 1988). The amino acid composition of the 65-kDa protein (data not shown) is also very similar to that of l-plastin.

Matsushima et al. (1987) identified a cytosol 65-kDa protein in glucocorticoid-pretreated human peripheral blood mononuclear cells whose serine phosphorylation was rapidly induced by IL 1. They purified the protein from human leukocytes and determined its partial amino acid sequence (Matsushima et al., 1988), which has recently been suggested to be identical with l-plastin (Lin et al., 1988). Thus, it is now evident that serine phosphorylation of a common cytosol protein, l-plastin, is involved in the intracellular signaling pathways of IL 1 and IL 2.

What is the function of the 65-kDa protein, l-plastin? The 65-kDa protein is a highly abundant protein in all leukocyte subpopulations (Table I; Matsushima et al., 1988). While this protein is not expressed in normal human fibroblasts, it is a transformation-induced protein and is frequently expressed to a high level in human cancer cells of solid tissue (Leavitt & Kakunaga, 1980; Goldstein et al., 1985; Goldstein & Leavitt, 1985). The latter finding, in combination with the cytosol location of the 65-kDa protein (Figure 6), seems particularly interesting. One of the common changes observed in transformed cell is the disruption of the uniform organization of the cytoskeleton: actin microfilaments that extend the length of normal cells are either diffusely distributed or concentrated beneath the cell surface. Also, during cell division, the entire cytoskeleton is reorganized as part of a controlled sequence of events. Taken together all described above, the 65-kDa protein, l-plastin, might be a kind of protein which interacts with cytoskeletal proteins. In fact, the amino acid compositions of the 65-kDa protein and l-plastin are very similar to that of an actin-binding protein, acumentin (Southwick & Stossel, 1981; its amino acid sequence information has not been reported). Acumentin has been reported to exist in cytosol of leukocytes in abundance and inhibit the polymerization of actin. Our preliminary experiments have suggested that the 65-kDa protein inhibits the polymerization of α-actin (Namba et al., unpublished results) as acumentin does. Therefore, it is highly probable that the 65-kDa protein, l-plastin, and acumentin are the same protein. We speculate that the 65-kDa protein dissociates from actin by phosphorylation and enhances the polymerization of actin. In fact, UW-4B cells spread and pseudopod formation occurs within 30 min after the addition of IL 2 (unpublished observation). The enhanced phosphorylation of some cytoskeletal proteins has been recently regarded as a subsequent process to transmit the initial transmembrane signal. For example, phosphorylation of vinculin in chick embryo fibroblast and Swiss 3T3 (Werth & Pastan, 1984), myosin light chain in BALB/c 3T3 (Bockus & Stiles, 1984), and microtubule-associated protein (MAP-1) in rat embryo fibroblast (3YI-B) (Sato et al., 1985) have been reported as early events after stimulation by phorbol esters or growth factors. The phosphorylation of the 65-kDa protein might be a subsequent process to transmit the initial transmembrane signal in T lymphocytes.

In order to understand the molecular mechanism of the intracellular signaling pathway and regulation of cell proliferation in the IL 2–IL 2 receptor system, it seems important to clarify whether the phosphorylation of p65 modulates the interaction between this protein and actin and how this modulation triggers cell proliferation.

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