Characterization of interleukin 2-stimulated phosphorylation of 67 and 63 kDa proteins in human T-cells

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We have investigated rapid and marked phosphorylation of cellular proteins induced by interleukin 2 (IL-2) in both phytohaemagglutinin-stimulated normal peripheral blood leucocytes, and IL-2-dependent or -independent human T-cell lines bearing human T-cell leukaemia (lymphotropic) virus type I. Two-dimensional electrophoretic analysis showed that the IL-2-induced phosphoprotein was of M_r 67 000 with a pI of 5.8 (pp67) and was distinct from the IL-2 receptor. IL-2 also stimulated phosphorylation of four other proteins, with an M_r of 63 000 and pI values 5.3–6.1 (pp63s). The stimulation of pp67 phosphorylation was observed within 5 min after addition of IL-2 and was maximal after 15 min. The maximal phosphorylation was more than 10-fold that observed initially. In IL-2-dependent cells, IL-2 dose responses of pp67 phosphorylation and cell proliferation were exactly correlated. Phosphoamino acid analysis showed that the phosphorylation site of pp67 and pp63s was a serine residue. Subcellular-fractionation studies indicated that pp67 was localized in cytosol, whereas pp63s phosphorylation was induced by IL-2 in nuclear and cytosol fractions. Similar phosphorylation of pp67 and pp63s was observed when the cells were treated with phorbol 12-myristate 13-acetate instead of IL-2. These results suggest (i) that IL-2–IL-2-receptor interaction leads to activation of protein kinase(s), resulting in phosphorylation of certain cellular proteins such as pp67 and pp63s, and (ii) that this phosphorylation could be an early event in the transmission of intracellular growth signalling from the IL-2 receptors.

INTRODUCTION

Interleukin 2 (IL-2) is a growth factor for T-, B- and other lymphocytes. These cells express receptors for IL-2 on their surface, and, on addition of IL-2, trap it rapidly and proliferate. Recent studies demonstrate two types of receptors for IL-2: high- and low-affinity [1]. The cell-growth signal is known to be transduced from the high-affinity IL-2 receptors [2–4], and we have lately suggested a possible relationship between transduction of the growth signal and IL-2 internalization, which is also mediated by the high-affinity receptors [5]. However, little is known about the mechanism of intracellular signalling induced by IL-2.

A variety of other growth factors induce phosphorylation of some cellular proteins. Some of them are thought to be controlled by tyrosine-specific protein kinases associated with receptors for epidermal growth factor (EGF) [6–9], platelet-derived growth factor (PDGF) [7–9] and insulin [10]. The activity of these kinases is regulated by interaction of the ligands with the receptors [11]. Furthermore, phosphorylation at serine/threonine residues of some cellular proteins such as ribosomal S6 protein [12–15], 62 kDa nuclear protein and 27 kDa cytosoluble protein of hamster lung fibroblasts [14,15], 22 kDa [16,17] and 80 kDa [17,18] proteins of 3T3 cells, is also known to be dependent on growth factors. The kinases responsible for such phosphorylation are yet to

be characterized. A favourite candidate is phospholipid/ Ca^{2+} -dependent protein kinase (C-kinase), which is known to be a receptor for the tumour promoter PMA [19,20], catalysing serine/threonine phosphorylation of various substrates (see reviews [21,22]). Recently, many studies using either growth factors or C-kinase activators have provided evidence that the activation of kinases and phosphorylation could play an important role in the intracellular signal transduction leading to cell proliferation [21–23].

In T-cells, the receptor for IL-2 seems not to have kinase activity, whereas C-kinase has been suggested to regulate phosphorylation and expression of the IL-2 receptor [24–26] and production of IL-2 [27], i.e. T-cell activation [28]. Furthermore, IL-2-dependent cell growth was suggested to be closely related to the activation of C-kinase [29]. In this context we recently found that IL-2 rapidly induced serine-specific phosphorylation of a 67 kDa cellular protein in IL-2-dependent murine cells, and it could be involved in the signalling of cell growth [30]. The very recent study also reported IL-2-dependent phosphorylation of IL-2-receptor molecules in murine T-cells [31], although the relationship between phosphorylation of IL-2 receptor and IL-2-dependent cell growth has not been clarified.

In the present study we further demonstrate that phosphorylation of 67 and 63 kDa cellular proteins, but not of IL-2-receptor molecules, is stimulated by IL-2 in

Abbreviations used: IL-2, interleukin 2; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate (also known as 'TPA'); HTLV-I/ATLV, human-T-cell-leukaemia (lymphotropic) virus type I/adult-T-cell-leukaemia virus; PBL, peripheral blood leucocytes; PHA, phytohaemagglutinin; PBS, phosphate-buffered saline (136.9 mM-NaCl/2.7 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.2); PMSF, phenylmethanesulphonyl fluoride; NP-40, Nonidet P40; PAGE, polyacrylamide-gel electrophoresis.

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various human T-cells, and that the phosphorylation of these proteins is also stimulated by PMA.

MATERIALS AND METHODS

Cells

IL-2-dependent cell lines, ILT-Mor, ILT-Mat and ILT-Yan, and IL-2-independent cell lines, TL-Mor and TL-Oml [32] carry the pro-virus of HTLV-I/ATLV and express IL-2 receptors constitutively. Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum, 2 mM-L-glutamine and antibiotics. The IL-2-dependent cells were cultured in the presence of

1 nM human recombinant IL-2 (obtained from Shionogi Co., Osaka, Japan). Peripheral-blood leucocytes (PBL) from a normal donor were treated with 1.0% PHA-M (Difco, Detroit, MI, U.S.A.) for 3 days *in vitro* and maintained in the culture medium with 1 nM-IL-2 for 7 days.

Radiolabelling of cells

Before radiolabelling, the IL-2-dependent cells and PHA-activated PBL were preincubated in the medium without IL-2 for 14 and 10 h respectively. Radiolabelling procedure of cells was carried out as described previously [30]. Briefly, 2×10^6 cells were incubated in

Fig. 1. IL-2-induced phosphorylation in PHA-stimulated normal PBL and human T-cell lines

PHA-stimulated PBL (a,b), IL-2-dependent ILT-Mor (c,d) and IL-2-independent TL-Mor (e,f) were incubated with (b,d,f) or without (a,c,e) IL-2 for 30 min before termination of ^{32}P labelling. Cell lysates were prepared from 2×10^6 cells and subjected to two-dimensional PAGE as described in the Materials and methods section. Gels were fixed, stained, dried, and exposed to X-ray film for 6 h at -70°C using intensifying screens. Arrowheads a and b show pp67 and pp63s respectively. Phosphorylase b (M_r 94000), bovine serum albumin (67000), ovalbumin (43000) and carbonic anhydrase (30000) were used as M_r markers. C-Phycocyanin (*Aspergillus nidulans*) (pI 4.85), azurin (*Pseudomonas aeruginosa*) (5.65), trifluoroacetylated metmyoglobin (porcine) (5.92) and metmyoglobin (porcine) (6.45) were used as pI markers.

1 ml of phosphate-free medium containing 1 mCi of [32 P]P_i (Amersham International, Amersham, Bucks., U.K.) for 3 h at 37 °C in plastic dishes. The cells were then harvested and washed with chilled PBS in a microtube. For treatment with IL-2, IL-2 (2 nM) was added to each culture 15 or 30 min before harvesting cells (unless otherwise stated).

Preparation of 32 P-labelled cell lysates

Cell lysates for isoelectric focusing were prepared from 32 P-labelled cells as described previously [9,33]. In brief, the pelleted cells were resuspended in 80 μ l of 20 mM-Tris/HCl, pH 8.8, containing 2 mM-CaCl₂,

Staphylococcus aureus nuclease (50 μ g/ml) (Cooper Biomedical, Malvern, PA, U.S.A.), 1% aprotinin (Sigma, St. Louis, MO, U.S.A.) and 100 μ M-sodium orthovanadate (Sigma), and were lysed in 50 mM-Tris/HCl, pH 7.0, containing 0.25% SDS, 1% 2-mercaptoethanol, 1 mM-PMSF, deoxyribonuclease (Cooper Biomedical) (100 μ g/ml), ribonuclease A (50 μ g/ml) (Cooper Biomedical) and 5 mM-MgCl₂. The lysates were kept on ice for 1 min and then mixed with (final concentrations of) 9.5 M-urea (Schwarz-Mann, Cambridge, MA, U.S.A.), 4% NP-40, 5% 2-mercaptoethanol and 2% Ampholine pH 5-8 (LKB-Produkter AB, Bromma, Sweden). The final volume of the sample was 250 μ l/2 $\times$ 10⁶ cells. The

Fig. 2. Kinetics of pp67 phosphorylation induced by IL-2 in ILT-Mor cells

ILT-Mor cells were arrested by incubation without IL-2 for 14 h and then treated with 2 nM-IL-2 for 0, 2, 5, 15, 30, 60 and 240 min. The lysates for 2 $\times$ 10⁵ cells were analysed by two-dimensional PAGE as in Fig. 1. Arrowheads indicate the position of pp67 (a), pp63s (b) and a marker protein 94 kDa (c) whose phosphorylation was unaffected by the IL-2 treatment. The amount of phosphorylation of pp67 (a) and 94 kDa protein (c) was estimated from the densities and areas measured with a densitometer, and the ratio a/c at each time is shown.

samples were rapidly frozen and stored at -70°C until use.

Two-dimensional gel electrophoresis

Portions of samples prepared as described above were subjected to two-dimensional gel electrophoresis. Isoelectric focusing in 9.2 M-urea for 7000V·h with Ampholine pH 5–8 was used in the first dimension and SDS/PAGE on 10 or 12.5% (w/v) acrylamide slab gels in the second [34]. Then the gels were fixed, stained with Coomassie Brilliant Blue R-250 and dried. Polypeptides labelled with ^{32}P were located by autoradiography using intensifying screens. pI marker proteins (BDH, Poole, Dorset, U.K.) in the first-dimension column gels and M_r markers (Pharmacia, Uppsala, Sweden) on the SDS/polyacrylamide slab gels were detected by staining with Coomassie Brilliant Blue.

The extents of phosphorylation were determined from the densities of spots in autoradiograms measured with a densitometer (model 620 video densitometer: Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Measurement of DNA synthesis in cells

ILT-Mat cells were preincubated in IL-2-free medium for 14 h and were seeded on 96-well plates at a concentration of 2.5×10^4 per well. After incubation with the indicated doses of IL-2 for 24 h at 37°C the cells were labelled with $1 \mu\text{Ci}$ of $[^3\text{H}]$ thymidine (Amersham International) for 4 h. Then cells were treated with 7% (w/v) trichloroacetic acid, washed on glass-fibre filters and acid-insoluble radioactivity on the filters was measured with a liquid-scintillation spectrometer in the presence of scintillator {0.5% PPO (2,5-diphenyloxazole)/0.05% POPOP[1,4-bis-(5-phenyloxazol-2-yl)-benzene] in toluene}.

Phosphoamino acid analysis

Pieces of two-dimensional gels containing ^{32}P -labelled spots of interest were incubated in 5.7 M-HCl for 1 h at 110°C to extract and hydrolyse phosphoproteins as described previously [30]. The phosphoamino acids were recovered on Dowex AG1-X8 resin (Cl^- form; Bio-Rad Laboratories) [35], and separated by thin-layer electrophoresis on a cellulose plate in pyridine/acetic acid/water (1:10:189, by vol.), pH 3.5, at 1000 V for 60 min, as described previously [9]. Samples containing about 500 c.p.m. were applied to the plates. Phosphoamino acid markers were detected by ninhydrin staining and ^{32}P -labelled phosphoamino acids were detected by autoradiography with intensifying screens.

Subcellular fractionation

Subcellular fractionation was carried out by the method of Farrar & Anderson [29] with some modifications. Briefly, ^{32}P -labelled cells were washed with PBS and then 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 resuspended in SEAT buffer $[(0.5-5) \times 10^7 \text{ cells/ml}]$ and were lysed by pipetting the suspension 30 times with a disposable 26 G needle/syringe on ice. Nuclei and any unbroken cells were pelleted by centrifugation at 800 rev./min (200 g) for 5 min. The postnuclear supernatant was centrifuged at 100 000 g for 60 min to separate the membrane fraction of the resulting pellet from cytosol fraction of the supernatant. For the preparation of nuclear fraction,

^{32}P -labelled cells were lysed with 1% NP-40/50 mM-NaCl/15 mM- MgCl_2 /1 mM-PMSF/50 mM-Tris/HCl, pH 7.5 [TNM buffer; $(0.5-5) \times 10^7 \text{ cells/ml}]$ on ice for 10 min. The lysate was centrifuged through 60% (w/v) sucrose in TNM buffer in a centrifugation tube at 800 g for 20 min. Nuclei free from intact cells were collected at the bottom, lysed and treated with nuclease by the same method as before. To each subcellular fraction was added urea, NP-40, 2-mercaptoethanol and Ampholine as described above, and it was stored at -70°C until use for electrophoresis.

RESULTS

IL-2-stimulated protein phosphorylation

Protein phosphorylation in PHA-stimulated normal PBL treated with IL-2 was analysed by two-dimensional PAGE. Phosphorylation of some cellular proteins increased on IL-2 treatment. Among them, marked phosphorylation of a 67 kDa protein with an apparent pI of 5.8 (pp67), was reproducibly demonstrated (Figs. 1a and 1b, arrowhead b). A similar increase in pp67 phosphorylation was always observed in various types of human T-cell lines, the IL-2-dependent cell lines ILT-Mor (Figs. 1c and 1d), ILT-Mat and ILT-Yan (not shown) and the HTLV-I-transformed cell lines TL-Mor (Figs. 1e and 1f) and TL-Oml (not shown). These results suggest that the increased phosphorylation of pp67 could be a common response to IL-2 in human T-cells expressing the receptor for IL-2. IL-2 also stimulated phosphorylation of other proteins, which appeared as a series of four phosphoproteins (pp63s) with an apparent M_r of 63 000 and apparent pI values of 5.3–6.1 (Fig. 1, arrowhead b), but the extents of phosphorylation of these pp63s stimulated by IL-2 were less than that of pp67.

Next we added cycloheximide (100 $\mu\text{g/ml}$) to cultures of ILT-Mor cells before 30 min of IL-2 treatment to

Fig. 3. IL-2 dose responses of pp67 phosphorylation and cell proliferation

The pp67 phosphorylation of ILT-Mat at various doses of IL-2 was examined as described in Figs. 1 and 2. Cell proliferation (●) was measured by incorporation of $[^3\text{H}]$ thymidine in triplicate. The extent of pp67 phosphorylation (○) was measured with a densitometer and was normalized as the ratio a/c (see the legend of Fig. 2).

determine whether the phosphorylation required protein synthesis *de novo*. The IL-2-stimulated phosphorylation was not affected by the addition of cycloheximide (not shown), suggesting that IL-2-IL-2-receptor interaction triggers the activation of protein kinase(s) directing phosphorylation of pre-existing 67 and 63 kDa proteins.

Kinetics of IL-2-stimulated phosphorylation of pp67

Phosphorylation of pp67 in ILT-Mor cells increased within 5 min after addition of IL-2 and reached a maximum after 15 min. Then the extents of phosphoryl-

ation gradually decreased (Fig. 2, arrowhead a). The phosphorylation of pp67 was quantified by measuring the density of spots in autoradiograms. The density of pp67 in each film was standardized by comparison with that of a 94 kDa phosphoprotein (Fig. 2, arrowhead c), which was chosen as a reference because it showed little difference in phosphorylation with or without IL-2. The ratios of pp67 to the 94 kDa phosphoprotein are indicated in Fig. 2. The maximal phosphorylation of pp67 at 15 min was approx. 13 times that initially (0 min). The extents of pp63s phosphorylation of (Fig. 2,

Fig. 4. Phosphorylation of pp67 and pp63s induced by PMA

PMA at 50 ng/ml (instead of IL-2) was added to the cell culture 15 min before termination of ^{32}P -labelling. Lysates of 2×10^5 cells were analysed as described for Fig. 1. Arrowheads indicate pp67 (a), pp63s (b) and 94 kDa protein (c). The ratios a/c (see the legend to Fig. 2) were shown. The basal extents of pp67 and pp63s phosphorylation in each of PHA-stimulated PBL, ILT-Mor and TL-Mor cells were shown in Figs. 1(a), 1(c) and 1(e). Values of a/c in each cell without PMA were 0.09 (a), 0.09 (b), 0.09 (c), 0.06 (d) and 0.07 (e). (a), TL-Mor; (b), TL-OmI; (c), ILT-Mor; (d), ILT-Yan; (e), PHA-stimulated normal PBL.

arrowhead b) reached the maximum 30 min after exposure of cells to IL-2.

IL-2 dose responses of pp67 phosphorylation and cell proliferation

The kinetic results suggest that the pp67 phosphorylation is possibly linked to cell proliferation. To test this possibility, cell proliferation and pp67 phosphorylation were examined by using various doses of IL-2. ILT-Mat cells, whose growth is completely dependent upon IL-2, constitutively express high (600 sites/cell; $K_d = 14$ pM)- and low (50 000 sites/cell; $K_d = 26$ nM)-affinity IL-2 receptors. Cell proliferation and phosphorylation of pp67 increased in parallel over the IL-2 dose range 2^{-8} (0.0039) nM– 2^{-4} (0.0625) nM (Fig. 3). The maximal cell proliferation was seen in the presence of 2^{-3} (0.125) nM-IL-2, which is enough to saturate the high-affinity receptors. At the same dose or higher, phosphorylation of pp67 reached the maximum.

Absence of absorption of pp67 and pp63s with anti-(IL-2 receptor) antibody

The observation that pp67 and pp63s were specifically phosphorylated at a very early stage of IL-2-IL-2-receptor interaction is reminiscent of phosphorylation induced by other growth factors, such as EGF, PDGF, insulin, insulin-like growth factor I and colony-stimulating factor I. In these cases, the growth factors induced phosphorylation of their own receptor molecules. It was therefore of interest to determine whether the receptor for IL-2 could serve as a phosphorylation substrate. To test this possibility we treated a 32 P-labelled TL-Mor lysate with murine monoclonal antibody (H-31)

to human IL-2 receptor [36] and then analysed it by two-dimensional PAGE. The antibody did not absorb pp67 and pp63s from the cell lysate. Under the same conditions, H-31 could remove IL-2 receptor molecules from the lysate as an immunoprecipitated form, which was analysed by SDS/PAGE (results not shown). These results show that pp67 and pp63s molecules are distinct from the IL-2 receptor.

Effect of PMA on phosphorylation of pp67 and pp63s

To gain some insight into the biochemical mechanism of IL-2-stimulated phosphorylation, we studied the effect of PMA in the system. On treatment with PMA (5–50 ng/ml), phosphorylation of pp67 increased in HTLV-I-carrying T-cell lines as well as PHA-stimulated normal PBL (Fig. 4). In all the cells treated, the extents of pp67 phosphorylation induced by PMA seemed to be the same, or higher, than those induced by IL-2 (Fig. 4, arrowhead a), whereas the extents of pp63s phosphorylation induced by PMA were greater than those induced by IL-2 (Fig. 4, arrowhead b). In ILT-Mat cells, PMA dose responses for stimulating proliferation as well as for phosphorylation of pp67 were observed over the PMA concentration range 0.2–6.25 ng/ml, and higher doses of PMA (up to 100 ng/ml) did not affect the level of proliferation and pp67 phosphorylation compared with that of 6.25 ng/ml treatment (results not shown).

Phosphoamino acid analysis of pp67 and pp63s

The phosphorylated residues in pp67 and pp63s were examined by cellulose electrophoresis of hydrolysed phosphoamino acids. As shown in Fig. 5, a spot corresponding to phosphoserine, but not to phospho-

Fig. 5. Phosphoamino acid analysis of pp67 and pp63s

ILT-Mor cells were labelled with 32 P in the presence of 2 nM-IL-2 for 15 min before harvesting. Cells were lysed and lysates were subjected to two-dimensional PAGE. The piece of gel containing pp67 (a) and each of pp63s (b–e; acidic to basic) was cut out and incubated in 5.7 M-HCl at 110 °C for 1 h. The resultant phosphoamino acids were separated on a cellulose plate by electrophoresis (1000V, 60 min) at pH 3.5 in pyridine/acetic acid/water (1:10:189, by vol.). Phosphoamino acid markers were located by ninhydrin staining. Abbreviations used: P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Fig. 6. Subcellular localization of pp67 and pp63s

Subcellular fractions of membrane, cytosol and nuclei were prepared from ^{32}P -labelled ILT-Mat cells with (b,d,f) or without (a,c,e) IL-2 as described in the Materials and methods section. Membrane fraction (a,b) of 5×10^6 cells, or a nuclear (c,d) or cytosol (e,f) fraction of 2.5×10^6 cells was subjected to two-dimensional PAGE, and dried gels were exposed to X-ray film for 12 h (e,f) or 40 h (a-d) at -70°C with intensifying screens. Arrowheads a and b indicate pp67 and pp63s respectively.

threonine or phosphotyrosine, was demonstrated for each phosphoprotein.

Subcellular localization of pp67 and pp63s

The subcellular localization of pp67 and pp63s was examined by using ^{32}P -labelled ILT-Mat-cell lysate. pp67 was located in cytosol and very little of it was found in membrane and nuclear fractions (Fig. 6, arrowhead a). In contrast with this, pp63s appeared in all three fractions after IL-2 treatment. In particular, marked enhancement by IL-2 was found in nuclear and cytosol fractions. Similar results were obtained with PMA instead of IL-2.

DISCUSSION

We previously reported that IL-2 induced phosphorylation of a cellular protein, pp67, in IL-2-dependent

murine cells [30]. The present study also demonstrated similar and novel phosphoproteins in various human T-cells. Phosphorylation of pp67 was rapidly and markedly induced by IL-2 in the human T-cells tested, including PHA-treated normal PBL and HTLV-I-antigen-negative TL-OmI cells. These results clearly indicate that pp67 is a cellular protein and not a product of HTLV-I. Moreover, pp67 is not the IL-2 receptor itself, because a monoclonal antibody to the IL-2 receptor failed to absorb pp67 in the cell lysates and because the M_r (67000) and pI (5.8) of pp67 were clearly different from those of the IL-2 receptor [37,38]. This is also supported by the experimental results showing that both IL-2 and PMA significantly induced phosphorylation of pp67, whereas phosphorylation of the IL-2 receptor was significantly induced by PMA, but not by IL-2, in our experiment.

In IL-2-dependent cells, IL-2 induced pp67 phosphoryl-

ation rapidly and transiently, and the IL-2-dose-dependence of pp67 phosphorylation was exactly correlated with that of cell proliferation. A similar correlation has been also observed in murine IL-2-dependent cells [30]. Furthermore, the correlation between pp67 phosphorylation and cell proliferation was also observed in the cells treated with PMA. Therefore it is suggested that phosphorylation of pp67 may be an early event in the growth-signal transmission of IL-2 receptors. IL-2-stimulated phosphorylation of pp67 was similarly detected in IL-2-independent HTLV-I-transformed T-cell lines which express the high- and low-affinity receptors for IL-2. In contrast with IL-2-dependent cells, the growth of these cells is inhibited by the addition of IL-2 [39], and we have already proved that the growth-inhibitory signal is transduced from the high-affinity IL-2 receptors by analogy to the IL-2 growth signal [40]. MT-1 cells express only the low-affinity IL-2 receptors constitutively [37] and do not respond to IL-2 with regard to cell-growth promotion and inhibition [39]. Interestingly the phosphorylation of pp67 (and pp63s) in MT-1 was not enhanced by IL-2 at any dose (T. Ishii & K. Sugamura, unpublished work), so it is suggested that the pp67 phosphorylation signal may be transduced from the high-affinity IL-2 receptors.

In some growth-factor-receptor systems the receptor-associated kinases and their putative substrates are thought to be important in intracellular signalling of growth regulation, and the known kinases of receptors are tyrosine-specific [11]. We could not detect any protein kinase activity associated with IL-2 receptor molecules (M. Fujii, T. Ishii & K. Sugamura, unpublished work), and the cytoplasmic domain of the IL-2 receptor molecule has only 13 amino acid residues, which seems to be too short a sequence to harbour kinase activity, and this region does not have homologous sequences with the kinase portion of EGF and insulin receptor [41,42]. What kinase, then, stimulates IL-2-dependent phosphorylation of pp67? The phosphorylation of pp67 was also induced by treatment of cells with PMA or the synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol, instead of IL-2 in a dose-dependent manner, and these reagents are known to activate C-kinase [19,20,43]. Recently, IL-2-IL-2-receptor interaction was found to induce rapid and transient activation of C-kinase [29]. As the kinetics of C-kinase activation by IL-2 bear a resemblance to that of serine-specific phosphorylation of pp67 induced by IL-2, C-kinase may be one of the possible kinases directing the phosphorylation of pp67. Kaibuchi *et al.* [44] detected three major target proteins of C-kinase *in vivo* and *in vitro* in activated T-cells: 82 kDa C-kinase itself and 56 kDa and 45 kDa proteins. In addition to those, several proteins, including a 67 kDa protein, seemed to be phosphorylated by C-kinases although Kaibuchi *et al.* [44] did not mention them. We made a preliminary attempt to see whether C-kinase directly phosphorylates pp67 and pp63s. Incubation of the cytosol fraction with partial purified C-kinase showed no phosphorylation of pp67 and pp63s, although this may not exclude the possibility that C-kinase directs these phosphorylations. Other growth factors, such as PDGF [45], and EGF [46], are also known to activate phosphatidylinositol turnover and C-kinase. C-kinase is thought to phosphorylate a 40 kDa protein in platelets [47], a 80 kDa protein in Swiss 3T3 cells [18] and some other proteins *in vivo* [22]. However, pp67 has not been

demonstrated in any of these growth-factor-receptor systems. It is still possible that the protein kinase directing the pp67 phosphorylation is distinct from C-kinase. Alternatively, it is also possible that IL-2- or PMA-stimulated phosphorylation is due to the inactivation of phosphatase(s) specific for pp67 by IL-2 or PMA.

In addition to pp67, we also detected four novel phosphoproteins with the same M_r of 63000 and pI values of 5.3–6.1 in the present study. Although PMA markedly induced phosphorylation of pp63s, the stimulation of its phosphorylation by IL-2 seemed to be less than that of pp67. The phosphoamino acid analysis showed that each of these four pp63s contained phosphoserine, but not phosphothreonine or phosphotyrosine. The pp63s were not the IL-2 receptor. Very similar phosphoproteins were previously detected in rodent fibroblasts stimulated with a variety of growth factors [9,48]. These findings suggest that the phosphoproteins like pp63s are common to fibroblasts and lymphocytes of human and rodent and could contribute to intracellular transmission of growth signals induced by various growth factors. An interesting possibility is that pp63s may serve as signal molecules and may directly associate with DNA and regulate gene expression induced by IL-2 treatment, because they are present in cytosol and nuclei.

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