Purification and Characterization of a Cytosolic 65-Kilodalton Phosphoprotein in Human Leukocytes Whose Phosphorylation Is Augmented by Stimulation with Interleukin 1†

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ABSTRACT: We have recently shown that glucocorticoids dramatically increase the number of interleukin 1 (IL 1) receptors on human peripheral blood mononuclear cells (PBMC) and that IL 1 selectively induces the phosphorylation of a cytosolic 65-kilodalton (kDa) protein (pp 65) in glucocorticoid-pretreated PBMC. We describe here the purification and biochemical characteristics of pp 65. ^{32}P-Labeled pp 65 was purified to homogeneity from the cytosol fraction of IL 1 stimulated [^{32}P]orthophosphate-labeled PBMC by sequential chromatography on Sephacryl S-200, high-performance liquid chromatography (HPLC) anion exchange, and hydroxyapatite HPLC. The purified pp 65 was homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The unphosphorylated 65-kDa protein (p 65) was also purified to homogeneity in a similar way. About 40 μg of purified 65-kDa protein was recovered from 5×10^8 PBMC. Analysis of the amino-terminal sequence of the purified pp 65 revealed the amino terminus of pp 65 to be blocked. Amino acid sequence analysis of a cyanogen bromide cleaved peptide showed pp 65 to be a unique protein whose protein sequence has not yet been reported. Studies of the distribution of p(p) 65 based on Western blotting using specific polyclonal rabbit antibody to p(p) 65 showed that p(p) 65 exists in a variety of cells such as neutrophils, monocytes, B lymphocytes, and myeloid cells. It could not be detected in the T cell leukemia cell line (MOLT), melanoma cells, and fibroblasts. Although the role of the pp 65 has not yet been established, the protein sequence should provide useful information for the cloning of cDNA encoded for pp 65. The purified pp 65 and antibody to pp 65 will enable us to study the physiological roles of pp 65.

Interleukin 1 (IL 1)[1] is a thymocyte comitogenic factor which is produced by a variety of cells. IL 1 has multiple biological activities [as reviewed by Oppenheim et al. (1986)]. Dower et al. (1985) have first described the existence of specific IL 1 receptors on various types of cells. We also described the existence of specific IL 1 receptor on human Epstein Barr virus transformed B lymphocytes and the identity of the receptor for IL 1α and IL 1β (Matsushima et al., 1986a). Our recent studies have shown that IL 1 itself down-regulates the expression of IL 1 receptor with internalization of receptor-bound IL 1 molecules (Matsushima et al., 1986b) and that glucocorticoids, whose synthesis can be augmented by IL 1 in vivo, up-regulate the expression of IL 1 receptor on normal human peripheral blood mononuclear cells (PBMC) without changing the dissociation constant (2.6×10^{-10} M) (Akahoshi et al., 1987). By using a glucocorticoid-pretreated human PBMC, we have observed that carrier-free purified recombinant human IL 1α selectively and rapidly induces the phosphorylation of a cytosolic 65-kDa protein at serine residues (Matsushima et al., 1987). We now report here the purification of the pp 65 to homogeneity and the biochemical properties of the purified pp 65 including a partial amino acid sequence.

Materials and Methods

Radiolabeling of Human PBMC with [^{32}P]Orthophosphate and Preparation of Cytosol. Approximately 5×10^6 human PBMC/mL of RPMI 1640 medium (Advanced Biotechnologies, Inc., Silver Spring, MD) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, VT) were cultured with 10^{-5} M prednisolone (Sigma, St. Louis, MO) for 5 h at 37 °C. Cells were then pelleted by centrifugation and washed twice with phosphate-free RPMI 1640 medium. Furthermore, 10^8/mL cells in phosphate-free RPMI 1640 medium were incubated first in the presence of 100 μCi/mL [^{32}P]orthophosphate (New England Nuclear, Boston, MA) for 1 h at 37 °C and then stimulated with 1 μg/mL carrier-free purified recombinant human IL 1α (Dainippon Pharmaceutical Co., Osaka, Japan) for 15 min. After stimulation, cells were washed twice with cold phosphate-free RPMI 1640 medium. To extract cells, cells were suspended in 1 mL of homogenizing

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[1] Abbreviations: IL 1, interleukin 1; pp 65, 65-kDa phosphoprotein; kDa, kilodalton(s); PBMC, peripheral blood mononuclear cell(s); HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; p 65, unphosphorylated 65-kDa protein; FCS, fetal calf serum; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 2-ME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; D-PBS, Dulbecco's phosphate-buffered saline; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NaPB, sodium phosphate buffer; PTH, phenylthiohydantoin.

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buffer consisting of 25 mM 8 *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 4 mM β-mercaptoethanol (2-ME), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Calbiochem, La Jolla, CA), 1 μg/mL leupeptin (Boehring Manheim, Indianapolis, IN), 50 mM sodium fluoride (Sigma), and 1 mM sodium vanadate (Sigma) at pH 7.5 and sonicated for 30 s by a Vibra cell (Somco and Materials Inc., Danbury, CT). The cytosol fraction was obtained by fractionating cell extracts at 100000*g* for 60 min at 4 °C.

Sources of Human Cell Lines. Epstein Barr virus transformed B lymphocyte cell lines [FMO and ORS; see Matsushima et al. (1985)] were generously provided by Dr. G. Tosato (FDA, Bethesda, MD). A human large granular lymphocyte cell line [YT; see Yodoi et al. (1985)] was generously provided by Dr. J. Yodoi (Kyoto University, Kyoto, Japan). A human myelomonocytic cell line [THP-1; see Tsuchiya et al. (1980)], a human melanoma cell line [A 375; see Onozaki et al. (1975)], and dermal fibroblasts were obtained from American Type Culture Collection (Rockville, MD). Two other human myelomonocytic cell lines, HL 60 and U937, were kindly provided by Dr. F. Ruscetti (LMI, BRMP, NCI, Frederick, MD). Human T lymphocyte leukemic cell lines, Jurkat and Molt, were generously provided by Dr. J. Ortaldo (LEI, BRMP, NCI, Frederick, MD). Every cell line except for fibroblasts was cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μg/mL streptomycin, and 100 units/mL penicillin. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Advanced Biotechnologies, Inc.) supplemented with 10% FCS and antibiotics. Cell extracts were prepared by adding 200 μL of 9 mM 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) (Pierce, Rockford, IL) in PBS/(1×10^7) cell pellets. After the cells were kept for 30 min on ice, insoluble materials and nuclei were removed by centrifugation at 10000*g* for 1 min.

Gel Chromatography. The 5-mL PBMC cytosol was applied to a 2.6 × 90 cm column of Sephacryl S-200 gel (Pharmacia, Uppsala, Sweden) equilibrated with Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 0.01% sodium azide at 4 °C. The flow rate was adjusted to 24 mL/h, and 4-mL fractions were collected. The column was calibrated with bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), chymotrypsinogen (M_r 25000), and cytochrome *c* (M_r 12500).

HPLC. Every HPLC manipulation was performed at room temperature using an LKB 2150 HPLC system (LKB, Uppsala, Sweden).

(*A*) *Anion-Exchange HPLC.* The samples were dialyzed against 0.02 M Tris-HCl buffer, pH 7.4 at 4 °C, and applied to a 250 × 4.1 mm AX300 column (Synchrom, Linden, IN) equilibrated with the same buffer at room temperature. The starting buffer was 0.02 M Tris-HCl, pH 7.4, and the limiting buffer was 0.02 M Tris-HCl buffer, pH 7.4, with 0.3 M NaCl. The flow rate was 1.0 mL/min, and 1.0-mL fractions were collected.

(*B*) *Hydroxyapatite HPLC.* The samples were dialyzed at 4 °C against 0.01 M sodium phosphate buffer (NaPB), pH 7.0, and 0.3 mM $CaCl_2$ and applied to a 100 × 7.8 mm hydroxyapatite column (Bio-Rad, Richmond, CA) equilibrated with the same buffer at room temperature. The starting buffer was 0.01 M NaPB, pH 7.0, with 0.3 mM $CaCl_2$, and the limiting buffer was 0.3 M NaPB, pH 7.0, with 0.01 mM $CaCl_2$. The flow rate was 1.0 mL/min, and 2.0-mL fractions were collected.

(*C*) *Reverse-Phase HPLC.* The cyanogen bromide cleaved peptides of pp 65 were separated on a 250 × 4.6 mm Hi-Pore reverse-phase column (Bio-Rad). The starting solvent was water with 0.01% trifluoroacetic acid (Pierce), and the limiting solvent was 60% acetonitrile (J. T. Baker Chemical Co., Phillipsburg, NJ) with 0.01% trifluoroacetic acid.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Electrophoreses were carried out with a veritcal slab gel of 0.75-mm thickness, 12.5% or 10% (w/v) acrylamide, in a discontinuous Tris–glycine buffer system as reported (Laemmli, 1970). Samples were applied after boiling for 3 min in 0.06 M Tris base, 10% glycerol, 5% 2-ME, and 2.3% SDS, pH 6.8. As molecular weight standards, phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20000), and α-lactalbumin (M_r 14400) from Pharmacia were used. After electrophoreses at 10 mA/gel for about 6 h, gels were stained with Coomassie brilliant blue G-250 or the silver staining method followed by autoradiography at −70 °C.

Antibody Formation to Purified pp 65 and Western Blotting Analysis of pp 65. A rabbit was first immunized with 10 μg of purified pp 65 in Freund's complete adjuvant. Two weeks later, the rabbit was further immunized 3 times weekly with 10 μg of pp 65 in Freund's incomplete adjuvant, and 1 week later, the rabbit was bled, and the serum was prepared by centrifugation after clotting of the blood. For Western blotting analysis of cell extracts, the samples were first separated on 10% or 12.5% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane for 16 h at 100 mA using 0.025 M Tris–glycine buffer, pH 8.3. After the proteins were transferred to a membrane, pp 65 was detected by using the rabbit anti-pp 65 was the first antibody (1:50 dilution) followed by ^{125}I-labeled protein A (4 μCi/20 mL). After the membrane was washed extensively, the membrane was dried and exposed to X-Omat AR film (Kodak, Rochester, NY) for 1–3 days at −70° C.

Amino Acid Composition Analyses of Purified pp 65. After being desalted into distilled water, an aliquot of purified pp 65 was lyophilized and then hydrolyzed with 6 N HCl containing 0.1% phenol, at 110 °C for 24 h, and analyzed on a Durrum D-500 amino acid analyzer (Copeland et al., 1980).

Cyanogen Bromide Cleavage of Purified pp 65 and Amino Acid Sequence Analyses of Cleaved Peptides. Purified pp 65 protein and cyanogen bromide cleaved peptides, which were prepared by digesting purified pp 65 in 70% formic acid by an excess amount of cyanogen bromide (Sigma) for 24 h at 37 °C and purified by reverse-phase chromatography, were subjected to gas-phase automated Edman degradation in an Applied Biosystems sequenator, Model 470A (Forter City, CA), as described. Conversion to the phenylthiohydantoin (PTH) derivative of each amino acid was accomplished with 25% (v/v) trifluoroacetic acid. PTH amino acids were then characterized and quantitated on a Waters phenylalkyl reverse-phase column (Henderson et al., 1980).

Determination of Protein Concentration and Radioactivity in Column Eluates. Protein concentration in column eluates was monitored at 280 nm by a spectrophotometer. The protein concentration of pooled fractions from each purification step was measured by a Bio-Rad dye protein assay kit with bovine serum albumin as a standard. ^{32}P was determined by scintillation counting of Cerenkov radiation.

Results

We first confirmed the rapid and selective induction of phosphorylation of 65-kDa protein in prednisolone-pretreated

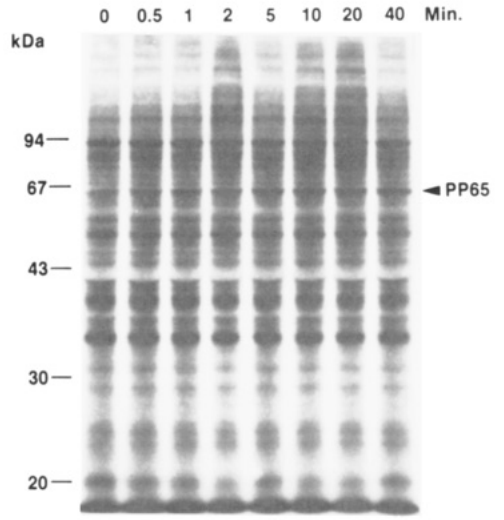

FIGURE 1: Time course of the induction of phosphorylation on 65-kDa protein in PBMC after stimulation with IL 1. PBMC at 5×10^6 cells/mL were cultured for 5 h at 37 °C in the presence of 10^{-5} M prednisolone. Cells were washed and resuspended in phosphate-free RPMI medium, added with 100 μCi/mL [^{32}P]orthophosphate, and incubated additionally for 2 h. Then IL 1α (1 μg/mL) was added, and cells were collected after different time periods. ^{32}P incorporation into cell-associated proteins was visualized by SDS–PAGE followed by autoradiography.

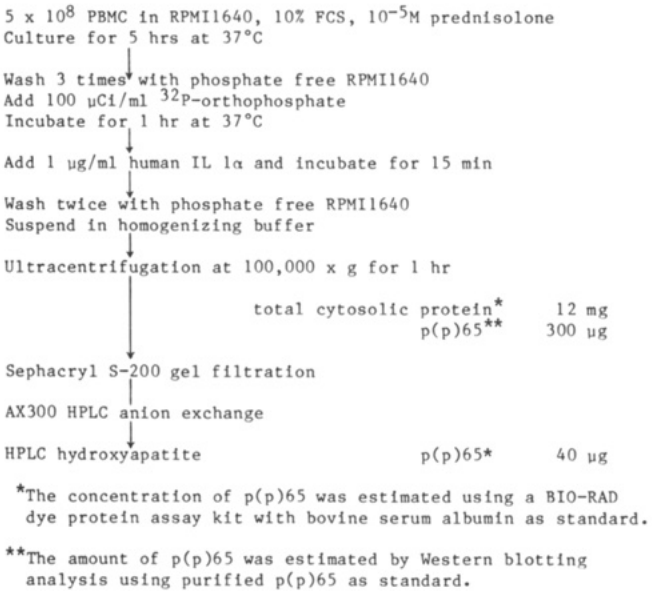

FIGURE 2: Flow sheet for the purification of p(p) 65.

human PBMC after stimulation with IL 1α. As shown in Figure 1, a significant increase in the phosphorylation of 65-kDa protein was already observed at 1 min and reached maximal levels at 10–20 min. As summarized in the purification flow sheet in Figure 2, to purify this 65-kDa phosphoprotein, half-billion PBMC pretreated with 10^{-5} M prednisolone were first labeled with [^{32}P]orthophosphate and stimulated with 1 μg/mL recombinant human IL 1α for 15 min. Since our previous study of the intracellular localization of pp 65 showed that pp 65 was exclusively located in the cytosol fraction of PBMC (Matsushima et al., 1987), cells were disrupted by sonication, and only cytosol which was freed of membranes and particulates by ultracentrifugation at 100000g for 60 min was used as a starting material for the purification of pp 65. As shown in Figure 3, although the overall protein contents of the cytosols from IL 1 nonstimulated and stimulated PBMC were the same (Figure 3, lanes 1 and 2), 65-kDa protein was selectively and predominantly phosphorylated in

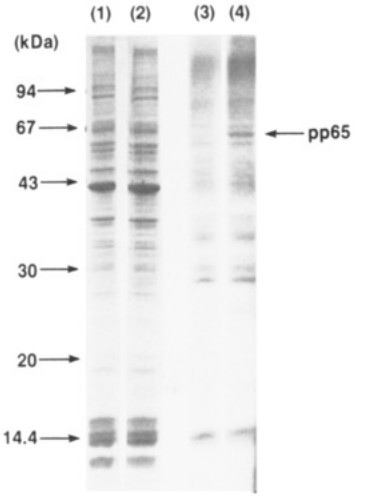

FIGURE 3: SDS–PAGE and autoradiograph of cytosol from PBMC. (1) Coomassie brilliant blue staining of cytosol from prednisolone (10^{-5} M)-pretreated IL 1 nonstimulated PBMC. (2) Coomassie brilliant blue staining of cytosol from prednisolone-pretreated IL 1 stimulated PBMC. (3) Autoradiograph for SDS–PAGE gel of nonstimulated ^{32}P-labeled PBMC cytosol. (4) Autoradiograph for SDS–PAGE gel of IL 1 stimulated ^{32}P-labeled PBMC cytosol.

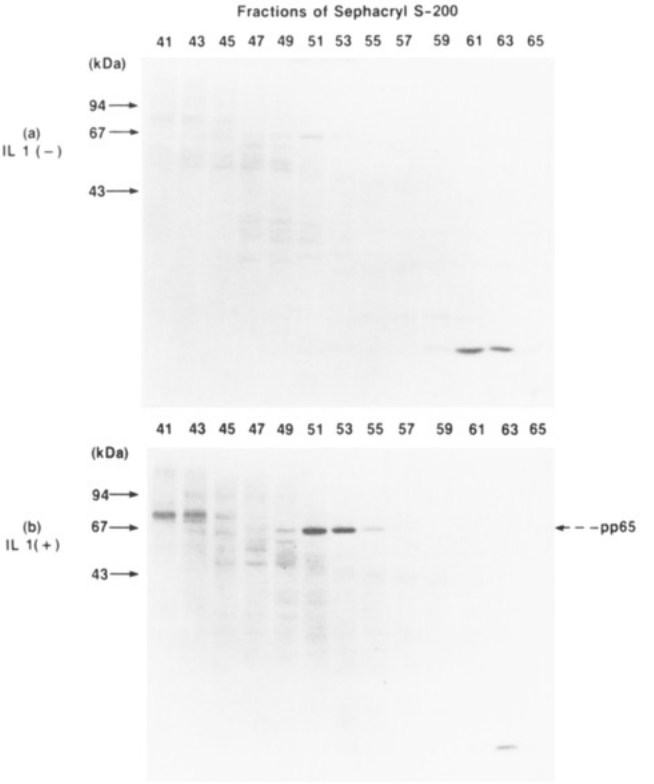

FIGURE 4: Autoradiograph of [^{32}P]orthophosphate-labeled PBMC cytosol fractionated by Sephacryl S-200 gel filtration. (a) Autoradiograph for SDS–PAGE gel of fractions of nonstimulated PBMC cytosol. (b) Autoradiograph for SDS–PAGE gel of fractions of IL 1 stimulated PBMC cytosol.

the cytosol of IL 1 stimulated PBMC (Figure 3, lanes 3 and 4). Cytosol fractions from IL 1 nonstimulated and stimulated PBMC were first chromatographed by Sephacryl S-200 gel filtration, and each fraction was analyzed by SDS–PAGE followed by autoradiography. As shown in Figure 4, fractions 49–55 contained pp 65. The pp 65 was more heavily labeled with [^{32}P]orthophosphate in IL 1 stimulated PBMC (Figure 4b) than in IL 1 nonstimulated PBMC (Figure 4a). pp 65 containing fractions were pooled and applied to an HPLC anion-exchange (AX300) column. As shown in Figure 5, only

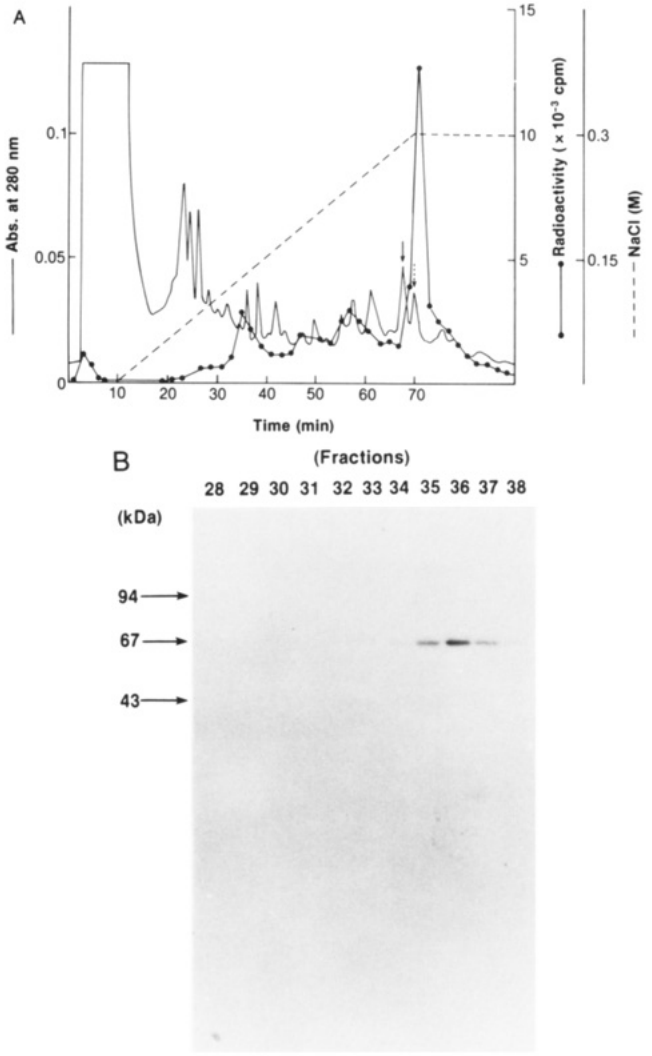

FIGURE 5: (A) HPLC AX300 anion exchange of ^{32}P-labeled pp 65 after S-200 gel filtration. The solid arrow indicates the position of the unphosphorylated form of p 65. The broken arrow indicates the position of ^{32}P-labeled pp 65. (B) Autoradiograph for SDS–PAGE gel of AX300 fractions.

one major ^{32}P-containing peak was detected by counting the radioactivity of each fraction (Figure 5A), and these fractions contained pp 65 (Figure 5B). Since at least three minor contaminants were detected by SDS–PAGE analysis when stained with Coomassie brilliant blue (data not shown), pp 65 containing fractions from AX300 were pooled and applied to a hydroxyapatite HPLC column. As shown in Figure 6A, this yielded one dominant absorbance peak with two minor absorbance peaks. SDS–PAGE analysis of fractions corresponding to the fractions containing most of the radioactivity showed only one protein band corresponding to 65 kDa (Figure 6B, lanes 3 and 4), and the autoradiograph indicated complete identity between the protein band and radiolabeled material (Figure 6B, lanes 5 and 6). In addition, since the peak eluting at 67 min from the AX300 anion-exchange HPLC column (as pointed by a solid arrow), which adjoined the pp 65 at 70 min, also contained predominantly 65-kDa protein (p 65) on SDS–PAGE analysis and since the amount of p 65 was much greater in IL 1 nonstimulated PBMC cytosol than in IL 1 stimulated PBMC cytosol (data not shown), we speculated that p 65 at 67 min is probably an unphosphorylated form of pp 65. This hypothesis was supported by the fact that pp 65 is a little more acidic than p 65, probably due to phosphorylation. Therefore, we also purified the p 65 to homogeneity by further

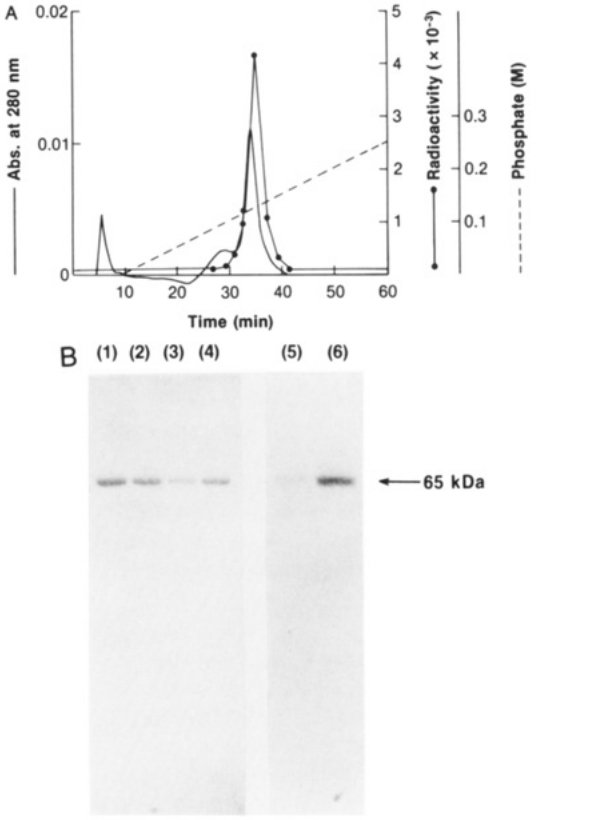

FIGURE 6: (A) Hydroxyapatite HPLC of ^{32}P-labeled pp 65 after HPLC anion exchange. (B) SDS–PAGE for (1 and 2) purified unphosphorylated form of p 65, (3 and 4) purified phosphorylated form of pp 65, and (5 and 6) autoradiograph of purified ^{32}P-labeled pp 65.

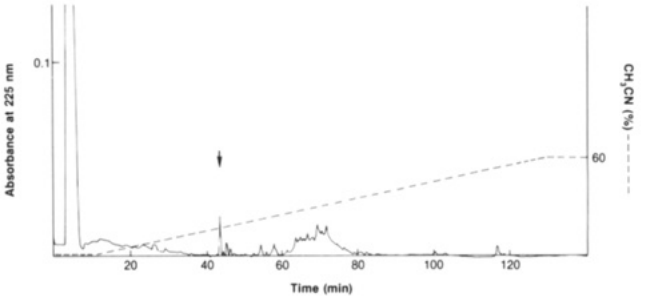

FIGURE 7: Reverse-phase HPLC of CNBr-cleaved peptides derived from purified pp 65. The arrow indicates the fragment which was used for amino acid sequence analysis.

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1   2   3   4   5   6   7   8   9   10  11
Ala-Arg-Lys-Ile-Gly-Ala-Arg-Val-Tyr-Ala-Leu
12  13  14  15  16  17  18  19  20  21  22
Pro-Glu-Asp-Leu-Val-Glu-Val-Asn-Pro-Lys-(Thr)
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FIGURE 8: Amino acid sequence of the fragment derived from CNBr-cleaved peptides of pp 65.

applying this fraction to hydroxyapatite HPLC (Figure 6B). As shown in Table I, the amino acid compositions of p 65 and pp 65 were essentially identical, confirming that pp 65 is a phosphorylated form of p 65. Since the amino-terminal end of the purified pp 65 could not be detected by the Edman degradation method, pp 65 was digested with cyanogen bromide, and the digested peptide fragments were separated by reverse-phase column chromatography (Figure 7). The amino acid sequence of the peptide at 44 min is shown in Figure 8. A computer-assisted search for protein sequence

Table I: Amino Acid Composition (Mole Percent) of Purified pp 65 and p 65 from Cytosol of PBMC

amino acid	pp 65	p 65	amino acid	pp 65	p 65
Asp	14.9	15.2	Ile	6.3	5.9
Thr	4.1	4.2	Leu	10.8	11.0
Ser	5.5	5.3	Tyr	2.3	2.5
Glu	11.5	11.6	Phe	3.5	3.3
Pro	1.9	3.5	His	1.8	1.6
Gly	9.5	9.7	Lys	7.8	7.4
Ala	7.4	7.1	Arg	4.7	4.6
Val	6.0	5.2	Cys	NT[a]	NT
Met	2.0	1.9	Trp	NT	NT

[a] NT, not tested.

Table II: Distribution of p(p) 65 in Various Types of Human Cells

PBMC	+	LGL, YT cell line	+
PMN	+	T lymphocyte lines	
monocytic cell lines		Jurkat	+
THP-1	+	MOLT	−
U937	+	myeloid cell lines	
HL60	+	K562	+
EBV-B lymphocyte lines		melanoma, (A375) cell line	−
ORS	+	skin fibroblasts, 1507	−
FMO	+	HeLa cells	−
Hodgkin cells	+		

homology of this peptide sequence with reported protein sequences revealed that pp 65 has a novel protein sequence.

In order to examine the levels of p(p) 65 in a variety of human cell line cells, polyclonal antibody to p(p) 65 was made by immunizing a rabbit with purified pp 65. Using this antibody, we could examine the distribution of p(p) 65 in different types of cells by Western blotting analysis as shown in Figure 9A. As shown in Figure 9B and summarized in Table II, p(p) 65 protein could be detected in a variety of human cells, including monocytes, T, B, and large granular lymphocytes, and myeloid cells. p(p) 65 was not detected in melanoma cells, dermal fibroblasts, HeLa cells, or MOLT cells, suggesting some selectivity of the presence of p(p) 65 in different types of cells.

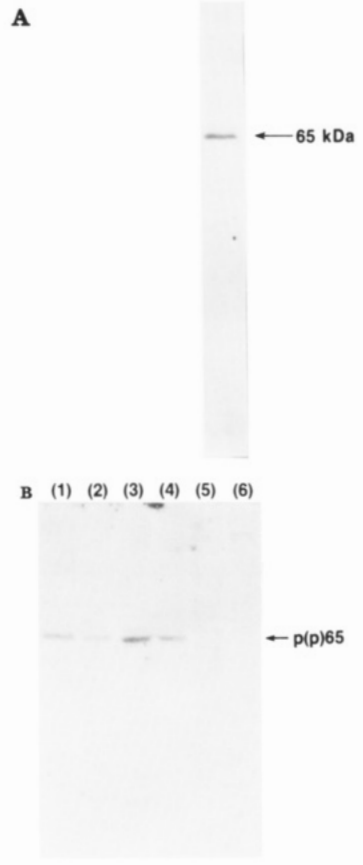

FIGURE 9: (A) Western blotting analysis of p(p) 65 from human PBMC cytosol using rabbit anti-human p(p) 65. (B) Western blotting analysis of p(p) 65 from other cell line extracts: (1) ORS; (2) YT; (3) THP-1; (4) FMO; (5) A375; (6) fibroblast 1507.

Discussion

We have purified both phosphorylated and unphosphorylated forms of a cytosolic 65-kDa protein in normal human PBMC whose phosphorylation is selectively augmented by IL 1 stimulation. Although the amino terminus of pp 65 was blocked, we have determined the amino acid sequence of one of the CNBr-cleaved peptides. A computer-assisted search for sequence homology of pp 65 with known protein sequences revealed that pp 65 has a unique protein sequence. By repeated and cumulative purification of p(p) 65, we could reproducibly purify about 40 μg of p 65 and pp 65 from 5×10^8 PBMC. In IL 1 stimulated glucocorticoid-treated PBMC cytosol, about 30–50% of 65-kDa protein is in the form of pp 65, whereas in nonstimulated PBMC cytosol only 5% is pp 65 based on the absorption and elution positions of p 65 and pp 65 on AX300 anion-exchange HPLC as shown in Figures 3 and 5A. Although the molecular weight of IL 1 receptor on glucocorticoid-treated PBMC (60–67 kDa) (Matsushima et al., 1987) is close to the molecular weight of p(p) 65, pp 65 is not the IL 1 receptor itself, because pp 65 localizes exclusively in cytosol. Western blotting analysis of particulates/membrane and cytosol-enriched fractions from unstimulated PBMC also showed that p(p) 65 was located exclusively in the cytosol (data not shown).

Phosphoamino acid analysis of purified ^{32}P-labeled pp 65 by thin-layer chromatography showed that only serine residues are phosphorylated as previously described (Matsushima et al., 1987). In preliminary experiments, purified p 65 could be phosphorylated in vitro by cAMP-dependent protein kinase A but not by protein kinase C (data not shown). This was consistent with the observation that the induction of the phosphorylation of p 65 could be inhibited in vivo by HA1004 and W-7 but not by H-7. However, we could not observe significant induction of intracellular cAMP by IL 1 stimulation of PBMC with and without prednisolone treatment. Recently, we have been able to identify an IL 1 inducible serine kinase in the cytosol fraction of prednisolone-pretreated PBMC using p65 as an in vitro substrate (Shiroo et al., unpublished data). Purification and characterization of this kinase are in progress.

Chaplin et al. (1980) have described phosphorylation of a similar 65-kDa protein in human PBMC following stimulation with lectins, such as phytohemagglutinin, concanavalin A, or pokeweed mitogen. However, no further biochemical characterization has been performed on that protein. In addition, although p(p) 65 could be detected in a variety of human cells, we could not detect p(p) 65 in at least two IL 1 responding cells [a melanoma cell line, A375 (Onozaki et al., 1985), and skin fibroblasts (Schmidt et al., 1982)], suggesting that the activation pathway by IL 1 is not uniform. We also could not detect the increase in phosphorylation of p 65 in YT cells after stimulation with IL 1 although YT cells have both IL 1 receptors and p 65. This suggests that the means by which IL 1 stimulates different types of cells may be highly variable. Consequently, phosphorylation of p 65 may play an important role only in regulation of activities of PBMC (especially B cells). This hypothesis is supported by the observations that IL 1 induced phosphorylation of p 65 concomitant with increased immunoglobulin production in prednisolone-treated PBMC (G. Tosato et al., unpublished observation). At present,

we do not have any evidence for the existence of G proteins coupled with IL 1 receptor, but it is also reasonable to speculate that several different types of G proteins are coupled with IL 1 receptor in different cell types. These different G proteins may regulate the subsequent intracellular biochemical events as proposed in other systems (Burch et al., 1986).

In conclusion, although the physiological function of pp 65 has not yet been established, the purified p 65, purified pp 65, and antibody can be used to study the physiological roles of this moiety. Antibody can also be useful for immunocytochemical analysis. With the specific antibody to pp 65 and the protein sequence of pp 65, we should be able to clone the cDNA to determine the entire amino acid sequence of pp 65.

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Thiol- and pH-Modulated Slow Conformational Changes and Cooperativity of Phenol-Binding Sites in Phenol Hydroxylase†

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ABSTRACT: Spectrophotometric titration of phenol hydroxylase (EC 1.14.13.7) with phenol indicated interacting sites for phenol binding. In the absence of added thiol, the cooperativity was positive up to a pH around 8.0 but negative at higher pH values. With added thiol–ethylenediaminetetraacetate, the cooperativity was negative at all investigated pH values. Conversely, a corresponding titration of an enzyme preparation that had been selectively modified in its two most reactive SH groups indicated positive cooperativity at all studied pH values. This selective modification affects the activity of the enzyme to a very minor degree, in contrast to more extensive SH blocking, which displaces flavin adenine dinucleotide with a corresponding loss of activity [Neujahr, H. Y., & Gaal, A. (1975) *Eur. J. Biochem. 58*, 351–357]. The reactivity of SH groups in the enzyme was significantly decreased after turnover. Thiol treatment restored it to that of the native enzyme. Adding phenol prior to reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the assay of phenol hydroxylase gave immediate linearity and higher initial rates than when NADPH was added first. In the absence of added thiol, there was then a shift of the pH optimum. The results indicate slow conformational changes limiting the rate of the overall reaction. The two most reactive SH groups of phenol hydroxylase, though not participating in any obvious redox reactions, are important for these slow conformational changes and for the cooperativity of phenol-binding sites, wherein the anionic S^- forms may be involved (pK_a for cysteine is 8.35).

The prokaryotic flavin-containing aromatic hydroxylases, which employ reduced pyridine nucleotides as electron-donating cosubstrates, undergo extensive changes in conformation upon binding of their phenolic substrates [for a review, see Ballou (1984) and Massey and Hemmerich (1975)]. These changes affect absorption, fluorescence, and circular dichroism spectra. Perturbation of absorption spectra or quenching of flavin fluorescence upon binding of phenolic substrates to these enzymes has been used to measure binding parameters. No

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