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Characterization of the 47-Kilodalton Autosomal Chronic Granulomatous Disease Protein: Tissue-Specific Expression and Transcriptional Control by Retinoic Acid

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A full-length cDNA clone was isolated for the 47-kilodalton (kDa) subunit of the NADPH oxidase system, whose absence is responsible for the most common form of autosomally inherited chronic granulomatous disease (CGD). It encodes a 44.7-kDa polypeptide, which contains two *src* homology (SH3) domains and several possible sites for phosphorylation by protein kinase C. We speculate that the SH3 domains may interact with the Rap1 protein associated with cytochrome b_{-245} (M. T. Quinn, C. A. Parkes, L. Walker, S. Orkin, M. Dinauer, and A. Jesaitis, Nature [London] 342:198–200, 1989). An antiserum raised to the predicted C terminus of the protein detects a polypeptide with an apparent molecular mass of 47 kDa in normal neutrophil granulocytes but not in those from patients with autosomal CGD. The antibody has been used to show that the protein associates with the vacuolar membrane and is phosphorylated in response to phorbol ester treatment. Analysis of a number of tissue types and cell lines shows that expression of the gene is confined to phagocytic cells and B lymphocytes. This observation suggests that patients with CGD may also have a defect in lymphocyte function. p47 protein and mRNA levels increase during retinoic acid-induced neutrophil differentiation of HL60 cells. Nuclear run-on transcription assays show that the gene for p47 is induced at the transcriptional level in a cycloheximide-insensitive manner. These data indicate that this gene is a primary target for regulation by retinoic acid.

Human chronic granulomatous disease (CGD) is an inherited syndrome characterized by the inability of phagocytic cells to destroy many of the organisms which they ingest. This disorder makes the patients prone to prolonged and sometimes fatal infections. The dysfunction of phagocyte killing results from an inability to generate superoxide (O_2^-) via the NADPH oxidase system (reviewed in reference 41). Inheritance of the disease takes both X-linked (65%) and autosomal (35%) forms. The gene for X-linked CGD has been isolated and shown to code for the large subunit of the membrane-bound heterodimeric cytochrome b_{-245} (15, 40, 46), which is tightly associated with the Rap1 protein (38). The development of in vitro assays for NADPH oxidase activity (6) has allowed two classes of autosomal CGD (A-CGD) (35) which lack cytosolic proteins of 67 and 47 kilodaltons (kDa) to be defined (50). p47 is a phosphoprotein which is absent in the majority of patients with A-CGD. Upon activation of the cell, this protein is phosphorylated by protein kinase C (21, 26, 42) and is thought to stimulate the NADPH oxidase in the vacuolar membrane. Cell fractionation studies have shown that in response to phorbol ester, the 47-kDa phosphoprotein translocates to a membrane fraction in normal cells but not in cells from patients with X-linked CGD which lack the cytochrome (9, 22). The study of p47 has been hampered by the lack of a monospecific antibody, although an antiserum has been made against a population of neutrophil proteins, including p47 and p65, isolated by GTP-agarose chromatography (50). Recently two groups of researchers have used this antiserum to isolate p47 cDNA clones (30, 51).

We have been studying the means by which retinoic acid (RA) induces granulocyte differentiation in the promyelocytic leukemia cell line HL60. Using a differential cDNA hybridization screen, we attempted to isolate genes regulated during this differentiation process. The sequence of one gene induced by RA turned out to be almost identical to the p47 sequences published by Lomax et al. (30) and Volpp et al. (51). There were, however, a number of single-base discrepancies between our sequence and the two others, which resulted in three different predictions for the open reading frame encoding the C-terminal portion of the protein. In this report we establish the reading frame of the C terminus by raising an antipeptide antibody and showing that it recognizes a 47-kDa phosphoprotein absent from cells of patients with A-CGD. Our cDNA sequence is in agreement with the recent revisions of the previously reported sequences (30, 51). The anti-C-terminus antibody is highly specific for p47 and has been used to investigate the protein by immunoprecipitation and immunofluorescence.

The results show a restricted pattern of gene expression limited to three cell types: granulocytes, monocytes, and B lymphocytes. We have investigated the regulation of the gene during neutrophil differentiation of HL60 cells and demonstrated a rapid transcriptional activation in response to RA.

MATERIALS AND METHODS

Cell culture. Leukemic cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (20% for KG1a cells) at 37°C and 10% CO_2. Differentiation of

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HL60 cells was induced by treatment with 1 μM RA (Sigma Chemical Co., St. Louis, Mo.) (4).

cDNA cloning. The cDNA was isolated as a gene whose expression is increased when HL60 promyelocytic leukemia cells are induced to differentiate into neutrophils. A lambda *gt*10 library of cDNA from HL60 cells induced with RA for 3 days was constructed. Duplicate filter lifts were probed with ^{32}P-labeled cDNA either from uninduced HL60 cells or from HL60 cells induced with RA for 6 days. Plaques which showed increased hybridization with cDNA from differentiated cells were picked and rescreened by hybridization to Northern blots of RNA from induced and uninduced HL60 cells.

Sequencing the cDNA. The cDNA was subcloned into the modified Bluescribe vector pVZ1 (20), and deletion series were made by the exonuclease III-S1 method of Henikoff (19). The full sequence of the cDNA was determined on both strands by the dideoxy-chain termination method with Sequenase (United States Biochemical Corp., Cleveland, Ohio), with dITP or 7′-deaza-GTP used at sites of compressions. Computer analysis of the sequences and database searches were performed with the following programs: Prosearch (J. Collins and A. Coulson, University of Edinburgh, Edinburgh, United Kingdom), AMPS (G. Barton and M. Sternberg, Imperial Cancer Research Fund, London, United Kingdom), Genepro (Riverside Scientific, Seattle, Wash.), and Multalin (11).

RNA extraction and analysis. Total RNA was extracted either by the RES (2) or by the guanidinium isothiocyanate-CsCl (8) method. For primer extension analysis and S1 mapping, poly(A)$^+$ RNA from RA-induced HL60 cells was prepared by oligo(dT) cellulose chromatography.

RNase protection analysis (34) was performed on 5 μg of total RNA in most cases. The actin probe was complementary to the most 3′ *Hin*f1 fragment of the human γ-actin cDNA (17). The p47 probe was transcribed from a plasmid containing a 3′ deletion of the p47 cDNA and was complementary to the first 236 bases of the cDNA plus about 70 bases of vector.

For primer extension analysis, a 20-base oligonucleotide complementary to bases 27 to 47 of the cDNA was 5′-end labeled with ^{32}P by using T4 polynucleotide kinase, annealed to 1 μg of poly(A)$^+$ RNA, and extended with avian myeloblastosis virus reverse transcriptase.

The probe for S1 protection analysis was produced by primer extension with the same oligonucleotide on a cloned, single-stranded genomic DNA containing the 5′ end of the cDNA. The probe extended to a *Pst*I site 187 bases upstream of the cDNA start site (data not shown).

Nuclear run-on transcription. Transcriptional elongation (run-on) assays were performed on nuclei from HL60 cells which had been treated for various times with 1 μM RA. A 12-h time point assay was also performed on cells treated with 100 μg of cycloheximide per ml. The protein synthesis inhibitor was present for 1 h before RA treatment as well as throughout the induction. The method used was essentially that described in reference 29, with the exception that the nuclei (2×10^7 for each time point) were suspended in a glutamate buffer [125 mM potassium glutamate, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 8.0), 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 40% glycerol] and frozen until required. Elongation (15 min at 37°C) was carried out in 50 μl of the same buffer supplemented with 200 μCi of [^{32}P]UTP, 1 mM ATP, 0.25 mM each GTP and CTP, 10 mM creatine phosphate, 1 U of RNasin per μl, and 50 μg of creatine kinase per ml. The RNA synthesized was fragmented by treatment with 0.3 M NaOH for 10 min on ice before hybridization. The c-*myc* probes were described previously (1). The histone H2B probes contained a 650-base fragment encompassing the entire chicken H2B coding region derived from pSP62-H2B (27) subcloned in both orientations in M13mp18 and mp19.

Raising antipeptide antiserum. A peptide corresponding to the C-terminal 20 residues of the predicted protein product was synthesized, coupled to Keyhole Limpet Hemocyanin, and injected in incomplete Freund adjuvant into two rabbits. One rabbit showed a significant immune response, as assayed by solid-phase enzyme-linked immunosorbent assay (18).

Immunoprecipitation. Immunoprecipitation was performed as described by Harlow and Lane (18). In vivo labeling was performed by incubating HL60 cells (5×10^6 to 10×10^6/ml) overnight in RPMI 1640 medium minus methionine with 4% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine per ml. Cells (10^7) were lysed in 1% Nonidet P-40 or 1% Triton X-114 (which enables hydrophilic and amphiphilic fractions to be separated) (3) containing 50 mM NaCl and 50 mM Tris hydrochloride (pH 8.0) and supplemented with the proteinase inhibitors phenylmethylsulfonylfluoride (100 μg/ml) and benzamidine (1 mM). For $^{32}P_i$ labeling, 10^8 HL60 cells were incubated with 400 μCi of $^{32}P_i$ for 4 h in 1 ml of RPMI 1640 medium minus phosphate plus 5% fetal calf serum followed by 5 min of stimulation with 1 μM TPA (12-*O*-tetradecanoylphorbol-13-acetate) or mock stimulation with the solvent (dimethyl sulfoxide) only. The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibody was blocked by adding 5 μg of peptide per μl of serum. The reaction mixture was incubated for 10 min at room temperature in phosphate-buffered saline plus 0.3% bovine serum albumin, centrifuged for 15 min in a microcentrifuge to remove immune complexes, and used immediately.

Western immunoblot analysis. Neutrophils were isolated from the blood of a healthy donor and from four unrelated patients with A-CGD. Isolation of cytoplasm and immunoblotting were performed as described elsewhere (47).

Immunofluorescence. Cells were surface stained with phycoerythrin-coupled anti-CD19 (B4; Coulter Electronics, Inc., Hialeah, Fla.) or anti-CD8 (SK2; Becton Dickinson and Co., Paramus, N.J.) monoclonal antibodies, fixed with 2% paraformaldehyde, and then permeabilized with 0.2% Nonidet P-40. These cells were then stained with the antipeptide antiserum (immunoglobulin G fraction) and fluorescein isothiocyanate-conjugated goat anti-rabbit F(ab′)$_2$ (TAGO Inc.). Stained cells were analyzed with a Facscan apparatus.

For immunofluorescence microscopy, neutrophils were allowed to phagocytose 2.95-μm latex beads by coincubation for 2 h in RPMI 1640 medium plus 10% fetal calf serum at 37°C. The cells were fixed and then permeabilized with a methanol-acetone (1:1) mixture for 2 min before being stained for p47 with affinity-purified antipeptide antibody.

RESULTS

Sequence analysis. The p47 cDNA (Fig. 1) was isolated from HL60 cells as a gene that was switched on during RA-induced differentiation. The clone is 1,380 bases long, including a poly(A) tail of 31 bases. This is consistent with the size of the major hybridizing species seen on Northern blots (1.4 kilobases [data not shown]). A minor hybridizing

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                                                                G      1
AGC ACT GGA GGC CAC CCA GTC ATG GGG GAC ACC TTC ATC CGT CAC           46
                            M   G   D   T   F   I   R   H
ATC GCC CTG CTG GGC TTT GAG AAG CGC TTC GTA CCC AGC CAG CAC           91
I   A   L   L   G   F   E   K   R   F   V   P   S   Q   H
TAT GTG TAC ATG TTC CTG GTG AAA TGG CAG GAC CTG TCG GAG AAG          136
Y   V   Y   M   F   L   V   K   W   Q   D   L   S   E   K
GTG GTC TAC CGG CGC TTC ACC GAG ATC TAC GAG TTC CAT AAA ACC          181
V   V   Y   R   R   F   T   E   I   Y   E   F   H   K   T
TTA AAA GAA ATG TTC CCT ATT GAG GCA GGG GCG ATC AAT CCA GAG          226
L   K   E   M   F   P   I   E   A   G   A   I   N   P   E
AAC AGG ATC ATC CCC CAC CTC CCA GCT CCC AAG TGG TTT GAC GGG          271
N   R   I   I   P   H   L   P   A   P   K   W   F   D   G
CAG CGG GCC GCC GAG AAC CGC CAG GGC ACA CTT ACC GAG TAC TGC          316
Q   R   A   A   E   N   R   Q   G   T   L   T   E   Y   C
AGC ACG CTC ATG AGC CTG CCC ACC AAG ATC TCC CGC TGT CCC CAC          361
S   T   L   M   S   L   P   T   K   I   S   R   C   P   H
CTC CTC GAC TTC TTC AAG GTG CGC CCT GAT GAC CTC AAG CTC CCC          406
L   L   D   F   F   K   V   R   P   D   D   L   K   L   P
ACG GAC AAC CAG ACA AAA AAG CCA GAG ACA TAC TTG ATG CCC AAA          451
T   D   N   Q   T   K   K   P   E   T   Y   L   M   P   K
GAT GGC AAG AGT ACC GCG ACA GAC ATC ACC GGC CCC ATC ATC CTG          496
D   G   K   S   T   A   T   D   I   T   G   P   I   I   L
CAG ACG TAC CGC GCC ATT GCC GAC TAC GAG AAG ACC TCG GGC TCC          541
Q   T   Y   R   A   I   A   D   Y   E   K   T   S   G   S
GAG ATG GCT CTG TCC ACG GGG GAC GTG GTG GAG GTC GTG GAG AAG          586
E   M   A   L   S   T   G   D   V   V   E   V   V   E   K
AGC GAG AGC GGT TGG TGG TTC TGT CAG ATG AAA GCA AAG CGA GGC          631
S   E   S   G   W   W   F   C   Q   M   K   A   K   R   G
TGG ATC CCA GCA TCC TTC CTC GAG CCC CTG GAC AGT CCT GAC GAG          676
W   I   P   A   S   F   L   E   P   L   D   S   P   D   E
ACG GAA GAC CCT GAG CCC AAC TAT GCA GGT GAG CCA TAC GTC GCC          721
T   E   D   P   E   P   N   Y   A   G   E   P   Y   V   A
ATC AAG GCC TAC ACT GCT GTG GAG GGG GAC GAG GTG TCC CTG CTC          766
I   K   A   Y   T   A   V   E   G   D   E   V   S   L   L
GAG GGT GAA GCT GTT GAG GTC ATT CAC AAG CTC CTG GAC GGC TGG          811
E   G   E   A   V   E   V   I   H   K   L   L   D   G   W
TGG GTC ATC AGG AAA GAC GAC GTC ACA GGC TAC TTT CCG TCC ATG          856
W   V   I   R   K   D   D   V   T   G   Y   F   P   S   M
TAC CTG CAA AAG TCG GGG CAA GAC GTG TCC CAG GCC CAA CGC CAG          901
Y   L   Q   K   S   G   Q   D   V   S   Q   A   Q   R   Q
ATC AAG CGG GGG GCG CCG CCC CGC AGG TCG TCC ATC CGC AAC GCG          946
I   K   R   G   A   P   P   R   R   S   S   I   R   N   A
CAC AGC ATC CAT CAG CGG TCG CGG AAG CGC CTC AGC CAG GAC GCC          991
H   S   I   H   Q   R   S   R   K   R   L   S   Q   D   A
TAT CGC CGC AAC AGC GTC CGT TTT CTG CAG CAG CGA CGC CGC CAG         1036
Y   R   R   N   S   V   R   F   L   Q   Q   R   R   R   Q
GCG CGG CCG GGA CCG CAG AGC CCC GGG AGC CCG CTC GAG GAG GAG         1081
A   R   P   G   P   Q   S   P   G   S   P   L   E   E   E
CGG CAG ACG CAG CGC TCT AAA CCG CAG CCG GCG GTG CCC CCG CGG         1126
R   Q   T   Q   R   S   K   P   Q   P   A   V   P   P   R
CCG AGC GCC GAC CTC ATC CTG AAC CGC TGC AGC GAG AGC ACC AAG         1171
P   S   A   D   L   I   L   N   R   C   S   E   S   T   K
CGG AAG CTG GCG TCT GCC GTC TGA GGC TGG AGC GCA GTC CCC AGC         1216
R   K   L   A   S   A   V   *
TAG CGT CTC GGC CCT TGC CGC CCC GTG CCT GTA CAT ACG TGT TCT         1261
ATA GAG CCT GGC GTC TGG ACG CCG AGG GCA GCC CCG ACC CCT GTC         1306
CAG CGC GGC TCC CGC CAC CCT CAA TAA ATG TTG CTT GGA GTG GpolyA      1380
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FIG. 1. Nucleotide sequence of p47 cDNA and derived protein sequence. The site of translocation initiation is at base 22. Underlined regions indicate the two SH3 domains. Serine residues which could be substrates for protein kinase C are in boldface type and are double underlined.

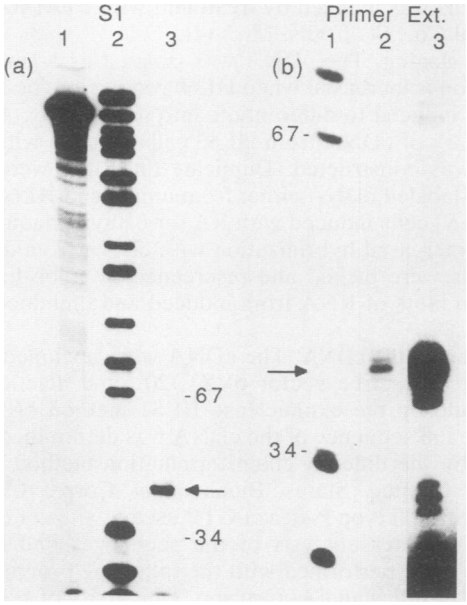

FIG. 2. Mapping of the 5′ end of the p47 mRNA. (a) S1 nuclease protection by mRNA from RA-treated HL60 cells of a 235-base-pair probe derived from a cloned genomic fragment covering the 5′ end of the cDNA sequence. Lanes: 1, undigested probe; 2, *Msp*I-digested pBR322 size markers; 3, S1 digestion products. (b) Primer extension on mRNA from RA-treated HL60 cells. Lanes: 1, *Msp*I-digested pBR322 size markers; 2, primer extension products; 3, overexposed autoradiograph of lane 2.

species of 2.1 kilobases was also observed in Northern blots of HL60 cells (data not shown). The first methionine codon is followed by an open reading frame coding for a 390-amino-acid protein with a predicted molecular mass of 44.7 kDa. Its calculated isoelectric point of 9.93 is consistent with that of the unmodified 47-kDa A-CGD factor as estimated by two-dimensional gel analysis (12, 36).

Mapping the mRNA 5′ end. Our cDNA sequence extends slightly further in the 5′ direction than the two previously described clones (30, 51), but it does not include an in-frame upstream termination codon. We could not, therefore, eliminate the possibility that the open reading frame extended upstream of the first Met codon in the cDNA. In order to address this question, we mapped the 5′ end of the mRNA. Primer extension analysis was performed with a 5′-end-labeled oligonucleotide corresponding to bases 27 to 47 of the cDNA. The major extension product of 45 to 50 bases (Fig. 2b, lane 2) maps the 5′ end of the RNA to within 3 bases of the 5′ end of the cDNA clone. A long exposure shows minor extension products up to 70 bases long which may correspond to minor start sites (Fig. 2b, lane 3). The 5′ end corresponding to the major primer extension product was confirmed by an S1 nuclease protection assay. For this experiment, a genomic DNA fragment that includes the 5′ end of the cDNA was isolated and used to prepare a 220-base 5′-end labeled probe extending from base 47 of the cDNA to an upstream *Pst*I site. The protected fragments of 45 to 50 bases (Fig. 2a, lane 3) confirm the primer extension result. Sequencing of the genomic fragment showed that the start site mapped by S1 protection does not correspond to a splice acceptor site. Furthermore, an in-frame termination codon is found 24 bases upstream of the transcription initiation site mapped in Fig. 2. This evidence proves that the first Met codon in the cDNA does in fact correspond to the N terminus of the protein.

Antipeptide antiserum to p47. A rabbit antiserum was raised to a peptide corresponding to the C-terminal 20 residues of the predicted protein. RNA was synthesized in vitro from the cDNA and translated in a rabbit reticulocyte lysate, and the product was immunoprecipitated with the antiserum. The major precipitated translation product migrated with an apparent molecular mass of 47 kDa (data not shown). This result confirms the reading frame assignment of the C terminus of the protein. Confirmation that the antiserum does indeed recognize the 47-kDa factor missing in cells of patients with A-CGD was provided by probing immunoblots of protein from neutrophils of a normal donor and patients with A-CGD. Whereas an immunoreactive band at 47 kDa was detected in the normal sample (Fig. 2, lane 5), no such band could be detected in samples from four patients with A-CGD (Fig. 3, lanes 1 to 4). The faint 36-kDa species

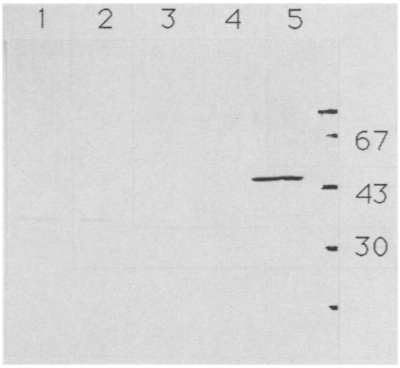

FIG. 3. Western blot analysis of cytoplasmic proteins from normal neutrophils (lane 5) and neutrophils from four unrelated patients with A-CGD (lanes 1 to 4); the anti-C-terminal antiserum was used. The sizes of the relevant molecular weight standards are shown in thousands.

detected in this assay is apparently a cross-reacting protein and was not detected by immunoprecipitation from HL60 cells (Fig. 4a).

SH3 domains and intracellular localization. The predicted protein contains two copies of a 50-amino-acid domain of homology to the *src* superfamily, named SH3 (37) (Fig. 5). In addition to the nonreceptor protein-tyrosine kinases, these domains are found in a number of other cytoplasmic proteins (33, 39, 44), including the 67-kDa cytosolic NADPH oxidase factor (28). We have previously speculated that SH3 domains are involved in protein association with the inner face of the plasma membrane (see reference 39 and Discussion). A subset of SH3-containing proteins, including GAP and CDC25 of *Saccharomyces cerevisiae* (5, 39), is thought to interact directly with members of the Ras protien family. In

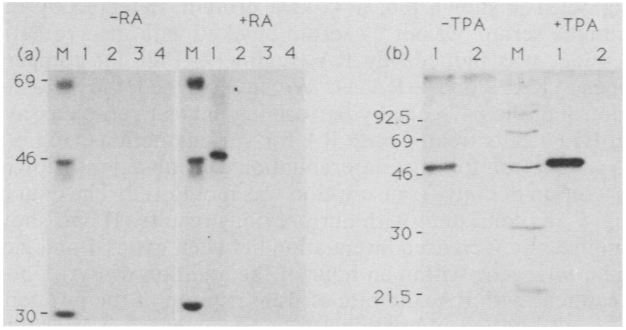

FIG. 4. Immunoprecipitations of p47 protein from HL60 cells. (a) p47 is induced by RA treatment of HL60 cells and partitions into the hydrophilic phase after Triton X-114 fractionation. [^{35}S]methionine-labeled HL60 cells were lysed with 1% Triton X-114, separated into hydrophilic and amphiphilic fractions, and immunoprecipitated. Lanes: M, molecular weight markers (shown in thousands at left); 1, hydrophilic fraction, immune antiserum; 2, hydrophilic fraction, peptide blocked; 3, amphiphilic fraction, immune antiserum; 4, amphiphilic fraction, peptide blocked. The HL60 cells were either undifferentiated (−RA) or induced to differentiate with 1 μM RA for 48 h (+RA). (b) p47 phosphorylation increases in response to phorbol ester treatment. p47 was immunoprecipitated from $^{32}P_i$-labeled cells. Lanes: 1, immune serum; 2, peptide-blocked serum; M, molecular weight markers (shown in thousands at left). The cells, which had been induced to differentiate with 1 μM RA for 48 h, were treated with 1 μM TPA for 5 min (+TPA) or mock treated (−TPA) with dimethyl sulfoxide.

Fig. 5 we show that two other proteins implicated in the control of Ras function also contain SH3 domains: STE6 of *Schizosaccharomyces pombe* (24) and IRA1 of *S. cerevisiae* (45). The dendrogram in Fig. 5 shows that the closest relatives of the N- and C- terminal SH3 domains of p47 are fodrin and GAP, respectively. The closest relative of the SH3 domains in p67 is STE6. The SH3 domains of p47 and p67 may be of importance with regard to potential interaction with the Rap1 protein that is associated with cytochrome b_{-245} (38).

Previous cellular fractionation studies have detected p47 in a membrane fraction in activated neutrophils (9, 22). This has been assumed to correspond to a translocation of the protein to the membrane of phagocytic vacuoles, where it is thought to interact directly or indirectly with cytochrome b_{-245} to activate electron transport. A specific antibody allows us to test directly this scheme for p47 function by immunofluorescent localization of the protein. Neutrophils were allowed to ingest latex beads, stained with antiserum, and viewed with a confocal microscope. All neutrophils were stained by the antiserum but with different intensities. Figure 6 shows confocal immunofluorescence micrographs of three particularly brightly stained cells (Fig. 6B, D, and F) and corresponding phase-contrast images (Fig. 6A, C, and E). These optical sections show rings of p47 staining around ingested beads, indicating that the protein associates with the vacuolar membrane. No staining was observed with an equal concentration of preimmune immunoglobulin G from the same rabbit or if the permeabilization step was omitted.

Although p47 associates with membranes, it contains no extensive hydrophobic region. We looked for hydrophobic properties of the protein which might result from posttranslational modifications by fractionating cell extracts with Triton X-114. When heated above the cloud point, the lysate separates into detergent and aqueous phases with amphiphilic and hydrophilic proteins partitioning between them (3). Figure 4a shows that the vast majority of p47 in HL60-derived neutrophils partitions to the hydrophilic phase. The same results were obtained with TPA-stimulated cells (data not shown). It is concluded that the protein is probably not modified to become intrinsically lipophilic and that it may therefore interact with membranes by protein-protein interaction.

Phosphorylation by protein kinase C. p47 was originally identified as a band on gels of total ^{32}P-labeled proteins in neutrophils activated by phorbol ester (42). The cDNA sequence predicts a highly basic region (residues 292 to 340) containing a number of serines, flanked by arginine residues, which could act as substrates for protein kinase C during activation of NADPH oxidase (16, 23, 53). Immunoprecipitation of $^{32}P_i$-labeled extracts indicated that the protein is phosphorylated in unstimulated HL60-derived neutrophils and that this phosphorylation increases in response to phorbol ester treatment, presumably by activation of protein kinase C (Fig. 4b). The basal level of p47 phosphorylation in unstimulated cells may indicate that the protein is also a substrate for kinases other than protein kinase C.

Tissue specificity of p47 expression. An RNase protection assay was used to investigate p47 mRNA levels in a number of cell types (Fig. 7). The RNA probe used in these experiments was complementary to the first 236 bases of the cDNA sequence and included about 70 bases of vector sequence. In some cases, incomplete RNase digestion resulted in a doublet of protected bands. The results in Fig. 7 showed, as expected, that the mRNA was strongly induced in HL60 cells differentiated with RA or dimethylformamide compared

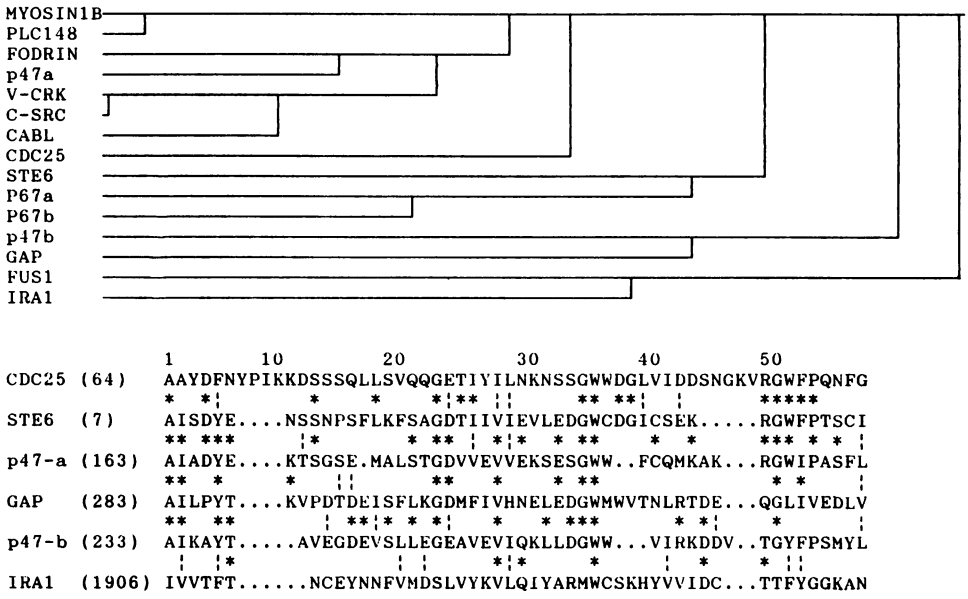

FIG. 5. The upper part of the figure is a dendrogram illustrating the relationship between 15 different SH3 amino acid sequences (see reference 39 for references to sequences). The lower part of the figure is a comparison of the SH3 domains of p47, IRA1, and CDC25 of *S. cerevisiae* (5, 45), bovine *ras* GTPase-activating protein type 1 (GAP) (48), and *Schizosaccharomyces pombe* STE6 (24). Identical residues are indicated by asterisks; conservative substitutions are indicated by dashed vertical lines. The numbers in parentheses indicate the first residue of the alignment. Both parts of the figure were generated by the program Multalin (11).

with undifferentiated cells (lanes 1 to 3). The RNA was also found in phagocytes isolated from normal peripheral blood: polymorphonuclear cells, purified monocytes, and total mononuclear cells (lanes 11, 18, and 19, respectively). (The low signals in lanes 18 and 19 were partly due to underloading, as indicated by the level of the actin control.) Consistent with expression in monocytes is the increased mRNA level observed when U937 cells were induced to differentiate by treatment with TPA for 3 days (lanes 20 and 21). In addition to classical phagocytic cells, the gene was expressed in B-lymphoid cell lines, including Epstein-Barr virus-immortalized lymphoblasts (Bristol 8 and Grimm; lanes 8 and 17, respectively) and to a much lesser extent in Burkitt's lymphoma cells (Manca and Daudi; Fig. 7b, lanes 6 and 7, respectively). In contrast, we could detect no expression in the T-cell line Molt 4 (lane 9) or the primitive myeloid cell line KG1a (lane 5). Northern blot analysis of the T-cell lines J6 and HSB2 also showed a complete lack of p47 mRNA (data not shown). The gene was also silent in an erythroid-myeloid line (K562), epithelial cells (HeLa and 293), fibroblasts, and fetal liver and placenta cells (lanes 4 and 12 through 16). In summary, the RNA analysis showed a highly tissue-specific pattern of p47 gene expression confined to phagocytes and B lymphocytes.

The observation that mRNA for p47 is expressed in B-lymphoid cell lines led us to investigate whether the protein was present in normal peripheral blood lymphocytes. Two-color immunofluorescence was used to investigate this question. Peripheral blood mononuclear cells were stained with phycoerythrin-coupled anti-CD19, a B-cell marker, and then permeabilized and reacted with anti-p47 and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies. The results of fluorescence-activated cell sorter (FACS) analysis in Fig. 8 show that p47 was expressed in at least 75% of CD19-positive cells, thereby confirming the RNA analysis of B-cell lines. CD8-positive cytotoxic T cells were not stained by anti-p47 in a similar two-color study (data not shown). FACS analysis also confirmed the expression of p47 antigen in neutrophils and in monocytes purified by adherence to BHK extracellular matrix (data not shown).

Transcriptional control of the p47 gene. The results in Fig. 7 show that mRNA levels for p47 increased during granulocyte differentiation of HL60 cells as well as monocyte differentiation of U937 cells. This process was accompanied by an increased level of protein, as demonstrated by immunoprecipitation from metabolically labeled HL60 cells. In Fig. 4a it is shown that a 47-kDa protein, detected by the immune serum but not by serum blocked with the synthetic peptide, was induced by RA treatment (Fig. 4a; compare lanes +RA 1 and −RA 1). We investigated the level of control of the p47 gene by performing nuclear run-on assays on HL60 cells treated with RA for various periods (Fig. 9). As a control for the differentiation response, the down-regulation of c-*myc* transcription was monitored. The data in Fig. 9 are consistent with our previous results (1) and show significantly increased attenuation between exons 1 and 2 of the c-*myc* gene within an hour of the addition of RA. Upon treatment with RA, the rate of transcription of the p47 gene increased noticeably within 1 h and was maximal after 6 h. Significantly, this induction of transcription was not abolished by treatment of the cells with 100 μg of cycloheximide per ml, which inhibited >95% of protein synthesis, as determined by [^{35}S]methionine incorporation. This observation strongly implies that the p47 gene responds directly to RA.

DISCUSSION

The cloning and analysis of a cDNA coding for p47, the factor missing in most cases of A-CGD, is described in this report. An antibody raised to the C terminus of the protein encoded by the cDNA recognizes a protein of the correct size in normal human neutrophils but not in those from patients with A-CGD (Fig. 3). This evidence establishes the

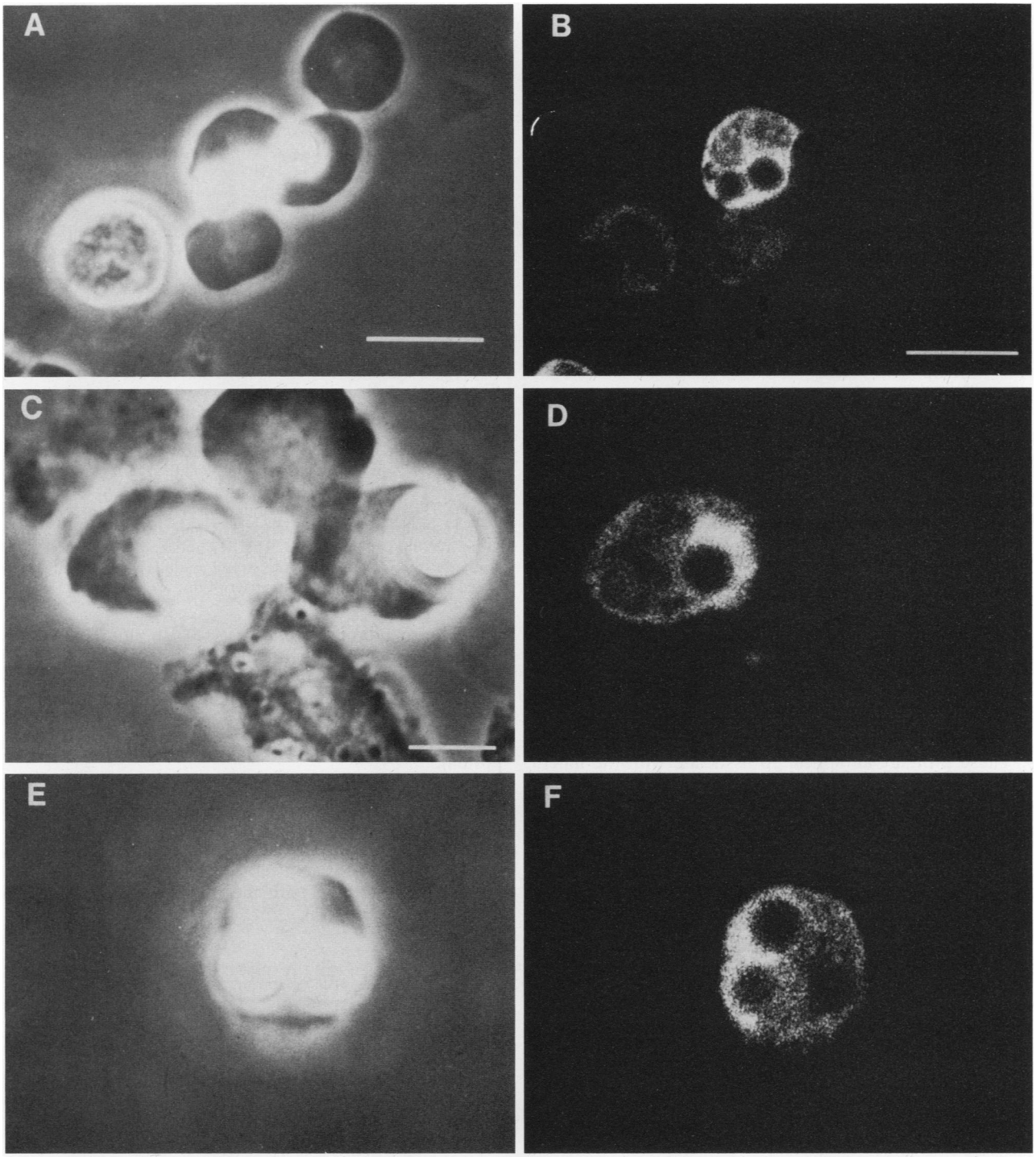

FIG. 6. Confocal immunofluorescence microscopy of p47 in neutrophils. Some cells have phagocytosed latex beads which appear as bright circles in the phase-contrast images (A, C, and E) . Corresponding fluorescence images are shown (B, D, And F). Optical sections were taken through the ingested beads. (A and B) Bar = 10 μm; (C) bar = 5 μm; (D to F) same magnification as panel C.

correct reading frame of the C-terminal portion of the protein, which was previously in doubt. Our cDNA sequence confirms the revised versions published by Volpp et al. and Lomax et al. (30, 51).

Antiserum made against a protein fraction isolated by GTP-agarose affinity chromatography recognizes p47 as well as several other proteins (50). However, contrary to a previous report (51), p47 does not contain a conventional NTP-binding motif (52), implying that p47 binds to GTP-agarose indirectly by associating with a GTP-binding protein. We have not, however, been able to demonstrate association of p47 with other proteins by coimmunoprecipitation (Fig. 4).

The predicted p47 protein sequence contains two copies of

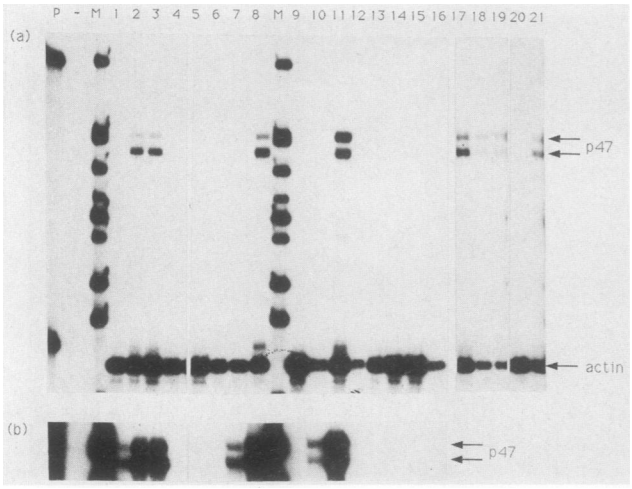

FIG. 7. (a) Tissue distribution of p47 mRNA. The results of an RNase protection assay of p47 and γ-actin mRNA levels in human cell lines and tissues are shown. The protected fragments are indicated by arrows. Lanes: P, undigested probes; M, *Msp*I-digested pBR322; 1, HL60 cells; 2, HL60 cells plus RA (24-h incubation); 3, HL60 cells plus dimethylformamide (24-h incubation); 4, K562 cells; 5, KG1a cells; 6, Manca cells; 7, Daudi cells; 8, Bristol 8 cells; 9, Molt 4 cells; 10, spleen cells; 11, normal neutrophils; 12, fetal liver cells; 13, 293 cells; 14, HeLa cells; 15, foreskin fibroblasts; 16, placental cells; 17, Grimm cells; 18, peripheral mononuclear cells; 19, monocytes; 20, U937 cells; 21, U937 cells plus TPA (3-day incubation). (b) Longer exposure of the p47 protected fragments in lanes 1 to 16, showing a low level of expression in undifferentiated HL60, Daudi, and spleen cell RNA (lanes 1, 7, and 10, respectively).

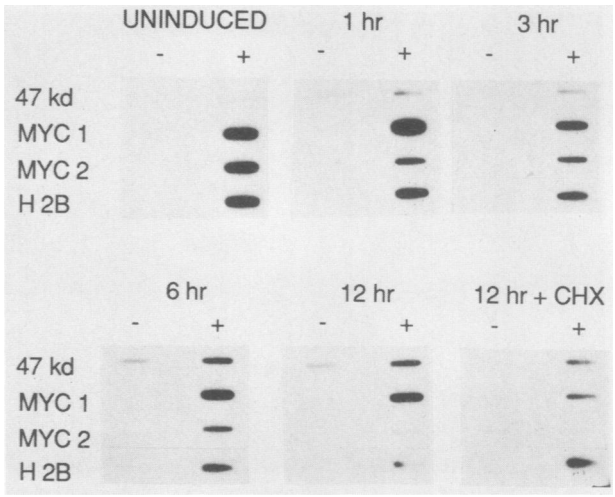

FIG. 9. Transcriptional induction of the p47 gene in response to treatment with RA (1 μM) for 1 to 12 h. Nuclear run-on transcription products were hybridized to the following slot-blotted single-stranded DNA probes: 47 kd, p47 full-length cDNA; MYC 1, human c-*myc* first exon; MYC 2, human c-*myc* second exon; H 2B, chicken histone H2B. − and + indicate probes for antisense and sense strand transcripts, respectively; CHX indicates treatment with 100 μg of cycloheximide per ml. The p47 antisense probe was slightly contaminated with the opposite strand, giving rise to a faint spurious signal of apparent antisense transcription.

the 50-amino-acid SH3 motif found in a heterogeneous group of cytoplasmic proteins, including the p67 A-CGD factor (28). These proteins all appear to associate with the inner face of the plasma membrane (39). The presence of these sequences in p47 is therefore consistent with our observation that the protein localizes to the vacuole membrane (Fig. 6) without being intrinsically hydrophobic (Fig. 4). Several SH3-containing proteins, including GAP, CDC25, STE6, and IRA1, are thought to interact with *ras* proteins which are normally membrane bound (Fig. 5). In this context, it is interesting to note that the Rap1 protein associated with cytochrome b_{-245} is a potential target for p47 and p67 interaction. The hypothesis that the SH3 domains are responsible for the membrane association of p47 could be tested by expression of genetically engineered mutants of the protein.

At both the mRNA and protein levels, p47 is expressed not only by phagocytic cells (granulocytes and monocytes) but also by B lymphocytes (Fig. 7 and 8). The availability of genomic clones will make it possible to investigate the determinants of this unusual pattern of expression, which includes members of both the myeloid and lymphoid lin-

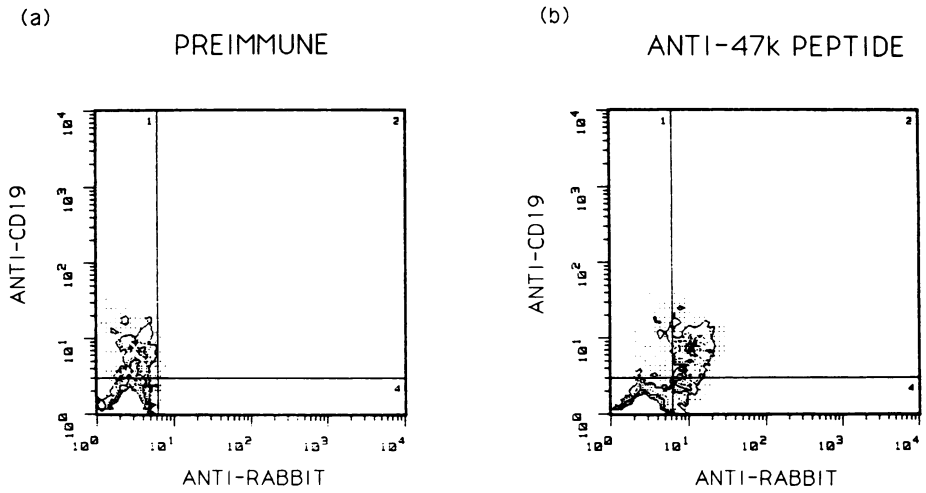

FIG. 8. FACS analysis of p47 expression in peripheral lymphocytes. Green fluorescence (*x* axis), Fluorescein-conjugated goat anti-rabbit serum (TAGO) in combination with either preimmune or anti-p47 rabbit serum. Red fluorescence (*y* axis), Phycoerythrin-conjugated anti-B-cell (CD19) monoclonal antibody (B4).

eages. The function of p47 in B cells is a mystery. It has previously been noted that some Epstein-Barr virus-transformed lymphoid cell lines (31, 32, 49) as well as tonsillar (32) and peripheral (25) B cells express a superoxide-generating system similar or identical to the phagocyte NADPH oxidase. Our results support these findings and provide evidence that the two superoxide-generating systems are indeed highly homologous. Possibly this system is involved in antibody-mediated internalization and processing of antigens. Superoxide may be directly involved in protein fragmentation, or the electrogenic NADPH oxidase may be necessary to produce the correct vacuolar pH for other peptidases to function (41). Alternatively, there is evidence that superoxide may have an immunomodulatory activity, being involved in the stimulation of T-cell activation and proliferation (7) and the suppression of lymphokine-activated killer activity by neutrophils (43). It will be interesting to investigate whether there are any defects in lymphocyte function in patients with CGD.

We have shown that the p47 gene responds to induction by retinoic acid at the transcriptional level by a pathway that does not require protein synthesis. It is probable that p47 transcription is directly activated by one of the nuclear RA receptors. The most abundant of these receptors in undifferentiated HL60 cells is RARα (14). The presence of this receptor has recently been shown to be necessary for RA-induced differentiation in HL60 cells (10). RARα is therefore the most probable candidate for the factor mediating RA-dependent p47 gene expression. The controlling elements of the p47 promoter are currently under investigation.

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