

manner to that seen in the presence of TPA (26). The dissociation of CD4 and p56<sup>lck</sup> that accompanies protein kinase C activation could be important during either T cell activation by antigen or T cell maturation in the thymus. Dissociation of CD4 and p56<sup>lck</sup> could have two effects: It might terminate regulation of the enzymatic activity of p56<sup>lck</sup> by CD4 (4), or it might alter the ability of p56<sup>lck</sup> to interact with specific polypeptide substrates.

Since dissociation of CD4 and p56<sup>lck</sup> appears to occur in concert with TPA-induced internalization of CD4, the differences between the properties of T cells that are induced to internalize CD4 by TPA and those that are not might be revealing as to the role of the dissociation of CD4 and p56<sup>lck</sup> in T cell function and development. Unfortunately, generalizations are difficult to make. Although TPA-induced internalization of CD4 is reported to occur in most human T cell lines (5, 27, 28), in human peripheral blood lymphocytes (6), in murine thymocytes (22), and in some murine CD4<sup>+</sup>-CD8<sup>+</sup> cell lines such as AKR1 (12), it does not occur in a number of mature, antigen-responsive murine T cells (12, 29).

The association of p56<sup>lck</sup> with both CD4 and CD8 is unlikely to be coincidental. The implication is that p56<sup>lck</sup> is important in the development or function, or both, of major histocompatibility complex class I-restricted and class II-restricted T cells. The properties of p56<sup>lck</sup> when it is bound to CD4 differ somewhat from those of the protein when it is bound to CD8. When bound to CD4, p56<sup>lck</sup> will undergo autophosphorylation in vitro and, in many types of T cells, dissociate when protein kinase C is activated. In contrast, when bound to CD8, p56<sup>lck</sup> undergoes less vigorous autophosphorylation in vitro and does not dissociate when protein kinase C is activated. It is possible that the regulation of p56<sup>lck</sup> by CD4 and by CD8 will thus be found to differ.

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## Recombinant 47-Kilodalton Cytosol Factor Restores NADPH Oxidase in Chronic Granulomatous Disease

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A 47-kilodalton neutrophil cytosol factor (NCF-47k), required for activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase superoxide (O<sub>2</sub><sup>-</sup>) production, is absent in most patients with autosomal recessive chronic granulomatous disease (AR-CGD). NCF-47k cDNAs were cloned from an expression library. The largest clone predicted a 41.9-kD protein that contained an arginine and serine-rich COOH-terminal domain with potential protein kinase C phosphorylation sites. A 33-amino acid segment of NCF-47k shared 49% identity with *ras* p21 guanine triphosphatase activating protein. Recombinant NCF-47k restored O<sub>2</sub><sup>-</sup>-producing activity to AR-CGD neutrophil cytosol in a cell-free assay. Production of active recombinant NCF-47k will enable functional regions of this molecule to be mapped.

ACTIVATED PHAGOCYtic CELLS PRODUCE superoxide anion (O<sub>2</sub><sup>-</sup>), which is converted to hydrogen peroxide and other microbicidal oxygen products. O<sub>2</sub><sup>-</sup> generation requires activation of a latent NADPH oxidase. A membrane-bound cytochrome b<sub>558</sub> (1), and both 47-kD and 65-kD neutrophil cytosol factors (NCF-47k and NCF-65k) (2-4), are necessary for activation of the NADPH oxidase. It is unclear how NADPH oxidase is activated, how many additional components are required, or how all of the components interact to achieve a functional enzyme complex.

Several genetic forms of chronic granulomatous disease (CGD) have been identified, in which phagocytic cells are defective in cytochrome b<sub>558</sub> (1), NCF-47k, or NCF-65k (2, 3). We have isolated cDNA clones that encode NCF-47k and demonstrated

that recombinant NCF-47k (rNCF-47k) restores O<sub>2</sub><sup>-</sup>-producing activity to NCF-47k-deficient AR-CGD polymorphonuclear neutrophil (PMN) cytosol in a cell-free reconstitution system.

We obtained NCF-47k cDNA clones using rabbit antiserum B-1, which recognizes NCF-47k and NCF-65k (3), to screen a Lambda-ZAP expression library of cDNA inserts derived from differentiated HL-60 cells (5, 6). NCF-47k cDNA clones were identified based on the ability of their recombinant fusion protein to inhibit B-1 antibody detection of NCF-47k on immu-

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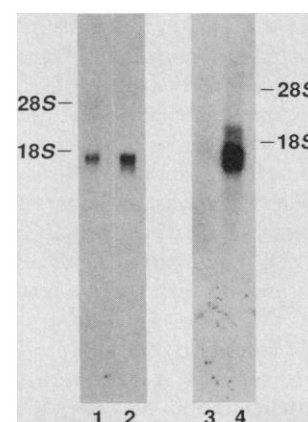
noblots of PMN cytosol (7). The identity of NCF-47k cDNA was further confirmed using antiserum obtained from rabbits immunized with partially purified rNCF-47k (7, 8). This rNCF-47k antiserum detected NCF-47k in normal PMN cytosol but failed to detect this protein in AR-CGD PMN cytosol known to be deficient in NCF-47k activity (2), similar to results with B-1 antiserum (3).

The NH<sub>2</sub>-terminus of recombinant fusion protein produced by Lambda-ZAP-derived plasmids contained a 3-kD segment of  $\beta$ -galactosidase. The NCF-47k clone 8a produced a 50-kD fusion protein and was sequenced in its entirety (9, 10) (Fig. 1A). An open reading frame was identified that was in frame with the fused  $\beta$ -galactosidase sequence. A short 5' sequence precedes the first Met codon with flanking sequences that conform to Kozak consensus criteria (11). This is followed by a 1119-bp open reading frame coding for a 373-amino acid protein of 41.9 kD. A polyadenylation signal at base pair 1303 (12) is followed by a polyadenylated tail.

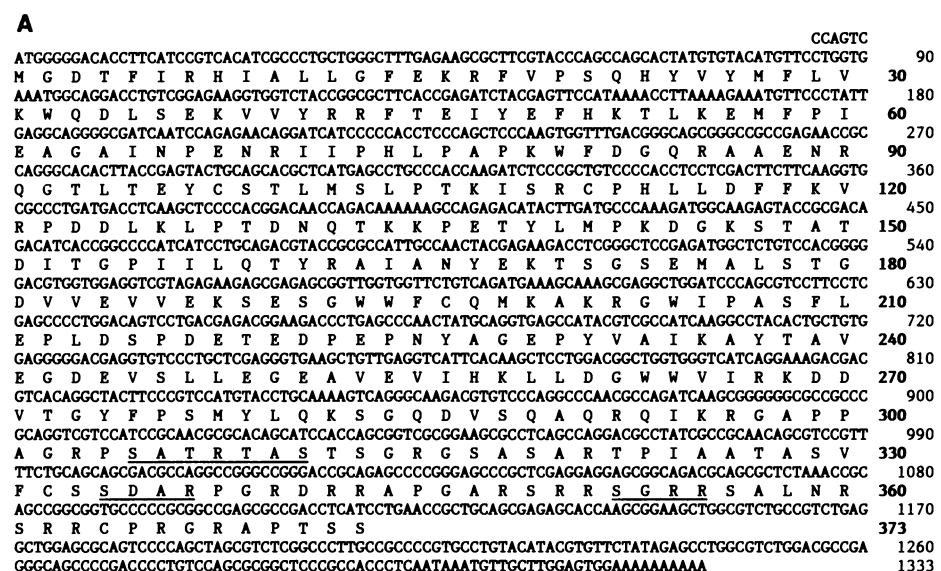
We analyzed the NCF-47k cDNA sequence with University of Wisconsin Ge-

netics Computer Group (UWCGC) and other software (13). The last 82 residues (292 to 373) at the COOH-terminus contain 20 arginines (24%) and 14 serines (17%) (Fig. 1A). This 82-residue segment is unusually basic with a net charge of +19. It is flanked by a short, highly acidic region, residues 211 to 254, with a net charge of -13. The sequence preceding residue 211 has a relatively balanced charge distribution. The overall net charge of NCF-47k is +9, consistent with the inability of the native protein from PMNs to bind to anion exchange resins (2) and its basic *pI* as determined by two-dimensional gel electrophoresis and immunoblot analysis (14).

PMNs from NCF-47k-deficient AR-CGD patients do not phosphorylate a 47-kD protein seen in normal activated PMNs (2, 15, 16). The existence of several *pI* forms of the 47-kD phosphoprotein after PMNs are activated suggests multiple phosphorylation of the same protein (15). Phosphoamino acid analysis of the PMN 47-kD phosphoprotein detects only phosphoserine (15). Protein kinase C phosphorylates a 47-kD protein in PMN cytosol fractions that contain NCF-47k (17). Also, antisera that bind



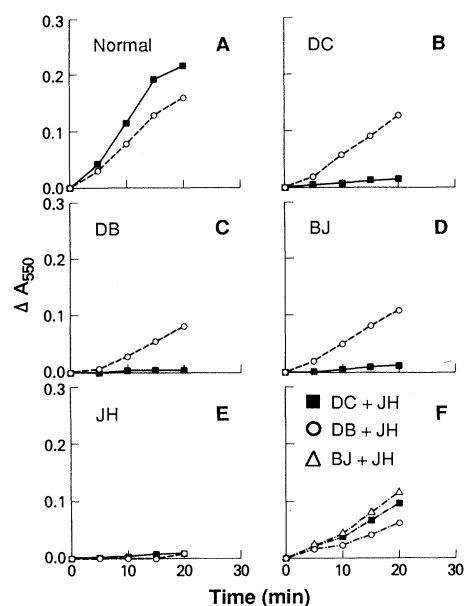
**Fig. 2.** Northern blot analyses of total cellular RNA (10 µg) probed with <sup>32</sup>P-labeled clone 8a cDNA. Lanes 1 and 2, respectively: blood monocytes from a normal volunteer and an AR-CGD patient (R.H.) whose PMNs are deficient in NCF-47k (2). Lanes 3 and 4, respectively: HL-60 cells that were uninduced or induced for 3 days with retinoic acid (1 µM). These experiments are representative of several similar RNA blots in which the average size of the transcript detected was ≈1.5 kb. With intense labeling of the ≈1.5-kb NCF-47k transcript as in lane 4 a fainter band of uncertain significance is seen consistently at ≈2.2 kb in induced HL-60 cells.



**Fig. 1.** (A) The nucleotide and deduced amino acid sequence of clone 8a cDNA with the most 5' nucleotide of the open reading frame designated as nucleotide 1, and the NH<sub>2</sub>-terminal methionine of the predicted protein designated as residue 1. Amino acid sequences conforming to substrate specificity requirements for phosphorylation of serine residues by protein kinase C (19) are underlined. Sequence from other NCF-47k clones was identical to that shown for clone 8a except for three consistent base pair differences. Adenosine<sup>496</sup>, adenosine<sup>558</sup>, and guanine<sup>621</sup> were replaced in two clones by guanine or adenosine, resulting in substitution of Asn<sup>166</sup> by Asp, while Val<sup>186</sup> and Ala<sup>207</sup> remain unchanged. This may represent two types of NCF-47k proteins, two allelic forms, or a cloning artifact. The nucleotide sequence has been deposited at GenBank (accession number M25665). Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (B) Alignment of a region of similarity between NCF-47k and *ras*-p21 GAP (22). Amino acid residue numbers correspond to bovine brain GAP. The same 35-residue sequence occurs in human placental GAP at positions 286 to 320. Solid lines indicate identities and double dots indicate conservative residue changes. This alignment was calculated using the program BESTFIT (UWCGC) (13).

NCF-47k immunoprecipitate the 47-kD phosphoprotein (18). Within the COOH-terminal region of NCF-47k is a cluster of four serines (residues 305, 311, 334, and 352) that occur in the context of Arg-X-X-Ser or Ser-X-X-Arg motifs (underlined in Fig. 1A) associated with the substrate target regions of protein kinase C (19). The overall basic charge characteristics of this COOH-terminal region further favor phosphorylation by protein kinase C (19). Thus, this COOH-terminal region may be involved in functions of NCF-47k that are regulated by phosphorylation events.

Comparison of NCF-47k with proteins in the National Biomedical Research Foundation data bank (release 19.0, December 1988) (13) revealed no significant regions of identity. The respiratory burst oxidase system involves guanosine triphosphate (GTP) regulatable components (3, 20) and phosphorylation of NCF-47k can be augmented by nonhydrolyzable GTP analogs (21). Because of these observations we compared NCF-47k with a number of proteins, not in the data bank, that are involved in GTP-regulated signal transduction, including GTP-binding subunits of heterotrimeric GTP-regulatory proteins, *ras* type G proteins, and the cytoplasmic *ras* p21 GTPase activating protein (GAP) (22). A 35-residue region of GAP (22) shares 49% identity with NCF-47k (Fig. 1B). This restricted region of similarity may indicate some functional relationship between NCF-47k and GAP.



**Fig. 3.** Results of cell-free assays (2, 24) of  $O_2^-$  production in which *E. coli*-derived rNCF-47k extract (7) was used to restore function to defective PMN cytosol from AR-CGD patients deficient in NCF-47k. The ordinate indicates  $\Delta A_{550}$  as a measure of  $O_2^-$  generation ( $\Delta A_{550}$  of 0.021 equals 1 nmol  $O_2^-$  produced) and the abscissa indicates time in minutes. All assay mixtures contain  $10^6$  cell equivalents of normal PMN membranes and  $10^5$  cell equivalents of test PMN cytosol or mixtures of cytosols in the standard assay mixture (2, 24), while some assays also contained 0.5  $\mu$ l of rNCF-47k extract (7). In panels (A-E) results without rNCF-47k are shown as solid lines (filled circles), while results following addition of rNCF-47k extract are shown as dashed lines (open circles). (A) Normal PMN cytosol, (B, C, and D) PMN cytosol from each of three patients with NCF-47k-deficient AR-CGD, (E) PMN cytosol from a patient with NCF-65k-deficient AR-CGD. (F) PMN cytosols from each NCF-47k-deficient AR-CGD patient combined as a 1:1 mixture with PMN cytosol from the NCF-65k-deficient AR-CGD patient.

We probed Northern blots (23) with  $^{32}$ P-labeled clone 8a cDNA to demonstrate that both normal monocytes and monocytes from an AR-CGD patient with NCF-47k protein deficiency contain a single transcript of about 1.5 kb (Fig. 2, lanes 1 and 2, respectively). Four other patients missing NCF-47k protein (2, 3) had variable amounts of apparently normal sized NCF-47k mRNA transcripts present in monocytes. This suggests that the NCF-47k protein deficiency in these patients is not the result of a failure of transcription. In HL-60 cells NCF-47k transcripts were only detected after differentiation (Fig. 2, lanes 3 and 4).

We added an extract of *Escherichia coli* that expressed rNCF-47k (7) to a cell-free  $O_2^-$  production assay (2, 24) that contained PMN cytosol from controls or AR-CGD patients combined with normal PMN membranes. At the concentration of rNCF-47k extract used,  $O_2^-$  production was slightly inhibited by normal cytosol and membranes (Fig. 3A). PMN cytosol from three unrelated patients with NCF-47k-deficient AR-CGD did not support  $O_2^-$  production in the cell-free assay with normal PMN membranes, but activity was restored in all cases by addition of rNCF-47k extract to the assay mixture (Fig. 3, B, C, and D). In contrast, the activity of cytosol from a patient with a deficiency of NCF-65k (2, 3) was not restored by addition of rNCF-47k extract (Fig. 3E). Previous studies had demonstrated that cytosols from NCF-47k-deficient PMNs and NCF-65k-deficient PMNs were complementary and active when mixed together in the cell-free assay (2). As a control to demonstrate the latent complementary activity of the specific preparations of PMN cytosols used in these studies, complementa-

tion was demonstrated between the NCF-65k-deficient cytosol and each of the NCF-47k-deficient cytosol preparations (Fig. 3F).

In summary, we have cloned the cDNA that encodes the 47-kD phosphoprotein, NCF-47k, that is missing from the phagocytic cells of patients with the most common form of AR-CGD. The unphosphorylated form of NCF-47k is a basic protein with an arginine- and serine-rich COOH-terminus that has a cluster of potential phosphorylation sites. NCF-47k shares a limited region of similarity with *ras*-p21 GAP. The rNCF-47k is functionally competent at restoring  $O_2^-$  production to defective PMN cytosol from NCF-47k-deficient AR-CGD patients. The ability to produce functional recombinant protein will aid in the delineation of phosphorylation sites and the significance of the similarity to GAP. It also allows the development of strategies for genetic correction of this form of CGD.

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Perry, Gaithersburg, MD), then detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indole phosphate (6). The cDNA inserts were rescued by an automatic excision procedure in which phage purified from positive clones were used to coinfect XL-1 Blue (Stratagene) host bacteria along with R408 helper phage. This resulted in the production of phagemids (Stratagene product protocol). Host bacteria (JM 109) were infected with phagemids harvested from the supernatants of these cultures. Transformants containing cDNA inserts in the Bluescript (Stratagene) plasmid vector were selected from LB/ampicillin plates.

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10. Sequence analysis of NCF-47k clones utilized the dideoxynucleotide chain termination sequencing method of Sanger (9). The 5' and 3' terminal sequencing reactions for each clone were primed with synthetic oligonucleotides complementary to the T3 and T7 RNA polymerase promoters flanking the cDNA cloning site. Internal sequence was obtained using 17 base oligonucleotide primers (synthesized with an Applied Biosystems 380A DNA Synthesizer) that annealed at intervals sufficient to obtain the entire nucleotide sequence from both strands of clone 8a.
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24. The cell-free microtiter plate assay of  $O_2^-$  production was performed with PMN membranes and cytosol as described (2) except that the system was activated with arachidonic acid (40  $\mu M$ ) augmented with GTP- $\gamma$ -S (5  $\mu M$ ). Studies on reconstitution of activity were done with the *E. coli*-derived rNCF-47k extract (7). Preliminary studies indicated that 0.5  $\mu l$  of this rNCF-47k extract only slightly inhibited  $O_2^-$  production when added to normal PMN membranes and cytosol, while consistently augmenting  $O_2^-$  production in assays that contained NCF-47k-deficient PMN cytosol. This amount of extract had minimal background ferricytochrome C-reducing activity when added to either control PMN cytosol or membranes in the cell-free assay.
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## In Vivo Activity Against HIV and Favorable Toxicity Profile of 2',3'-Dideoxyinosine

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The purine analog 2',3'-dideoxyinosine (ddI), which has anti-retroviral activity in vitro was administered for up to 42 weeks to 26 patients with acquired immunodeficiency syndrome (AIDS) or severe AIDS-related complex (ARC). Ten of these individuals were AZT-intolerant. Eight dose regimens were studied. The drug was orally bioavailable and penetrated into the cerebrospinal fluid (CSF). Comparatively little evidence of an effect against human immunodeficiency virus (HIV) was seen at the lowest four doses. However, patients in the four highest dose groups (ddI at 1.6 milligrams per kilogram intravenously and then  $\geq 3.2$  milligrams per kilogram orally at least every 12 hours or higher) had increases in their circulating  $CD4^+$  T cells ( $P < 0.0005$ ), increased  $CD4/CD8$  T cell ratios ( $P < 0.01$ ), and, where evaluable, more than an 80% decrease in serum HIV p24 antigen ( $P < 0.05$ ). The patients also had evidence of improved immunologic function, had reduced viremic symptomatology, and gained a mean of 1.6 kilogram with these comparatively infrequent dosing schedules (every 8 or 12 hours). The most notable adverse effects directly attributable to ddI administration at the doses used in this study included increases in serum uric acid (due to hypoxanthine release) and mild headaches and insomnia. These results suggest that serious short-term toxicity at therapeutic doses is not an inherent feature in the profile of agents with clinical anti-HIV activity. Further controlled studies to define the safety and efficacy of this agent may be worth considering.

SEVERAL DRUGS HAVE NOW BEEN shown to have clinical anti-retroviral activity against HIV (1-4), and one of these drugs, 3'-azido-2',3'-dideoxythymidine (called zidovudine, or AZT), has been demonstrated to reduce the morbidity and mortality of patients with AIDS or AIDS-related complex (2). Widespread use of AZT, the only anti-retroviral drug currently approved for the treatment of severe HIV infection, is likely to be one factor above and beyond prophylaxis against *Pneumocystis car-*

*inii* pneumonia that has contributed to the increased survival of AIDS patients (both gay men and intravenous drug users) diagnosed since the end of 1986 (5). However, AZT and each of the other agents shown to have activity against HIV in vivo may also cause substantial toxicity in some patients with severe HIV infection (1, 3, 4, 6). In particular, 40 to 80% of AIDS patients do not tolerate therapeutic doses of AZT for 26 weeks because of bone marrow suppression or other toxicities (6). Moreover, the im-

provements induced by AZT are only transient in many patients with advanced AIDS (1, 2, 7), and Larder *et al.* have recently reported that isolates of HIV from patients receiving long-term AZT therapy may have reduced sensitivity to this drug (8). For these reasons, improved drugs and drug combinations are needed for AIDS and its related diseases.

2',3'-Dideoxyinosine (ddI) (Fig. 1) is a purine dideoxynucleoside with potent activity against HIV in vitro in T cells (9) and monocytes (10). It is closely related to 2',3'-dideoxyadenosine (ddA), a compound first synthesized by Robins and Robins in 1964 (11). [Indeed ddA is rapidly converted to ddI by the ubiquitous enzyme adenosine deaminase (12), so these drugs can for many purposes be considered alternate forms.] In human cells, ddI is metabolized to its active moiety, 2',3'-dideoxyadenosine-5'-triphosphate (ddA-TP) (13). It is thought that ddA-TP inhibits HIV DNA polymerase (reverse transcriptase) activity preferentially, and thereby suppresses HIV infection by blocking the synthesis of a DNA copy of the viral genome. Its mechanism of action is thought to be chain termination, competitive inhibition of reverse transcriptase, or both (14). However, while ddI is a potent anti-retroviral agent, it is not a broad spectrum antiviral drug. Unlike the triphosphates of AZT or 2',3'-dideoxycytidine (ddC), ddA-TP has a long half-life (over 12 hours) in cells exposed to ddI (15). Mitsuya and Broder found that ddI has a relatively high therapeutic index in vitro as compared to other dideoxynucleosides (9), and it has relatively little in vitro toxicity for human marrow progenitor cells (16). These observations suggested that ddI was worth testing in patients with HIV infection.

A total of 26 patients (25 male and 1 female) with HIV infection, aged 23 to 51, were entered into an initial clinical trial of ddI (17). Ten patients had AIDS and 16 had ARC (18). All had antibodies to HIV and less than 300  $CD4^+$  T cells per cubic millimeter (median 60; range 6 to 266). Ten of the patients were AZT-intolerant by virtue of having developed nausea, malaise, anemia, or headaches (19). All but one patient had received no AZT or anti-HIV therapy during the preceding 4 weeks. Prophylaxis for *Pneumocystis carinii* pneumonia was permitted during this study (20). Patients were given ddI intravenously for 14 days at doses

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