10. A. Kontos *et al.*, *Mar. Turtle Newsl.* **42**, 10 (1988).
11. J. R. Hendrickson, *Proc. Zool. Soc. London* **130**, 455 (1958).
12. D. W. Owens, M. A. Grassman, J. R. Hendrickson, *Herpetologica* **38**, 124 (1982).
13. C. Gremone and J. L. Gomez, *Isla de Aves como Area de Desove de la Tortuga Verle* (Fundación para la Defensa de la Naturaleza, Caracas, Venezuela, 1984).
14. J. A. Mortimer and A. Carr, *Copeia* **1987(1)**, 103 (1987).
15. C. K. Dodd, *Brimleyana* 7, 39 (1982).
16. J. C. Avise, *Philos. Trans. R. Soc. London Ser. B* **312**, 325 (1986).
17. J. C. Avise *et al.*, *Annu. Rev. Ecol. Syst.* **18**, 489 (1987).
18. C. Moritz, T. E. Dowling, W. M. Brown, *ibid.*, p. 269.
19. W. M. Brown, M. George, A. C. Wilson, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1967 (1979).
20. R. A. Lansman, R. O. Shade, J. F. Shapira, J. C. Avise, *J. Mol. Evol.* **17**, 214 (1981).
21. B. W. Bowen, A. B. Meylan, J. C. Avise, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 573 (1989).
22. W. M. Brown, *ibid.* 77, 3605 (1980).
23. Nine additional endonucleases (Bam HI, Bgl I, Bgl II, Bst EII, Cla I, Kpn I, Pst I, Sac I, and Xba I) were also employed. Since each produced only zero or one mtDNA cut in our assays, they were uninformative and not included in the estimates of sequence divergence.
24. M. Nei and W.-H. Li, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5269 (1979).
25. M. Slatkin and W. P. Maddison, *Genetics* **123**, 603 (1989).
26. M. Slatkin, *ibid.* **121**, 609 (1989).
27. A. F. Carr, *Natl. Geogr. Mag.* **131(6)**, 876 (1967).
28. ———, *Copeia* **1975**, 547 (1975).
29. R. R. Sokal and F. J. Rohlf, *Biometry* (Freeman, San Francisco, CA, 1969).
30. G. Medina and G. Sole, personal communication.
31. We thank C. Lagueux, G. Medina, J. Ross, and R. Witham for help obtaining samples. G. Medina and G. Sole furnished unpublished data on Aves Island. Permits or logistic support were provided by B. Campbell, D. Carr, C. Carson, G. Cruz, E. Possardt, J. Woody, the U.S. Air Force, and the Caribbean Conservation Corporation. We thank A. Huff, L. French, and J. Leiby for comments on the manuscript. Supported by the National Geographic Society and by NSF grants to B.W.B. and J.C.A.

22 December 1989; accepted 27 March 1990

Cloning of a 67-kD Neutrophil Oxidase Factor with Similarity to a Noncatalytic Region of p60$^{c\text{-}src}$

THOMAS L. LETO,* KAREN J. LOMAX, BRYAN D. VOLPP, HIROYUKI NUNOI, JOAN M. G. SECHLER, WILLIAM M. NAUSEEF, ROBERT A. CLARK, JOHN I. GALLIN, HARRY L. MALECH

Chronic granulomatous diseases (CGDs) are characterized by recurrent infections resulting from impaired superoxide production by a phagocytic cell, nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase. Complementary DNAs were cloned that encode the 67-kilodalton (kD) cytosolic oxidase factor (p67), which is deficient in 5% of CGD patients. Recombinant p67 (r-p67) partially restored NADPH oxidase activity to p67-deficient neutrophil cytosol from these patients. The p67 cDNA encodes a 526–amino acid protein with acidic middle and carboxyl-terminal domains that are similar to a sequence motif found in the noncatalytic domain of *src*-related tyrosine kinases. This motif was recently noted in phospholipase C–γ, nonerythroid α-spectrin (fodrin), p21^{ras}–guanosine triphosphatase–activating protein (GAP), myosin-1 isoforms, yeast proteins cdc-25 and fus-1, and the 47-kD phagocyte oxidase factor (p47), which suggests the possibility of common regulatory features.

COMPLEMENTARY DNAS ENCODING three oxidase components defective in distinct forms of CGD have been reported; two encode subunits of the membrane-bound cytochrome b-558 (*1*) and one encodes p47 phagocyte cytosolic oxidase factor (*2, 3*). Complementary DNA clones encoding the p67 cytosolic factor were obtained as described (*2*) by screening a Lambda-ZAP (Stratagene) expression library containing cDNA inserts derived from HL60 cells differentiated for 2 days with dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cAMP). The library was screened with polyclonal rabbit antiserum B-1, which reacts against both p67 and p47 (*4, 5*). Several independent cDNA inserts encoding p67 were isolated, ranging in size from 2.2 to 0.5 kb (*6*).

To demonstrate that these clones encoded a protein antigenically similar to p67, we showed that fusion proteins encoded by two of the largest clones specifically blocked B-1 antibody detection of native p67 on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) immunoblots of neutrophil cytosol (*7*). Furthermore, rabbit antibodies to r-p67 (*8*) reacted specifically against a 67-kD polypeptide in neutrophil cytosol from all normal subjects and most CGD patients except one who was previously characterized as p67-deficient (Fig. 1a). The p67 cDNA detected an ~2.4-kb mRNA transcript in monocytes from normal individuals and all CGD patients examined including the one patient with p67 protein deficiency (Fig. 1b). The p67 transcript was not detected in uninduced HL60 cells, but a transcript identical in size to that seen in monocytes was present in HL60 cells induced to differentiate with retinoic acid for 5 days, consistent with earlier observations indicating that p67 protein is limited to cells capable of superoxide generation (*5*).

Further evidence was sought to confirm that these cDNAs encode a functional factor absent from p67-deficient autosomal recessive CGD patients. This was accomplished by demonstrating that r-p67 is functionally active in a cell-free superoxide-generating assay requiring neutrophil membranes and cytosol (Fig. 2) (*4, 9*). The r-p67 partially reconstituted nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)–dependent superoxide production by neutrophil cytosol from a p67-deficient CGD patient (Fig. 2b), but failed to restore activity to neutrophil cytosols from p47-deficient CGD patients (Fig. 2, c, d, and e). In control experiments oxidase activity was also partially restored when p47-deficient cytosols were mixed with the p67-deficient cytosol (Fig. 2f). Several factors account for the less than complete restoration, including lability of patient cytosol, presence of inhibitors in the *Escherichia coli*–derived r-p67 that diminish even normal cytosolic activity (Fig. 2a) and the inherent lability of r-p67 during isolation. (Less than maximal activity was seen in the complementation experiment in Fig. 2f.)

The nucleotide sequence of p67 cDNA (clone 10) predicts a 526–amino acid protein with a calculated size of 60,900 kD (Fig. 3a). The apparent size of the clone 10 recombinant fusion protein by SDS-PAGE was 70 kD (*6, 7*). A methionine codon occurs 25 bases from the 5′ end of clone 10 cDNA within a consensus sequence that conforms to translation initiation sites observed in eukaryotic mRNAs (*10*). After a stop codon at position 1579, there are another 557 bases of untranslated sequence, which include two polyadenylation signals (underlined in Fig. 3a).

Within the predicted peptide sequence, the COOH-terminal domain was noted for a large number of acidic residues, bearing a net charge of −12 in the last 65-residue segment. Comparison of the deduced amino acid sequence of p67 with sequences recorded in the National Biomedical Research Foundation (NBRF) database (version 23)

T. L. Leto, K. J. Lomax, H. Nunoi, J. M. G. Sechler, J. I. Gallin, H. L. Malech, Bacterial Diseases Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Building 10, Room 11N112, Bethesda, MD 20892.
B. D. Volpp, W. M. Nauseef, R. A. Clark, Department of Medicine, University of Iowa and Veterans Administration Medical Center, Iowa City, IA 52242.

*To whom correspondence should be addressed.

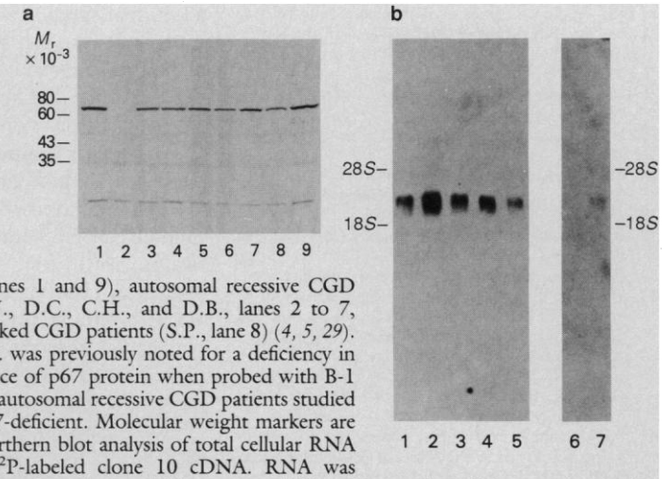


Fig. 1. Expression of p67 protein and mRNA by phagocytic cells from CGD patients. (**a**) SDS-PAGE immunoblot analysis of neutrophil cytosolic proteins with antibodies to r-p67 (*8*). Gels were loaded with peripheral blood neutrophil cytosolic proteins (2×10^6 cells per lane) from normal subjects (lanes 1 and 9), autosomal recessive CGD patients (J.H., R.H., B.J., D.C., C.H., and D.B., lanes 2 to 7, respectively), and an X-linked CGD patients (S.P., lane 8) (*4, 5, 29*). Cytosol from patient J.H. was previously noted for a deficiency in p67 activity and an absence of p67 protein when probed with B-1 antibody (*4, 5*). All other autosomal recessive CGD patients studied were characterized as p47-deficient. Molecular weight markers are given on the left. (**b**) Northern blot analysis of total cellular RNA (10 μg) probed with ^{32}P-labeled clone 10 cDNA. RNA was isolated, separated by electrophoresis on a 1.0% agarose gel, blotted onto nitrocellulose, and probed as previously described (*2*). Lanes 1 to 5, blood monocyte RNA from normal (lane 1), X-linked CGD (lane 2), p47-deficient autosomal recessive CGD (lanes 3 and 4), and p67-deficient autosomal recessive CGD (lane 5) subjects. Lanes 6 and 7, HL-60 RNA from uninduced cells (lane 6) and cells 3 days after induction with 1 μ*M* retinoic acid (lane 7). The single transcript detected was ~2.4 kb. Ribosomal RNA size markers are indicated.

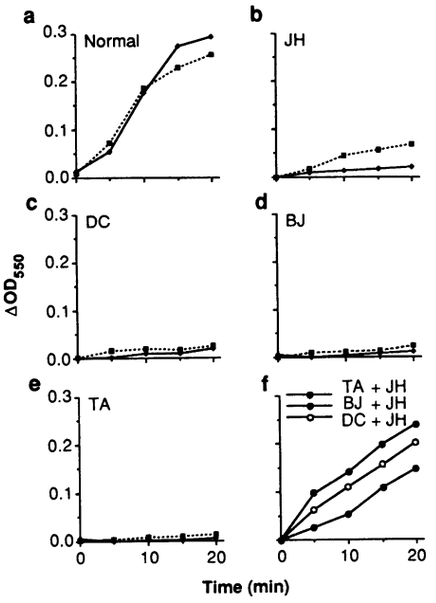


Fig. 2. Cell-free assay showing partial restoration of defective NADPH oxidase activity in p67-deficient neutrophil cytosol with r-p67 (*9*). (**a** to **e**) No added r-p67 shown as a solid line (♦), added r-p67 shown as a dashed line (□). (**a**) Normal subject cytosol; (**b**) cytosol from the p67-deficient patient (J.H.); (**c** to **e**) cytosol from each of three p47-deficient patients (D.C., B.J., and T.A.); (**f**) mixtures of cytosol from the p67-deficient patient with cytosol from each of the p47-deficient patients. Data represent the average of quadruplicate assays (difference in slope for JH cytosol with r-p67 versus without r-p67 significant at $P < 0.001$). Cytosolic activity from patients D.C., B.J., and T.A. was previously restored by r-p47, whereas J.H. cytosol activity was not (*2*).

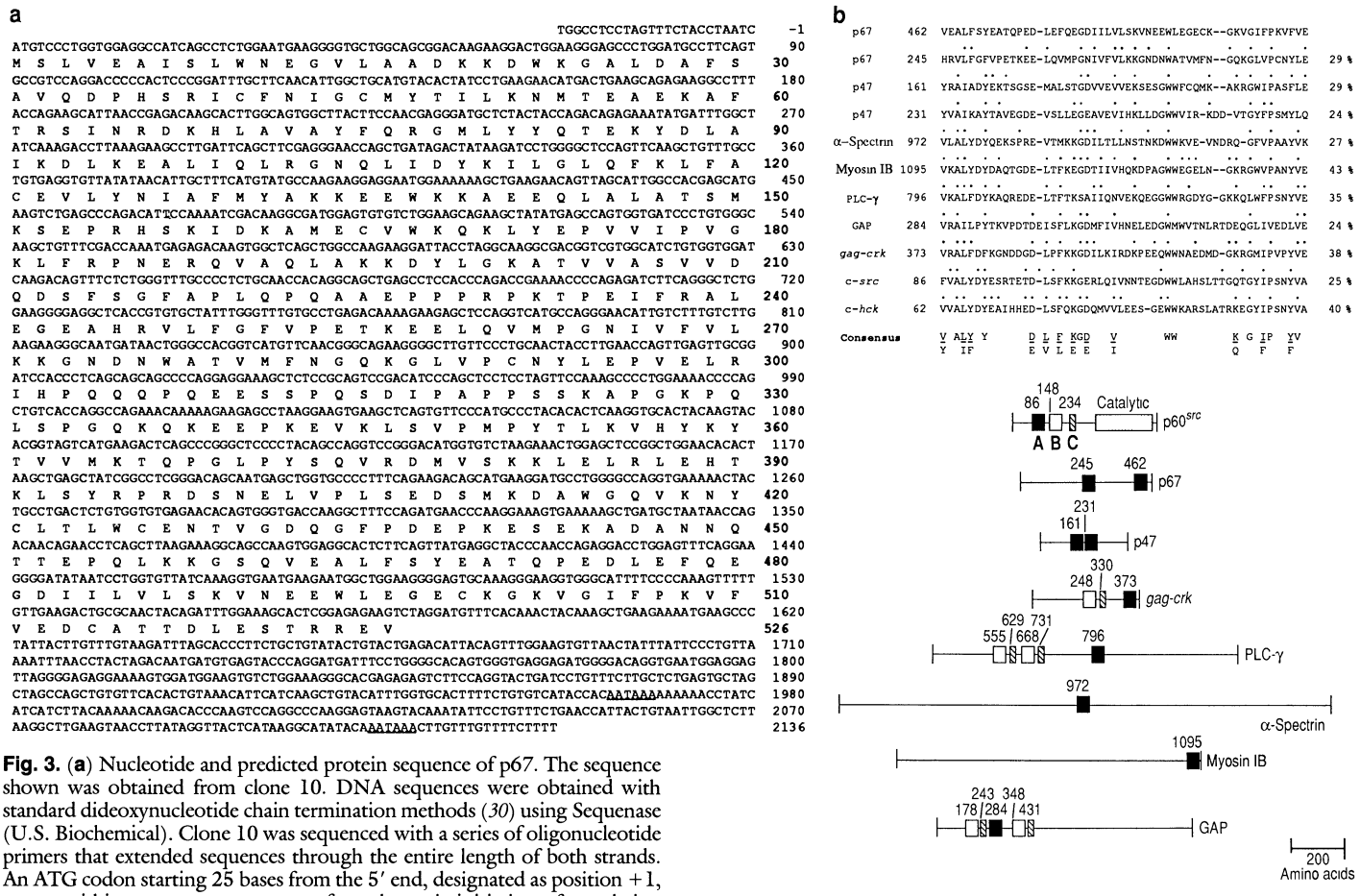
(*11*) has revealed significant structural similarities between this COOH-terminal domain and a limited portion of the noncatalytic domains [designated region A (*12*) or SH3 (*13*)] of a number of oncogene products in the nonreceptor tyrosine kinase family of which *src* is a prototype (Fig. 3, b and c) (*14*). These include *lyn*, *syn*, *yes*, *hck*, *lck*, *abl*, *fgr*, and *blk* (*14*). Further analysis revealed a second repeat of *src* region A–like sequence within the mid segment of p67 (residues 245 to 295). The degree of similarity that the p67 region A motifs exhibited to proteins in this family ranged from 18 to 40% amino acid identity within a 51-residue segment. This region of similarity encompasses exons 3 and 4 of c-*src*, a relatively well conserved segment in both the viral oncogene and cellular proto-oncogene sequences of the entire *src* family (*15*). Many of these *src*-related cellular proto-oncogene products accumulate in terminally differentiated tissues and have limited tissue distribution, suggesting that they may be important in cellular functions distinct from cell proliferation (*15*).

Whereas the deduced amino acid sequence of p67 provides little information concerning its precise role in the oxidase complex, its structural relation to the noncatalytic region A of proteins in the *src* family may provide important clues. Several recent reports describe structural similarities recognized between region A of the *src* family proteins and other diverse proteins that do not exhibit kinase activity. These include the other cytosolic oxidase factor p47 (*3*), the viral oncoprotein *gag-crk* (*16*), nonerythroid α-spectrin (*17*), a phosphoinositide-specific phospholipase C (PLC-γ) (*12, 18*), guanosine triphosphatase–activating protein (GAP) (*19*), myosin I–related isoforms in *Acanthamoeba*, *Dictyostelium*, and yeast (*20*), and other yeast proteins cdc25 and fus1 (*20*) (Fig. 3b). Although GAP, PLC-γ, and *gag-crk* contain sequences similar to two additional noncatalytic segments of *src*, regions B and C (SH2) (Fig. 3c), only region A is found in p67, p47, myosin I, and α-spectrin, suggesting that region A functions independently of *src*-like regions B and C (*12, 16–20*).

We find a tandem repeat of the region A motif in p47 that had not been previously recognized, though other regions of similarity between p47 and GAP (*2*) or *src* (*3*) had been identified. Thus, both phagocyte cytosol oxidase proteins, p47 and p67, contain two segments of *src* region A motifs. No other regions of similarity between p47 and p67 were noted.

The region A motif in these diverse proteins may subserve a common functional role. One possibility involves guanosine triphosphate (GTP) regulatory elements, since activation of the NADPH oxidase is modulated by GTP and the cytosolic factors bind to GTP matrices (*5*). Neither of the neutrophil cytosolic factors contains the three consensus sequence elements characteristic of several distinct GTP binding proteins (*21*). Their structural similarity to GAP and phospholipase C could reflect interactions with common guanine nucleotide regulated components, though the region A sequence of GAP lies outside the region that is thought to interact with p21^{ras} (*22*).

A more likely possibility involves the interaction of region A–containing proteins with the membrane cytoskeleton. All of these proteins localize to the cortical cytoplasm, suggesting that they recognize common membrane skeleton components. Both p47 and p67 appear to translocate from cytosol to the membrane skeleton with activation of the oxidative burst in phagocytic cells (*23*). Deletions within the noncatalytic region A of pp60$^{v\text{-}src}$ affect its association with the detergent-insoluble cytoskeletal matrix of extracted cells (*24*). Furthermore, mutations in this region affect the specificity of p60$^{c\text{-}src}$ for certain cytoskeletal proteins whose phosphorylation is thought to correlate with malignant transformation, suggesting that this region is involved in substrate recognition (*25*). One of these favored pp60$^{v\text{-}src}$ substrates, pp36 (also calpactin I or lipocortin II) exhibits calcium-dependent binding to lipid, actin, and spectrin (*26*). An adenosine triphosphate (ATP)–insensitive actin-binding site has been mapped to the COOH-terminal domain of myosin I (*27*), a region composed of a *src* region A motif and proline-rich sequences (*28*). The occurrence of similar proline-rich sequences together with *src* region A motifs in p47 and p67




```
a                                                                    TGGCCTCCTAGTTTCTACCTAATC   -1
ATGTCCCTGGTGGAGGCCATCAGCCTCTGGAATGAAGGGGTGCTGGCAGCGGACAAGAAGGACTGGAAGGGAGCCCTGGATGCCTTCAGT   90
M  S  L  V  E  A  I  S  L  W  N  E  G  V  L  A  A  D  K  K  D  W  K  G  A  L  D  A  F  S   30
GCCGTCCAGGACCCCCACTCCCGGATTTGCTTCAACATTGGCTGCATGTACACTATCCTGAAGAACATGACTGAAGCAGAGAAGGCCTTT  180
A  V  Q  D  P  H  S  R  I  C  F  N  I  G  C  M  Y  T  I  L  K  N  M  T  E  A  E  K  A  F   60
ACCAGAAGCATTAACCGAGACAAGCACTTGGCAGTGGCTTACTTCCAACGAGGGATGCTCTACTACCAGACAGAGAAATATGATTTGGCT  270
T  R  S  I  N  R  D  K  H  L  A  V  A  Y  F  Q  R  G  M  L  Y  Y  Q  T  E  K  Y  D  L  A   90
ATCAAAGACCTTAAAGAAGCCTTGATTCAGCTTCGAGGGAACCAGCTGATAGACTATAAGATCCTGGGGCTCCAGTTCAAGCTGTTTGCC  360
I  K  D  L  K  E  A  L  I  Q  L  R  G  N  Q  L  I  D  Y  K  I  L  G  L  Q  F  K  L  F  A  120
TGTGAGGTGTTATATAACATTGCTTTCATGTATGCCAAGAAGGAGGAATGGAAAAAAGCTGAAGAACAGTTAGCATTGGCCACGAGCATG  450
C  E  V  L  Y  N  I  A  F  M  Y  A  K  K  E  E  W  K  K  A  E  E  Q  L  A  L  A  T  S  M  150
AAGTCTGAGCCCAGACATTCCAAAATCGACAAGGCGATGGAGTGTGTCTGGAAGCAGAAGCTATATGAGCCAGTGGTGATCCCTGTGGGC  540
K  S  E  P  R  H  S  K  I  D  K  A  M  E  C  V  W  K  Q  K  L  Y  E  P  V  V  I  P  V  G  180
AAGCTGTTTCGACCAAATGAGAGACAAGTGGCTCAGCTGGCCAAGAAGGATTACCTAGGCAAGGCGACGGTCGTGGCATCTGTGGTGGAT  630
K  L  F  R  P  N  E  R  Q  V  A  Q  L  A  K  K  D  Y  L  G  K  A  T  V  V  A  S  V  V  D  210
CAAGACAGTTTCTCTGGGTTTGCCCCTCTGCAACCACAGGCAGCTGAGCCTCCACCCAGACCGAAAACCCCAGAGATCTTCAGGGCTCTG  720
Q  D  S  F  S  G  F  A  P  L  Q  P  Q  A  A  E  P  P  P  R  P  K  T  P  E  I  F  R  A  L  240
GAAGGGGAGGCTCACCGTGTGCTATTTGGGTTTGTGCCTGAGACAAAAGAAGAGCTCCAGGTCATGCCAGGGAACATTGTCTTTGTCTTG  810
E  G  E  A  H  R  V  L  F  G  F  V  P  E  T  K  E  E  L  Q  V  M  P  G  N  I  V  F  V  L  270
AAGAAGGGCAATGATAACTGGGCCACGGTCATGTTCAACGGGCAGAAGGGGCTTGTTCCCTGCAACTACCTTGAACCAGTTGAGTTGCGG  900
K  K  G  N  D  N  W  A  T  V  M  F  N  G  Q  K  G  L  V  P  C  N  Y  L  E  P  V  E  L  R  300
ATCCACCCTCAGCAGCAGCCCCAGGAGGAAAGCTCTCCGCAGTCCGACATCCCAGCTCCTCCTAGTTCCAAAGCCCCTGGAAAACCCCAG  990
I  H  P  Q  Q  Q  P  Q  E  E  S  S  P  Q  S  D  I  P  A  P  P  S  S  K  A  P  G  K  P  Q  330
CTGTCACCAGGCCAGAAACAAAAAGAAGAGCCTAAGGAAGTGAAGCTCAGTGTTCCCATGCCCTACACACTCAAGGTGCACTACAAGTAC 1080
L  S  P  G  Q  K  Q  K  E  E  P  K  E  V  K  L  S  V  P  M  P  Y  T  L  K  V  H  Y  K  Y  360
ACGGTAGTCATGAAGACTCAGCCCGGGCTCCCCTACAGCCAGGTCCGGGACATGGTGTCTAAGAAACTGGAGCTCCGGCTGGAACACACT 1170
T  V  V  M  K  T  Q  P  G  L  P  Y  S  Q  V  R  D  M  V  S  K  K  L  E  L  R  L  E  H  T  390
AAGCTGAGCTATCGGCCTCGGGACAGCAATGAGCTGGTGCCCCTTTCAGAAGACAGCATGAAGGATGCCTGGGGCCAGGTGAAAAACTAC 1260
K  L  S  Y  R  P  R  D  S  N  E  L  V  P  L  S  E  D  S  M  K  D  A  W  G  Q  V  K  N  Y  420
TGCCTGACTCTGTGGTGTGAGAACACAGTGGGTGACCAAGGCTTTCCAGATGAACCCAAGGAAAGTGAAAAAGCTGATGCTAATAACCAG 1350
C  L  T  L  W  C  E  N  T  V  G  D  Q  G  F  P  D  E  P  K  E  S  E  K  A  D  A  N  N  Q  450
ACAACAGAACCTCAGCTTAAGAAAGGCAGCCAAGTGGAGGCACTCTTCAGTTATGAGGCTACCCAACCAGAGGACCTGGAGTTTCAGGAA 1440
T  T  E  P  Q  L  K  K  G  S  Q  V  E  A  L  F  S  Y  E  A  T  Q  P  E  D  L  E  F  Q  E  480
GGGGATATAATCCTGGTGTTATCAAAGGTGAATGAAGAATGGCTGGAAGGGGAGTGCAAAGGGAAGGTGGGCATTTTCCCCAAAGTTTTT 1530
G  D  I  I  L  V  L  S  K  V  N  E  E  W  L  E  G  E  C  K  G  K  V  G  I  F  P  K  V  F  510
GTTGAAGACTGCGCAACTACAGATTTGGAAAGCACTCGGAGAGAAGTCTAGGATGTTTCACAAACTACAAAGCTGAAGAAAATGAAGCCC 1620
V  E  D  C  A  T  T  D  L  E  S  T  R  R  E  V                                             526
TATTACTTGTTTGTAAGATTTAGCACCCTTCTGCTGTATACTGTACTGAGACATTACAGTTTGGAAGTGTTAACTATTTATTCCCTGTTA 1710
AAATTTAACCTACTAGACAATGATGTGAGTACCCAGGATGATTTCCTGGGGCACAGTGGGTGAGGAGATGGGGACAGGTGAATGGAGGAG 1800
TTAGGGGAGAGGAAAAGTGGATGGAAGTGTCTGGAAAGGGCACGAGAGAGTCTTCCAGGTACTGATCCTGTTTCTTGCTCTGAGTGCTAG 1890
CTAGCCAGCTGTGTTCACACTGTAAACATTCATCAAGCTGTACATTTGGTGCACTTTTCTGTGTCATACCACAATAAAAAAAACCTATC 1980
ATCATCTTACAAAAACAAGACACCCAAGTCCAGGCCCAAGGAGTAAGTACAAATATTCCTGTTTCTGAACCATTACTGTAATTGGCTCTT 2070
AAGGCTTGAAGTAACCTTATAGGTTACTCATAAGGCATATACAAATAAACTTGTTTGTTTTCTTTT                          2136
```

Fig. 3. (**a**) Nucleotide and predicted protein sequence of p67. The sequence shown was obtained from clone 10. DNA sequences were obtained with standard dideoxynucleotide chain termination methods (*30*) using Sequenase (U.S. Biochemical). Clone 10 was sequenced with a series of oligonucleotide primers that extended sequences through the entire length of both strands. An ATG codon starting 25 bases from the 5′ end, designated as position +1, occurs within a consensus sequence for eukaryotic initiation of translation (*10*). Polyadenylation signals (AATAAA) in the 3′ untranslated region are underlined. The complete sequence is deposited with GenBank (accession number M32011). (**b**) Alignment of p60^{src}-like region A (*12*) motifs in p67, p47 (*2, 3*), *gag-crk* (*16*), human and chicken nonerythroid α-spectrin (*17*), bovine PLC-γ (*12, 18*), human GAP (*19*), *Acanthamoeba* myosin-IB (*28*), and the human *src*-related nonreceptor tyrosine kinases c-*src* and c-*hck* (*14*). Dots above sequences indicate residues identical to the COOH-terminal region A sequence in p67. Sequence identities range between 43% (myosin I) and 24% (GAP). The duplicated motif in p47 (*2, 3*) has an internal homology of 37% amino acid identity. The consensus given below denotes those residues occurring in >70% of the sequences compared above. Numbers on the left refer to the first position residue in these sequence motifs, while the numbers on the right give percent identities to the COOH-terminal region A sequence in p67. (**c**) Schematic representation of p60$^{c\text{-}src}$-like regions, originally defined as region A (~50 residues, closed box), region B (~45 residues, open box), and region C (~20 residues, hatched box) according to Stahl *et al.* (*12*). The middle region of p47 contains a tandem repeat of the region A motif, whereas the related sequence in p67 is found in middle and COOH-terminal domains. Numbers above boxes refer to the first residue in these motifs.

suggests that these proteins may also bind directly to actin. The ability to express functional recombinant cytosol oxidase proteins should enable a detailed assessment of these interesting structure-function relationships.

REFERENCES AND NOTES

1. M. C. Dinauer, S. H. Orkin, R. Brown, A. J. Jesaitis, C. A. Parkos, *Nature* **327**, 717 (1986); C. Teahan, P. Rowe, P. Parker, N. Totty, A. W. Segal, *ibid.*, p. 720; C. A. Parkos *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3319 (1988).
2. K. J. Lomax, T. L. Leto, H. Nunoi, J. I. Gallin, H. L. Malech, *Science* **245**, 409 (1989); *ibid.*, **246**, 987 (1989).
3. B. D. Volpp, W. M. Nauseef, J. E. Donelson, D. R. Moser, R. A. Clark, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7195 (1989); *ibid.*, p. 9563.
4. H. Nunoi, D. Rotrosen, J. I. Gallin, H. L. Malech, *Science* **242**, 1298 (1988).
5. B. D. Volpp, W. M. Nauseef, R. A. Clark, *ibid.*, p. 1295.
6. The p67 clone 10 encodes the largest fusion polypeptide, 70 kD by SDS-PAGE, and is thought to contain the entire coding sequence of p67. Shorter clones aligned with matching sequences within clone 10. All of the cDNAs contained long open reading frames fused with a vector sequence encoding the 35 NH_2-terminal residues of β-galactosidase. This accounts for the larger size of clone 10 fusion protein compared to native p67 by SDS-PAGE.
7. T. L. Leto, unpublished observations.
8. Recombinant p67 fusion protein was produced in *E. coli* and prepared for immunization of rabbits as described (*2*). Bacterial cells (*E. coli* strain JM 109), transformed with a Bluescript plasmid (Stratagene) bearing p67 cDNA (clone 10), were grown to an OD_{600} of 0.5, induced with 5 m*M* isopropyl-thiogalactopyranoside, and grown for an additional 2 hours. The induced *E. coli* cells were lysed in 20 m*M* tris-HCl (*p*H 8), 5 m*M* EDTA, 0.1 m*M* diisopropylfluorophosphate, 0.1% deoxycholate, and lysozyme (200 μg/ml) and sonicated to shear large DNA. The r-p67, obtained from the pelleted inclusion bodies from this lysate, used as antigen was judged >80% pure. Immunoblots were probed with 1000-fold diluted rabbit antiserum to r-p67, which was detected with alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G according to manufacturer's protocols (Bio-Rad).
9. The r-p67 was produced as described (*8*) and purified from the *E. coli* lysate supernatant fraction by Sepharose Q (Pharmacia) chromatography. A peak of activity was recovered from a fraction eluting at ~0.2*M* NaCl. The r-p67 (10 μl) was added to each assay mixture containing normal neutrophil membranes from 5×10^5 cells and patient cytosol from 10^6 cells (*4*). All other assay components were used as described (*2*). Superoxide generation is expressed as ΔOD_{550}, the superoxide dismutase–sensitive change in absorbance of cytochrome C upon reduction by superoxide (ΔOD_{550} of 0.1 equals 2.77 nmol of superoxide produced per assay mixture).
10. M. Kozak, *Nucleic Acids Res.* **12**, 857 (1984).
11. D. J. Lipman and W. R. Pearson, *Science* **227**, 1435 (1985); J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984). Software analysis was performed on a supercomputer maintained by the National Cancer Institute Advanced Scientific Computing Laboratory, Frederick Cancer Research Facility, Frederick, MD.
12. M. L. Stahl, C. R. Ferenz, K. L. Kelleher, R. W. Kriz, J. L. Knopf, *Nature* **332**, 269 (1988).
13. T. Pawson, *Oncogene* **3**, 491 (1988).
14. A. Tanaka *et al.*, *Mol. Cell. Biol.* **7**, 978 (1987); M. Quintrell *et al.*, *ibid.*, p. 2267; R. M. Perlmutter *et al.*, *J. Cell. Biochem.* **38**, 117 (1988); S. M. Dymecki, J. E. Niederhuber, S. V. Desiderio, *Science* **247**, 332 (1990).
15. R. M. Perlmutter *et al.*, *Biochim. Biophys. Acta* **948**, 245 (1988).
16. B. J. Mayer, M. Hamaguchi, H. Hanafusa, *Nature* **332**, 272 (1988).

17. V. M. Wasenius *et al.*, *J. Cell Biol.* **108**, 79 (1989); A. P. McMahon *et al.*, *Differentiation* **34**, 68 (1987).
18. P. G. Suh, S. H. Ryu, K. H. Moon, H. W. Suh, S. G. Rhee, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5419 (1988).
19. U. S. Vogel *et al.*, *Nature* **335**, 90 (1988); M. Trahey *et al.*, *Science* **242**, 1697 (1988).
20. A. R. F. Rodaway *et al.*, *Nature* **342**, 624 (1989); D. G. Drubin, J. Mulholland, Z. Zhu, D. Botstein, *ibid.* **343**, 288 (1990).
21. T. E. Dever, M. J. Glynias, W. C. Merrick, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1814 (1987).
22. M. S. Marshall *et al.*, *EMBO J.* **8**, 1105 (1989).
23. R. A. Clark, B. D. Volpp, K. G. Leidal, W. M. Nauseef, *J. Clin. Invest.* **85**, 714 (1990).
24. M. Hamaguchi and H. Hanafusa, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2312 (1987).
25. W. M. Potts, A. B. Reynolds, T. J. Lansing, J. T. Parsons, *Oncogene Res.* **3**, 343 (1988).
26. V. Gerke and K. Weber, *EMBO J.* **3**, 227 (1984); J. Glenney, *J. Biol. Chem.* **261**, 7247 (1986).
27. T. J. Lynch *et al.*, *J. Biol. Chem.* **261**, 17156 (1986).
28. G. Jung, C. J. Schmidt, J. A. Hammer, *Gene* **82**, 269 (1989).
29. R. A. Clark *et al.*, *N. Engl. J. Med.* **321**, 647 (1989).
30. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
31. We thank P. M. Murphy for providing the RNA used in the library construction and K. Chen for assistance in preparing the recombinant protein.

4 December 1989; accepted 2 March 1990

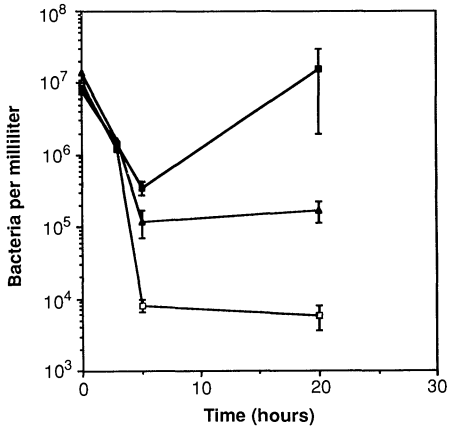


Fig. 1. Survival of *S. typhimurium* in J774 macrophages. Macrophages were infected (*5*) with *S. typhimurium* 14028 (■), MS4347 (▲), or MS7953(*phoP*) (□). The number of viable bacteria at each time point was determined by lysing the macrophages and plating. Results are expressed as the average of three wells plus standard error.

Induction of *Salmonella* Stress Proteins upon Infection of Macrophages

NANCY A. BUCHMEIER AND FRED HEFFRON

Regulated expression of bacterial genes allows a pathogen to adapt to new environmental conditions within the host. The synthesis of over 30 *Salmonella* proteins is selectively induced during infection of macrophages. Two proteins induced by *Salmonella* are the heat shock proteins GroEL and DnaK. Two avirulent, macrophage-sensitive mutants of *Salmonella* synthesize GroEL and DnaK but fail to synthesize different subsets of proteins normally induced within the macrophage. Enhanced expression of selected *Salmonella* proteins contributes to bacterial survival within macrophages and may also contribute to the apparent immunodominance of heat shock proteins.

COORDINATED REGULATION OF genes necessary for virulence enables a pathogen to respond to diverse conditions and environments encountered during the infectious process (*1*). One of the most hostile environments encountered by an invading microorganism is the intracellular environment of macrophages. To survive within macrophages, a pathogen must be resistant to such antimicrobial factors as acidic pH, toxic oxidative products, and lysosomal and granular proteins and peptides (*2*). Resistance to such varied stresses involves a number of genes, as evidenced by the isolation of many unrelated avirulent macrophage-sensitive mutants of *Salmonella typhimurium* (*3*). Wild-type *Salmonella* can grow in macrophages from many different sources, including the macrophage-like cell line J774, increasing over 30-fold after an initial decrease in numbers (*4, 5*) (Fig. 1). Little is known about the regulated expression of factors required for survival within macrophages or within other cells of the host. In this report, we provide evidence that *S. typhimurium* responds during infection of macrophages by the increased synthesis of a number of bacterial proteins that we call macrophage-induced proteins (MIPs). The MIPs are located within multiple regulatory networks. We have also observed that two of the major *Salmonella* proteins induced within macrophages are heat shock proteins GroEL and DnaK, immunodominant antigens for many infectious organisms.

To characterize the response of *Salmonella* during macrophage infection, we compared *Salmonella* proteins synthesized after infection of J774 macrophages with those synthesized during growth under identical culture conditions in the absence of macrophages. Macrophage protein synthesis was inhibited by cycloheximide and bacterial proteins were labeled with [^{35}S]methionine. One-dimensional polyacrylamide electrophoresis of the labeled proteins revealed at least three MIPs (Fig. 2A). The most prominent induced protein was about 58 kD (protein a), whereas the other two were approximately 68 and 27 kD (proteins b and c, respectively). We detected no protein synthesis in uninfected macrophages treated with cycloheximide or macrophages infected with heat-inactivated *Salmonella*.

Improved resolution of over 1000 *Salmonella* proteins was obtained with high-resolution two-dimensional electrophoretic gels of ^{35}S-labeled proteins and computer-aided analysis (Protein Databases Inc.). Of the 405 proteins analyzed on parallel gels, 34 *Salmonella* proteins showed four times more stimulation in synthesis during infection of macrophages compared to synthesis during growth in media. Of these, 12 MIPs appeared to be uniquely expressed in the macrophage environment, because they were absent in *Salmonella* grown in the same media without macrophages. These newly expressed proteins may be required only during in vivo infection and may correspond to genes identified by *Salmonella* mutants that are sensitive to macrophages but grow well in culture media (*3*). MIPs that are present at low concentrations in culture media may be essential under a variety of conditions but needed at higher concentration during macrophage infection [for example, enhanced expression of heat shock proteins at high temperature (*6*)]. Twenty of the most prominent *Salmonella* MIPs are shown in Fig. 3B compared to proteins synthesized by bacteria grown in medium (Fig. 3A). Synthesis of 136 *Salmonella* proteins during macrophage infection was reduced by 75%. Many of these proteins (about 52) were not synthesized after macrophage infection. Approximately half of the MIPs appear to be specific to the macrophage environment since they were not induced after infection of two epithelial cell lines, CACO.2 and MDCK (*7*).

We determined the kinetics of MIP induction after infection of macrophages (Fig. 2A, lanes 4 to 7). The increase in synthesis of the *Salmonella* MIPs was first detected from 30 to 60 min after infection. MIPs were synthesized during the next 3 hours and continued to be synthesized 20 hours after infection (*8*) during the active growth phase of *Salmonella* infection. The rapid in-

Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037.