ml of crude RLCM (10,000 DF units) intraperitoneally, beginning 8 hours after cell injection and continuing every 8 hours for a total of 14 doses. Group II (19 rats) received RLCM that had been rendered inactive by being heated at 70°C for 2 hours, and group III (10 rats) received serum-free medium. All rats in groups II and III died of leukemia within 16 days (Fig. 2). Autopsy in each animal showed leukemic ascites and grossly visible diffuse peritoneal leukemic infiltrates. Three animals in group I died of leukemia in 16 days; another four were dead at 23 days; and 12 animals (63%) were alive at 150 days without evidence of disease. Thus the development of leukemia was aborted in 63% of the rats by treatment with RLCM.

Finally, in order to demonstrate that RLCM exerts its action by inducing differentiation of the transplanted C51 cells, we placed diffusion chambers containing 1×10^5 C51 cells each in twelve 7-day-old rats and randomized them into two groups of six rats each. Group I received 0.2 ml of RLCM intraperitoneally every 8 hours beginning 8 hours after placement of diffusion chambers and continuing for a total of five doses. Group II received serum-free medium and served as controls. At 48 hours 94% of C51 cells in group I were differentiated with >90% viability but without growth, whereas cell number in the control rats increased approximately sevenfold with no evidence of differentiation. These results confirmed that RLCM exerted its action in vivo by inducing terminal differentiation of the transplanted leukemic cells. However, since differentiation is accompanied by loss of proliferative capacity, the presence of an additional independent growth-inhibiting activity in the RLCM cannot be excluded.

The results described herein strongly suggest that in the rat chloroleukemia model, the success or failure of transplantability of a given tumor load in young rats (≤21 days old) is a function of the amount of DF which the animal can generate. Rejection of the tumor can be accomplished through terminal differentiation of the injected cells. The relevance of these observations to human myelogenous leukemia remains uncertain at present. However, given the fact that human leukemic cells respond to DF in vitro, our observations in this rat model suggest the possibility that maintenance of the undifferentiated (leukemic) state in vivo may at least in part be due to the host's limited capacity to generate the appropriate level of DF. It is possible that some CSFs act primarily as proliferation factors in vivo whereas others like G-CSF stimulate differentiation. Proliferation predominates if the ratio of proliferation to differentiation activity is increased—particularly if the leukemic cell is less responsive to DF, thus requiring higher DF levels. A more attractive hypothesis involves a key role for the leukemic cell itself. The leukemia cell may help maintain itself in the undifferentiated state (and therefore at a proliferative advantage) by feedback inhibition of differentiation, perhaps through the production of soluble mediators. The soluble mediators could exert their action at the leukemia cell receptor level to competitively inhibit the action of DF. In this regard, when concentrated conditioned medium from C51 cells is added to cultured C51 cells along with DF, it does not block differentiation. However, this does not exclude the possible production of blocking factors in vivo. Alternatively, soluble factors from leukemic cells could act on DF-producing cells to block DF production. The nature of factors produced by the host and the leukemic cells and the interplay among the factors remain to be defined.

Whatever the exact mechanism involved in the maintenance of the undifferentiated (leukemic) state in vivo, our observations suggest that the administration of DF with or without cytoreductive chemotherapy is a promising treatment of human myelogenous leukemia.

REFERENCES AND NOTES

1. N. A. Nicola, D. Metcalf, M. Matsumoto, G. R. Johnson, *J. Biol. Chem.* **258**, 9017 (1983).
2. M. Tomida, Y. Yamamoto-Yamaguchi, M. Hozumi, *ibid.* **259**, 10978 (1984).
3. A. A. Yunis, G. K. Arimura, F.-M. Wu, M.-C. Wu, *Leukemia Res.* **11**, 673 (1987).
4. D. Metcalf, *Int. J. Cancer* **25**, 225 (1980).
5. Y. Ichikawa, *J. Cell. Physiol.* **76**, 175 (1970).
6. E. Fibach and L. Sachs, *ibid.* **86**, 22 (1975).
7. J. Lotem and L. Sachs, *Int. J. Cancer* **28**, 375 (1981).
8. J. Honma, T. Kasukabe, J. Okabe, M. Hozumi, *Cancer Res.* **39**, 3167 (1979).
9. D. Metcalf, *J. Natl. Cancer Inst. Monogr.* **60**, 123 (1982).
10. N. A. Nicola, C. G. Begley, D. Metcalf, *Nature (London)* **314**, 625 (1985).
11. H. Shay, M. Gruenstein, G. Harris, *Acta Haematol.* **14**, 337 (1955).
12. ———, L. Glazer, *Blood* **7**, 613 (1952).
13. W. C. Moloney *et al.*, *ibid.* **19**, 45 (1962).
14. A. A. Yunis, G. K. Arimura, H. G. Haines, R. J. Ratzan, M. A. Gross, *Cancer Res.* **35**, 337 (1975).
15. M.-C. Wu and P. M. Reuben, *Exp. Hematol.* **12**, 267 (1984).
16. S. Takasaki, F. Emling, L. Lieve, *J. Cell Biol.* **98**, 2198 (1984).
17. J. A. Vilpo, *Scand. J. Hematol.* **10**, 367 (1973).

18. Supported by the American Cancer Society Rochelle Wilderman Memorial Grant for Cancer Research (CH-280B) and by USPHS Grant AM 07114.

13 July 1987; accepted 20 October 1987

Signal for Attachment of a Phospholipid Membrane Anchor in Decay Accelerating Factor

INGRID W. CARAS, GREGORY N. WEDDELL, MICHAEL A. DAVITZ, VICTOR NUSSENZWEIG, DAVID W. MARTIN, JR.

Decay accelerating factor (DAF) belongs to a novel group of membrane proteins anchored to the cell surface by a glycophospholipid membrane anchor that is covalently attached to the carboxyl terminus of the protein. The last 37 amino acids of membrane DAF, when fused to the carboxyl terminus of a secreted protein, are sufficient to target the fusion protein to the plasma membrane by means of a glycophospholipid anchor. This approach provides a novel means of targeting proteins to the cell-surface membrane.

WHILE MOST INTEGRAL MEMbrane proteins are anchored to the lipid bilayer by a hydrophobic polypeptide transmembrane domain, a small class of proteins is held in the plasma membrane by an unusual mechanism involving covalent attachment of a complex phospholipid anchor to the carboxyl terminus of the protein. Proteins anchored in this way include Thy-1 (*1, 2*), the variant surface glycoproteins of African trypanosomes (*3*), acetylcholinesterase (*4*), 5′ nucleotidase (*5*), and decay accelerating factor (DAF) (*6, 7*). Attachment of the anchor, which contains glycosylated phosphatidylinositol (PI) and ethanolamine, apparently occurs after proteolytic removal of 17 to 31 carboxyl-terminal residues from the protein (*8, 9*). The signal that dictates processing and attachment is not known. In this report we show that the last 37 amino acids of DAF fused to the carboxyl terminus of a secreted protein (i) direct attachment of a PI-anchor and (ii) target the fusion protein to the plasma membrane. Use of this signal provides a novel means of anchoring a protein to the outer cell membrane in such a way that the entire protein projects into the extracellular space.

I. W. Caras, G. N. Weddell, D. W. Martin, Jr., Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.
M. A. Davitz and V. Nussenzweig, Department of Pathology, New York University School of Medicine, New York, New York 10016.

DAF binds activated complement fragments C3b and C4b, thereby inhibiting amplification of the complement cascade on host cell membranes (*10–12*). We recently identified two classes of DAF messenger RNA (mRNA) in HeLa cells, one apparently related to the other by a splicing event that causes a coding frameshift near the carboxyl terminus (*13*). Two DAF proteins are therefore possible, having divergent carboxyl-terminal domains that differ in their hydrophobicity. Expression of the two complementary DNAs (cDNAs) in Chinese hamster ovary (CHO) cells suggests that the spliced DAF mRNA (which predicts a hydrophobic carboxyl terminus) generates phospholipid-anchored, membrane-bound DAF (*13, 14*). In contrast, the protein encoded by the unspliced DAF mRNA has a hydrophilic carboxyl terminus and is secreted. These observations suggest that the carboxyl terminus of DAF plays a role in directing the attachment of a phospholipid membrane anchor.

To test this concept, we used gene manipulation to construct a fusion protein in which the last 37 amino acids of membrane DAF predicted by the spliced cDNA were fused in frame to the carboxyl terminus of a truncated form of glycoprotein D (gD-1) from herpes simplex virus type 1 (HSV-1). The truncated gD-1 is constitutively secreted to the culture medium since it lacks the carboxyl-terminal membrane-spanning domain (*15*). The resultant fusion protein (Fig. 1) contains 75% of gD-1 from the amino terminus (amino acids 1 to 300), including the signal sequence, and contains 10% of membrane DAF from the carboxyl terminus, including the 20–amino acid segment that is divergent between the two predicted DAF proteins plus 17 amino acids of adjacent common sequence. The gD-1–DAF fusion protein, native gD-1 (*16*), and the truncated gD-1 (*15*) were expressed in CHO cells and localized by indirect immunofluorescence. Internal labeling of permeabilized cells expressing either native gD-1 or the gD-1–DAF fusion protein showed similar localization of immunofluorescence in a perinuclear region, possibly the endoplasmic reticulum (Fig. 2, a′ and c′, respectively). Cells expressing truncated gD-1 showed intense fluorescence diffused throughout the cell cytoplasm (Fig. 2b′). Immunofluorescence of intact (nonpermeabilized) cells expressing full-length native gD-1 shows that this protein is expressed on the cell surface as expected from its transmembrane domain

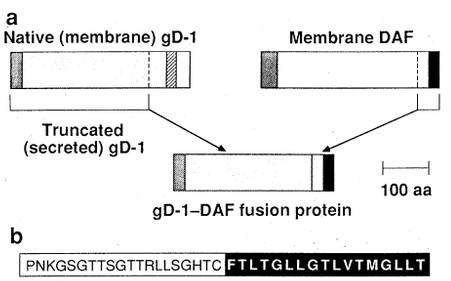

Fig. 1. (**a**) Schematic diagram showing the regions of gD-1 and DAF that are present in the gD-1–DAF fusion protein. Truncated (secreted) gD-1 was constructed from native (membrane) gD-1 (*14*) and comprises amino acids 1 to 300, including the hydrophobic signal sequence (residues 1 to 25, indicated as a dark gray area). The hydrophobic membrane-spanning domain (residues 340 to 360, cross-hatched region) and the carboxyl-terminal hydrophilic domain (residues 361 to 393) are excluded. The point of the truncation (residue 300) is indicated by a broken line. Truncated gD-1 was fused to residue 311 of membrane DAF. The gD-1–DAF fusion contains the last 37 residues of membrane DAF predicted from cDNA sequence (residues 311 to 347) and includes a carboxyl-terminal hydrophobic region (residues 331 to 347, depicted in black). A Hind III–Hinf I fragment encoding the first 300 amino acids of HSV gD-1 was ligated via a synthetic linker to a Xmn I–Eco RV fragment encoding the carboxyl terminus of DAF (amino acids 318 to 347). The synthetic Hinf I–Xmn I linker (5′-ATTCGCCAAATAAAGGAAGTGGAACC) encoded amino acid 301 of gD-1 and amino acids 311 to 317 of DAF and created an in-frame fusion. Size marker, 100 amino acids. (**b**) Amino acid sequence of the 37–amino acid segment of membrane DAF included in the gD-1–DAF fusion protein. The hydrophobic region is shown in black. The one-letter symbols for the amino acids are A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine; and X, unknown or "other."

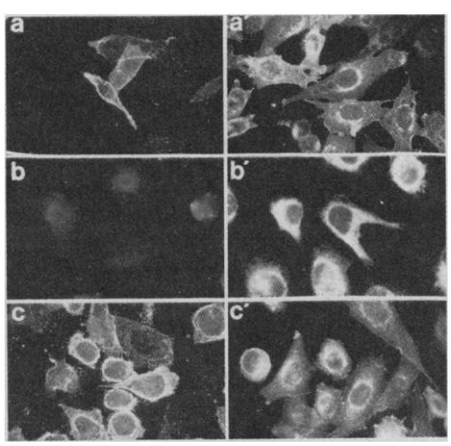

Fig. 2. Immunofluorescent labeling of transfected CHO cells, showing the cellular localization of expressed protein: (**a**, **b**, and **c**) cell surface labeling of intact (nonpermeabilized) cells and (**a′**, **b′**, and **c′**) permeabilized cells labeled internally. The cells are expressing (a and a′) native (membrane) gD-1, (b and b′) truncated (secreted) gD-1, and (c and c′) the gD-1–DAF fusion protein. The cDNA encoding the gD-1–DAF fusion protein was inserted into a mammalian expression vector between an RSV promoter and an SV40 polyadenylation sequence (*24*) and transfected into CHO cells by the calcium-phosphate coprecipitation method (*25, 26*). Mouse dihydrofolate reductase cDNA provided a selectable marker for gene expression (*26*). Stable cell lines derived from individual colonies were used for analysis. Cell lines expressing native gD-1 or truncated gD-1 were derived as described (*15, 16*). For internal labeling, cells were fixed for 4 minutes in 2% paraformaldehyde and then permeabilized with ethanol for 5 minutes. For surface labeling, cells were either fixed in paraformaldehyde or incubated directly with antibody (*13, 27, 28*). Cells were incubated with a monoclonal antibody against the amino terminus of gD-1 for 2 hours at room temperature, washed in phosphate-buffered saline, and exposed to rhodamine-conjugated goat antiserum to mouse immunoglobulin G for 2 hours.

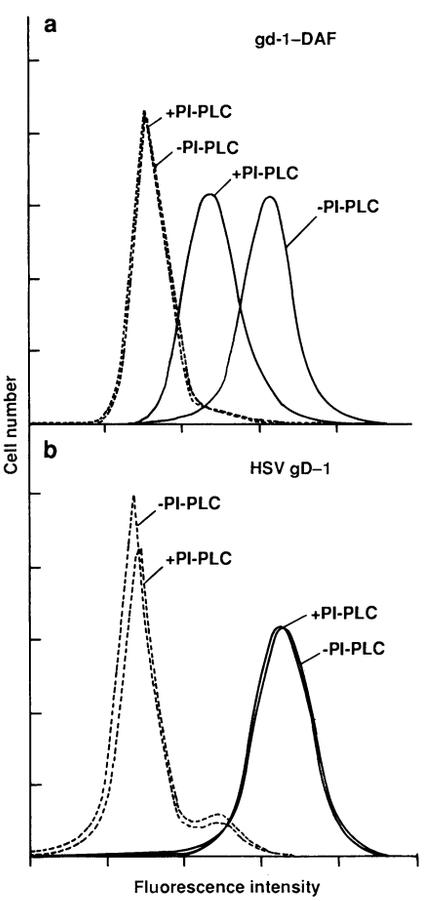

Fig. 3. Release of cell-surface gD-1–DAF by PI-PLC. CHO cells (2×10^6 per milliliter) expressing either (**a**) gD-1–DAF or (**b**) native gD-1 were incubated for 60 minutes at 37°C in Dulbecco's modified Eagle's medium containing 2% heat-inactivated fetal bovine serum. PI-PLC, 2μg/ml [purified from *S. aureus* culture supernatants (*17*)], was added as indicated. After treatment, cells were washed and analyzed by indirect immunofluorescence (with monoclonal antibody against gD-1) and flow cytometry. The background fluorescence (left, broken lines) represents staining with second-step fluorescein isothiocyanate–conjugated goat antiserum to mouse immunoglobulin G alone. Samples were run on a Becton Dickinson FACS IV.

(Fig. 2a). In contrast, no surface labeling was detected in cells expressing the truncated (secreted) form of gD-1 (Fig. 2b). Cells expressing the gD-1–DAF fusion protein also show surface staining (Fig. 2c), an indication that addition of the carboxyl-terminal domain of DAF redirects the secreted (truncated) gD-1 to the plasma membrane.

The carboxyl-terminal segment of DAF present in the gD-1–DAF fusion protein contains a 17–amino acid hydrophobic region at the carboxyl terminus that may act as a transient membrane anchor and that is thought to be removed post-translationally and replaced with a PI anchor (*8, 9, 13*). The above experiments do not distinguish whether the fusion protein is anchored by a phospholipid anchor or by the 17–amino acid hydrophobic region. Therefore, to determine the nature of the attachment, we

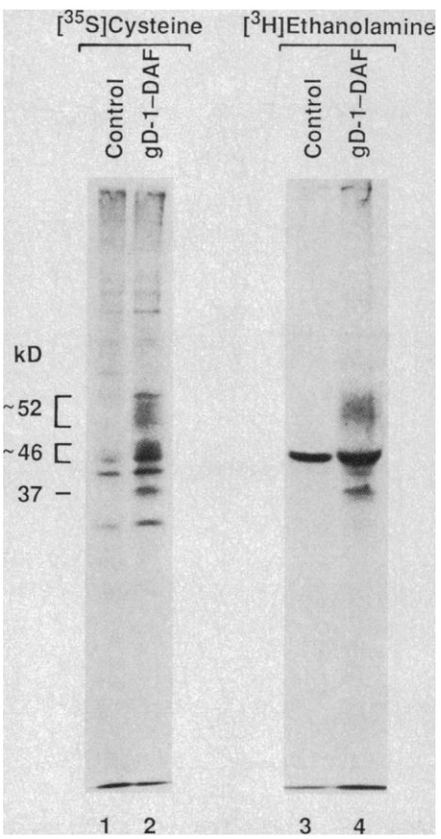

Fig. 4. Metabolic labeling and immunoprecipitation analysis of gD-1–DAF in transfected CHO cells. Cells were incubated with [^{35}S]cysteine (250 μCi per 60-mm plate) for 6 hours (lanes 1 and 2) or with [^{3}H]ethanolamine (1 mCi per 100-mm plate) for 16 hours (lanes 3 and 4). Lanes 1 and 3, control precipitations from cells transfected with a plasmid containing only dihydrofolate reductase cDNA; lanes 2 and 4, precipitation from cells expressing gD-1–DAF. Immunoprecipitations were carried out as described (*29*) with rabbit polyclonal antibodies to HSV-1 (DAKO). Similar results were seen with a mouse monoclonal antibody. Molecular sizes (in kilodaltons) of gD-1–DAF–specific bands are indicated.

incubated CHO cells expressing either native gD-1 or gD-1–DAF with purified PI-specific phospholipase C (PI-PLC) from *Staphylococcus aureus* (*17*) and analyzed them by indirect fluorescence and flow cytometry (FACS). Treatment with PI-PLC [which is free of proteolytic contaminants (*1*)] resulted in a substantial reduction in the amount of gD-1–DAF on the cell surface as indicated by the marked decrease in relative cell fluorescence displayed on a log scale (Fig. 3a). Typically, 70 to 80% of the cell-surface gD-1–DAF was released by PI-PLC as indicated by quantitative FACS analysis. In contrast, full-length native gD-1 expressed on the cell surface was unaffected by treatment with PI-PLC (Fig. 3b). The specificity of the release was further confirmed by the observation that the phospholipase C, from either *Clostridium perfringens* or *Bacillus cereus* [which does not hydrolyze PI (*18, 19*)], did not release gD-1–DAF from the plasma membrane.

The glycophospholipid anchor of DAF contains ethanolamine and glucosamine in addition to PI (*7*). The glycosylated phospholipid is thought to be linked to the protein through an amide bond between the terminal carboxyl group of the polypeptide and the amine group of ethanolamine (*8, 9, 20*). To confirm that the gD-1–DAF fusion protein is anchored by such a structure, we metabolically labeled cells with either [^{3}H]ethanolamine or [^{35}S]cysteine and analyzed the proteins by immunoprecipitation. Multiple forms of gD-1–DAF (a 37-kD species and at least two larger, highly diffuse species of approximately 46 kD and 52 kD, respectively) were detected by both polyclonal and monoclonal antibodies to HSV-1 only in cells expressing gD-1–DAF (Fig. 4, lane 2). Preliminary pulse-chase experiments and experiments with neuraminidase suggest that the 37-kD species is a precursor whereas the larger species represent mature, highly glycosylated forms of the protein. [^{3}H]Ethanolamine-labeled bands corresponding to all three species were specifically detected in cells expressing gD-1–DAF (Fig. 4, lane 4). Native gD-1 was not labeled with [^{3}H]ethanolamine.

We conclude that the gD-1–DAF fusion protein is linked to the plasma membrane via PI. This conclusion is supported by the following evidence: (i) gD-1–DAF on the cell surface was sensitive to digestion with highly purified PI-PLC, whereas native gD-1 was unaffected; (ii) broad specificity phospholipases were ineffective in releasing gD-1–DAF; and (iii) gD-1–DAF was specifically labeled by [^{3}H]ethanolamine, a component of the glycophospholipid anchor. We conclude further that the information or "signal" necessary for directing the attachment of a phospholipid membrane anchor is contained within the 37 carboxyl-terminal amino acids of DAF. The concept that the carboxyl-terminal sequence plays a role in directing the attachment of lipid is supported by recent identification of multiple classes of the neural cell adhesion molecule (N-CAM) mRNA, presumably resulting from differential mRNA splicing. The different forms of N-CAM encoded by these mRNAs have different carboxyl-terminal domains, apparently resulting in membrane attachment either via a hydrophobic membrane-spanning domain or via a phospholipid (*21*). Inspection of the carboxyl-terminal amino acid sequences available for PI-anchored proteins has revealed no obvious homology, the only common feature being the presence of a short hydrophobic peptide (15 to 20 residues) at the carboxyl terminus predicted by the cDNA sequence. This hydrophobic peptide, which could serve as a transient membrane anchor, is presumed to be removed during processing (*2, 8, 9, 23*). The lack of sequence conservation in the carboxyl-terminal region of PI-anchored proteins suggests that the processing signal may be conformational in character.

Experiments aimed at determining the importance of the hydrophobic region and the adjacent sequences may aid in elucidating the nature of this signal. Addition of a phospholipid membrane anchor by the means described above offers a novel mechanism for targeting soluble or secreted proteins to the cell-surface membrane. Further, this methodology provides a means of investigating the function of the anchor by comparing the properties of a protein anchored either by a transmembrane domain or by a PI anchor.

REFERENCES AND NOTES

1. M. G. Low and P. W. Kincade, *Nature (London)* **318**, 62 (1985).
2. A. G. D. Tse *et al.*, *Science* **230**, 1003 (1985).
3. M. A. J. Ferguson, M. G. Low, G. A. M. Cross, *J. Biol. Chem.* **260**, 14547 (1985).
4. A. H. Futerman *et al.*, *Biochem. J.* **226**, 369 (1985); W. L. Roberts and T. L. Rosenberry, *Biochemistry* **25**, 3091 (1986).
5. M. G. Low and J. B. Finean, *Biochim. Biophys. Acta* **508**, 565 (1978).
6. M. A. Davitz, M. G. Low, V. Nussenzweig, *J. Exp. Med.* **163**, 1150 (1986).
7. M. E. Medof *et al.*, *Biochemistry* **25**, 6740 (1986).
8. M. G. Low, *J. Biochem.* **244**, 1 (1987).
9. G. A. M. Cross, *Cell* **48**, 179 (1987).
10. A. J. Nicholson-Weller, J. Burge, D. T. Fearon, P. F. Weller, K. F. Austen, *J. Immunol.* **129**, 184 (1982).
11. M. E. Medof, T. Kinoshita, V. Nussenzweig, *J. Exp. Med.* **160**, 1558 (1984).
12. T. Kinoshita, M. E. Medof, V. Nussenzweig, *J. Immunol.* **136**, 3390 (1986).
13. I. W. Caras *et al.*, *Nature (London)* **325**, 545 (1987).
14. I. W. Caras, unpublished results.
15. L. A. Lasky, D. Dowbenko, C. C. Simonsen, P. W. Berman, *Bio/Technology* **2**, 527 (1984).
16. P. W. Berman, D. Dowbenko, L. A. Lasky, *Science* **222**, 524 (1983).
17. M. G. Low, *Methods Enzymol.* 71, 741 (1981).

18. C. Little, *ibid.*, p. 725.
19. T. Takahashi, T. Sugahara, A. Ohsaka, *ibid.*, p. 710.
20. A. A. Holder, *Curr. Top. Microbiol. Immunol.* **117**, 57 (1985).
21. J. J. Hemperly, G. M. Edelman, B. A. Cunningham, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9822 (1986).
22. A. G. D. Tse *et al.*, *Science* **230**, 1003 (1985).
23. J. C. Boothroyd *et al.*, *Nature (London)* **288**, 624 (1980).
24. C. M. Gorman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982).
25. M. Wigler *et al.*, *ibid.* **76**, 1373 (1979).
26. C. C. Simonsen and A. D. Levinson, *ibid.* **80**, 2495 (1983).
27. T. A. Gottlieb *et al.*, *J. Cell Biol.* **102**, 1242 (1986).
28. J. K. Rose and J. E. Bergmann, *Cell* **30**, 753 (1982).
29. D. J. Anderson and G. Blobel, *Methods Enzymol.* **96**, 111 (1983).
30. We thank P. Berman and L. Lasky for providing gD-1 cell lines and plasmids, M. Low of the Department of Physiology, Columbia University, for a gift of PI-PLC, P. Ng and P. Jhurani for oligonucleotide synthesis, and D. Standring for valuable discussions. This work was supported by Genentech, Inc. M.D. is supported by a Physician-Scientist award and V.N. by NIH grants AI-08499 and AI-23276.

7 August 1987; accepted 6 October 1987

Efflux of Chloroquine from *Plasmodium falciparum*: Mechanism of Chloroquine Resistance

DONALD J. KROGSTAD,* ILYA Y. GLUZMAN, DENNIS E. KYLE, AYOADE M. J. ODUOLA, SAMUEL K. MARTIN, WILBUR K. MILHOUS, PAUL H. SCHLESINGER

Chloroquine-resistant *Plasmodium falciparum* accumulate significantly less chloroquine than susceptible parasites, and this is thought to be the basis of their resistance. However, the reason for the lower accumulation of chloroquine was unknown. The resistant parasite has now been found to release chloroquine 40 to 50 times more rapidly than the susceptible parasite, although their initial rates of chloroquine accumulation are the same. Verapamil and two other calcium channel blockers, as well as vinblastine and daunomycin, each slowed the release and increased the accumulation of chloroquine by resistant (but not susceptible) *Plasmodium falciparum*. These results suggest that a higher rate of chloroquine release explains the lower chloroquine accumulation, and thus the resistance observed in resistant *Plasmodium falciparum*.

MALARIA IS A DISEASE OF IMmense importance, with an estimated 200 to 300 million cases and 2 million deaths each year (*1*). Chloroquine, the drug most widely used for the treatment of malaria, is effective against three of the four malaria species that infect humans. However, the species which poses the greatest risk of complications and death (*Plasmodium falciparum*) is often resistant to treatment with chloroquine (*2*). Chloroquine-resistant *P. falciparum* is now established in Southeast Asia and South America (*3*), and has recently spread westward across Africa from Kenya and Tanzania to Nigeria (*4*).

Beginning with the studies of Fitch (*5*), a number of investigators have shown that chloroquine-resistant *P. falciparum* accumulate significantly less chloroquine than do susceptible parasites. Our studies (*6*) and those of Yayon *et al.* (*7*) indicate that chloroquine is concentrated in the acid vesicular compartment of the parasite. Although the amount of chloroquine accumulated by resistant parasites is substantially less than that accumulated by susceptible parasites (*6*), the reason for this difference has been unknown.

Recent studies suggest that the accumulation of chloroquine in parasite acid vesicles inhibits parasite growth by raising the *p*H of the acid vesicle (*6, 7*). In mammalian cells, chloroquine enters and leaves the cell by passive diffusion and is concentrated as a weak base in the acid vesicle by the *p*H gradient across the vesicle membrane (*8*). In contrast, both the susceptible and resistant parasite accumulate more chloroquine in their vesicles than can be explained by its properties as a weak base (*6, 9*). We examined the kinetics of chloroquine accumulation and release by the parasite to determine whether they could account for the differences in chloroquine accumulation associated with susceptibility and resistance.

Parasites were grown in vitro in suspensions of O-positive red cells, with RPMI 1640; 25 m*M* Hepes, and $NaHCO_3$ at 0.2 g/100 ml, in a culture system devised by Trager and Jensen (*10*). The cultures were exposed to an atmosphere of 3% O_2, 3% CO_2, and 94% nitrogen in modular incubation chambers (Linde Division, Union Carbide, New York) and then maintained at 37°C (*11*). The Haiti 135 and Indochina I/CDC strains of *P. falciparum* were used because of their known susceptibility and resistance to chloroquine (median effective doses of 3 to 6 and 50 to 60 n*M*, respectively) (*12*).

[^{3}H]Chloroquine accumulation and release were calculated in two ways: (i) from the residual radioactivity of the culture medium supernatant after centrifugation of the parasitized red cell suspension through silicon oil (*6, 13*), and (ii) from the radioactivity of the pellet after alkali digestion of the cell pellet (*6*). Preliminary experiments showed excellent agreement between the two methods, and demonstrated that >95%

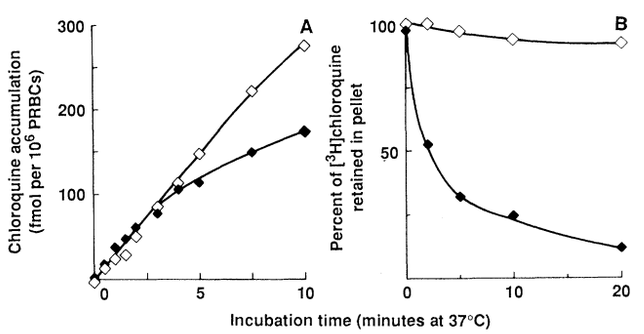

Fig. 1. Chloroquine accumulation and release by parasitized red cells. (**A**) The initial rates of chloroquine accumulation observed with susceptible (Haiti 135, open diamonds) and resistant (Indochina I, filled diamonds) *P. falciparum* were indistinguishable (28.6 ± 1.5 versus 29.1 ± 3.8 fmol per 10^6 parasitized red cells for the first 4 minutes). In these experiments suspensions of parasitized red cells (2×10^6 per milliliter) were suspended in culture medium containing 1 n*M* [^{3}H]chloroquine at zero time. Linear regression was used to calculate the rate of chloroquine accumulation for both parasites. PBRCs, parasitized red cells. (**B**) Chloroquine was released more rapidly from the resistant parasite than from the susceptible parasite. Similar values for the initial release $t_{1/2}$ were obtained when culture medium containing 1 n*M* [^{3}H]chloroquine was used with both parasites for the initial 60-minute incubation with [^{3}H]chloroquine (which produced different chloroquine accumulations) or different concentrations of [^{3}H]chloroquine (0.1 and 1.0 n*M*, respectively, for susceptible and resistant *P. falciparum*) to produce similar accumulations of chloroquine (174 to 180 fmol per 10^6 parasitized red cells) (B). Four separate experiments were performed in each case (A and B).

D. J. Krogstad and I. Y. Gluzman, Departments of Medicine and Pathology, Washington University School of Medicine, St. Louis, MO 63110.
D. E. Kyle, A. M. J. Oduola, W. K. Milhous, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307.
S. K. Martin, Division of Medicine, Walter Reed Army Institute of Research, Washington, DC 20307.
P. H. Schlesinger, Department of Biomedical Research at Washington University School of Dental Medicine, St. Louis, MO 63110.

*To whom correspondence should be addressed at the Department of Pathology, Box 8118, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.