droceramide from dihydrosphingosine (*16*). Our studies suggest that the introduction of the double bond may be required for ceramide to regulate cell growth and viability.

Because TNF-α may activate other signaling pathways, especially protein kinase C (*17, 18*), we investigated whether activation of protein kinase C resulted in apoptosis in U937 cells. We used dioctanoylglycerol (DiC_8) as a cell-permeable analog of endogenous diacylglycerols as well as phorbol 12-myristate 13-acetate (PMA), a pharmacologic activator of protein kinase C. Neither agent caused DNA fragmentation (Fig. 4, A and B). Although PMA causes internucleosomal DNA fragmentation in the U937 cell line, the effects occur after 12 hours and may be a consequence of cell differentiation (*19*). The faster effects of C_2-ceramide suggest that the sphingomyelin-ceramide pathway may mediate the action of TNF-α on apoptosis. In fact, the addition of PMA prevented the effects of TNF-α and C_2-ceramide on DNA fragmentation (Fig. 4, B and C), which is consistent with reports that PMA inhibits apoptosis (*20, 21*).

These studies identify ceramide as a possible mediator of apoptosis in response to the action of a number of extracellular agents and insults. For example, γ-interferon also causes sphingomyelin hydrolysis and ceramide generation (*4*), and ceramide may mediate its effects on apoptosis. Also, the presence of hypoxia, which induces apoptosis (*22, 23*), results in elevated ceramide concentrations in rat liver in vivo (*24*). Thus, ceramide may also mediate the effects of hypoxia on programmed cell death. Finally, in the human T cell line CEM, which undergoes DNA fragmentation in response to glucocorticoids (*25*), ceramide (3 and 6 μM) induced 57 ± 2% and 76 ± 1% DNA fragmentation, respectively (*15*). Preliminary data also indicate that primary mouse thymocytes undergo DNA fragmentation in response to C_2-ceramide (3 μM). Therefore, the effects of C_2-ceramide are not restricted to U937 cells, and ceramide may mediate apoptosis in response to multiple agents and injuries.

The mechanism of action of ceramide remains poorly understood. Ceramide and sphingosine have been shown to induce phosphorylation of the epidermal growth factor receptor, possibly by activating a protein kinase (*26*). We have recently identified a ceramide-activated protein phosphatase (*27*) that may be involved in transducing the effects of ceramide. The ability of PMA to counteract the effects of ceramide suggests that the effects of the diacylglycerol–protein kinase C pathway counteract those of the proposed ceramide-phosphatase pathway.

Note added in proof: Since submission of this study, Van Veldhoven and colleagues (*28*) have found a significant elevation of ceramide concentrations in human immunodeficiency virus (HIV)–infected CEM cells up to fourfold over concentrations in control uninfected cells. However, phospholipids showed no changes, and diacylglycerol showed only modest increases. Because HIV is known to induce programmed cell death (*29*), the results from the current study suggest that ceramide may mediate the effects of HIV on programmed cell death.

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13. Ceramide concentrations were measured by a modification of the diacylglycerol kinase assay, which quantitates the total cellular mass of ceramide (*5*). The changes in relative and absolute amounts of ceramide in U937 cells closely approximate changes that were observed in HL-60 cells and account for most of the hydrolyzed sphingomyelin (*5*).
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TECHNICAL COMMENTS

Ambiguities in Ab Initio Phasing

One of the main reasons that determining macromolecular crystal structures is difficult is the absence of phase information for the observed diffraction intensities or amplitudes. This phase problem is often overcome by exhaustive preparation and analysis of heavy atom derivatives of a crystallized molecule. Diffraction measurements on crystals in which heavy atoms have bound uniformly to a macromolecule, when taken with data from native crystals, can lead to approximate values for the missing phases.

Direct methods for determining the phases of a small molecule structures without the use of additional experimental data have been developed and are now the main method for obtaining phases for crystals of these molecules (*1*). Interest in similar ab initio phasing approaches for macromolecules has been great, but progress has been slow (*2*). A significant step toward ab initio phasing, in the absence of any experimental phase information, would be the direct determination of the protein-solvent boundary from diffraction intensities. S. Subbiah (*3*) describes an algorithm designed to produce such an envelope by modeling the contents of the unit cell with a hard-sphere gas, which maximizes the correlation between observed and calculated structure factor amplitudes. Although this idea seems feasible, the analysis by

Subbiah (3) of the results of test cases does not account for the possibility of enantiomeric structures or for the problem of equivalent origins. Structure factors calculated from models that differ from each other only in handedness differ in phase, but not in amplitude. Therefore, when only observed structure factor amplitudes are presented to a program such as the one described by Subbiah, possible structures that are related to each other by inversion symmetry are indistinguishable and are equally likely outcomes. This problem is not serious if it is recognized; a putative solution and its enantiomer must both be compared to the correct structure when test cases are evaluated.

A similar but conceptually more difficult problem arises from the presence of equivalent origins in most space groups (4). In $P2_12_12_1$, the space group of the two test cases described by Subbiah (3), there are eight equivalent origins: (0,0,0), (0,0,1/2), (0,1/2,0), (0,1/2,1/2), (1/2,0,0), (1/2,0,1/2), (1/2,1/2,0), and (1/2,1/2,1/2) in fractional coordinates. Structures related to each other by any of the above translational shifts give rise to identical structure factor amplitudes. Consequently, for every candidate solution, there are seven others that are indistinguishable on the basis of structure factor amplitudes but that are not equivalent in the crystallographic sense. Therefore, when assessing the correctness of a predicted structure in a test case in $P2_12_12_1$, one would have to compare the known structure with the predicted structure and with seven other structures related by differences in origin. In $P2_12_12_1$, the enantiomer ambiguity, taken together with the ambiguity in origin, gives 16 crystallographically distinct models with the same structure factor amplitudes; all of these would require inspection for possible agreement with the known structure. This inspection was not performed by Subbiah (3).

Suppose we stipulate that the algorithm Subbiah describes is capable of producing the correct protein-solvent boundary. Because the program starts from random sphere positions, when run repeatedly it would generate, arbitrarily, one of the 16 equally probable solutions in each run. Subbiah does not make this observation (3), but he does state that when he attempted to calculate an average from the multiple cycles, "the quality of the results was not significantly improved." In fact, one should expect different results from separate runs.

In the two test cases that Subbiah describes, the spheres usually, but not always, condensed in a region that is said to correlate with the solvent region, not with the protein region as expected. Although Subbiah (3) correctly points out that the two possibilities are similar, corresponding roughly to negative images of each other, the unexpected result is more probably due to one of two possibilities: (i) the program was successful, but the known structure was compared to the single model predicted by the program without necessary consideration of enantiomer or origin ambiguity or (ii) the program generated random correlations between the correct and predicted structures. While Subbiah's approach is clever and may indeed work, any correlations between the known structures and the calculated ones that have been found may well be accidental.

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Response: The concerns raised by Yeates and Zhang are legitimate: 32 possibilities (eightfold origin ambiguity, twofold enantiomer ambiguity, and twofold Babinet ambiguity) are available for condensation in $P2_12_12_1$. Babinet ambiguity was discussed in my report of the condensing protocol approach to ab initio phasing (*1*). Recent improvements to the methodology—the improved condensing protocol—on average allow both Babinet images to appear with equal probability (*2*). A new method that can resolve this ambiguity in the final condensed images has also been reported (*2*). In terms of the J index (the ratio of the number of correctly placed scatterers to those incorrectly placed relative to the true macromolecular envelope), the improved condensing protocol routinely produces values greater than 10 and even 20, in contrast to the original reports of 2. Moreover, although the original condensing protocol was already highly constrained to produce a single condensed cluster, in the improved protocol an additional requirement has been included to explicitly achieve this, and smaller subclusters do not occur.

Although enantiomer ambiguity was not discussed in my report (*1*), it does occur.

With regard to origin ambiguity, the condensing protocol as implemented in (*1*) can be arranged to select a given origin, as in test cases where the correct origin is known. I performed all calculations (*1*) in the lowest symmetry $P1$ space group and referenced all moves in the entire process to a particular origin. More important, in the real-life experimental situation, where the origin is unknown beforehand, neither the condensing protocol nor its improved form is capable of choosing the correct origin or enantiomer. Nevertheless, this residual ambiguity does not in anyway compromise the usefulness of the final Babinet-fixed envelopes in the real-life experimental situation. In general, there are three ways of using these low-resolution envelopes to proceed to atomic structures: (i) molecular replacement, (ii) rescue of heavy atoms by difference Fourier, and (iii) some future method of higher resolution ab initio phasing that would exploit these envelopes.

First, in practical molecular replacement, the origin is defined by the arbitrary choice of a search range. This is not an obstacle to attaining atomic resolution structures. Because an atomic model with the correct handedness is available, the condensed image can be discriminated from its enantiomer. Second, the traditional heavy atom method depends on the arbitrary origin choice of the first derivative. If some useful phase information can be obtained from these low-resolution envelopes, using these origin-specific phases will be equivalent to choosing the origin in standard heavy atom methodology. As in traditional heavy atom methods, an envelope-based approach will require either anomalous data or the correct handedness of molecular features to resolve the enantiomer ambiguity. Third, if a high-resolution ab initio phasing method is discovered that exploits a low-resolution envelope that has origin and enantiomer ambiguity, the correct structure could be traced with the use of a different but equivalent origin, and the enantiomer ambiguity could be settled as it has been before.

Note added in proof: The improved condensing protocol (*1*, *2*) has now been shown to work by other independent investigators, to work in several "blind" test cases from distant labs, and to correctly predict envelopes up to a year before the experimental answer was ascertained (*3*).

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