biotinylated complexes were incubated with streptavidin-coupled alkaline phosphatase for 1 hour and detected by incubation with the color reagents *p*-nitroblue tetrazolium (0.33 mg/ml) and BCIP (0.16 mg/ml) in 0.1 M tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ at room temperature.
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Intracellular Calcium Release Mediated by Sphingosine Derivatives Generated in Cells

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Soluble and hydrophobic lipid breakdown products have a variety of important signaling roles in cells. Here sphingoid bases derived in cells from sphingolipid breakdown are shown to have a potent and direct effect in mediating calcium release from intracellular stores. Sphingosine must be enzymically converted within the cell to a product believed to be sphingosine-1-phosphate, which thereafter effects calcium release from a pool including the inositol 1,4,5-trisphosphate–sensitive calcium pool. The sensitivity, molecular specificity, and reversibility of the effect on calcium movements closely parallel sphingoid base–mediated inhibition of protein kinase C. Generation of sphingoid bases in cells may activate a dual signaling pathway involving regulation of calcium and protein kinase C, comparable perhaps to the phosphatidylinositol and calcium signaling pathway.

A NUMBER OF PRODUCTS OF CELLULAR lipid breakdown have fundamental roles in signal transduction pathways. The soluble phosphoinositide headgroup, inositol 1,4,5-trisphosphate (IP_3) is a major Ca^{2+} signal mediator (*1*) and interacts with intracellular receptors now known to function as Ca^{2+} channels that allow Ca^{2+} release from intracellular pools (*2*). Hydrophobic lipid breakdown products are also major signal mediators, including diacylglycerol (*3*), arachidonic acid, prostaglandins and related derivatives (*4*), and the breakdown products of sphingolipids (*5, 6*). In the latter case, evidence has revealed sphingosine and its derivatives to be powerful inhibitors of protein kinase C (PKC) activity (*5–9*), opposing the action of diacylglycerol, and suggesting that they participate as endogenous modulators of cell function (*5*). Sphingosine is also a potent inhibitor of several calmodulin-activated enzymes, including Ca^{2+}-calmodulin–dependent protein kinase (*10*). Moreover, sphingosine mediates and modifies a multitude of responses in cells, including platelet aggregation, the neutrophil respiratory burst, killer cell activation, insulin- and growth factor–induced responses, and the growth and differentiation of many cell types (*6*). We now describe a new action of sphingosine derivatives in mediating rapid and profound translocation of Ca^{2+} from intracellular stores.

The movements of intracellular Ca^{2+}, the mechanisms of second messenger–activated Ca^{2+} transfer, and the nature of mobilizable Ca^{2+} pools have been studied extensively with saponin-permeabilized cells (*11, 12*). We used permeabilized cells of the DDT_1MF-2 smooth muscle cell line (*11–13*) and observed that addition of 30 μM sphingosine induced a large release of Ca^{2+} accumulated within intracellular Ca^{2+}-pumping organelles (Fig. 1A). The effect was seen after a lag of 30 s and thereafter was rapid. A similar release of Ca^{2+} was also induced by sphingosylphosphorylcholine (SPC); however, in this case the effect was almost instantaneous. In contrast, addition of up to 100 μM *N*-acetyl sphingosine did not induce any release of Ca^{2+}. The structures of these compounds are shown in Fig. 1B. Sphingosine and SPC are both highly effective in blocking PKC activity (*8*). The charged 2-amino position of sphingosine is important for PKC inhibition, no inhibition being observed if this position is acylated, as in *N*-acetyl sphingosine (*5, 7, 8, 14*); thus, we saw an identical specificity profile for Ca^{2+} release. This correspondence of specificity with PKC inhibition (*7, 14*) was extended by observations revealing no effect on Ca^{2+} release of ceramide or sphingomyelin (the respective long-chain *N*-acyl derivatives of sphingosine and SPC) and showing dihydrosphingosine to be equally effective as sphingosine.

The effect of sphingosine on Ca^{2+} release was potent (Fig. 1C); concentrations as low as 0.3 μM induced significant release; the 50% effective concentration (EC_{50}) for sphingosine was 1.6 μM. The EC_{50} for SPC was slightly higher, 2.8 μM. In vitro, the effects of sphingosine and its derivatives on PKC activity appear less potent (EC_{50} values of 80 and 120 μM for sphingosine and SPC, respectively). However, lipid dilution effects of these hydrophobic molecules in the mixed micelle assay contributed to a considerably reduced apparent sensitivity (*5, 7, 8*). In vivo, the blocking actions of sphingosine on activation of neutrophils by phorbol ester or on growth of Chinese hamster ovary cells (both effects believed mediated by PKC) are half-maximal between 1 and 2 μM (*14*). Such effects, measured under almost identical cell concentrations as we used here (5×10^5 cells per milliliter), have a sensitivity very similar to activation of Ca^{2+} release. If measurements could be performed after yet further dilutions of cells, the sensitivity

Table 1. Influence of SPH and SPC on mannose-6-phosphatase (Man-6-Pase) activity in isolated RER vesicles from DDT_1MF-2 cells. Membranes used were the B3 (RER-enriched) fraction isolated from cells (*17, 19*). Activity was assessed by colorimetrically measuring inorganic phosphate (P_i) released from glucose-6-phosphate (Glc-6-P) or mannose-6-phosphate in the intracellular medium used for Ca^{2+} transport assays (Fig. 1) containing RER vesicles (100 μg/ml), 0.8 mM glucose-6-phosphate or mannose-6-phosphate and either 50 μM sphingosine or SPC, or 0.25% Triton X-100 (TX-100). The assay proceeded for 12 min at 37°C, and liberated P_i was determined by the method of Chen *et al.* (*30*). Results are means ± SD of six determinations. The limit of sensitivity was approximately 1 nmol of P_i per milligram of membrane protein per minute.

Addition to RER vesicles	Enzyme activity (nmol/mg/min)	
	Glc-6-Pase	Man-6-Pase
None	38.3 ± 4.3	<1.0
SPC	35.0 ± 2.3	<1.0
SPH	28.3 ± 0.8	<1.0
TX-100	73.3 ± 1.8	71.6 ± 9.9
TX-100 + SPC	71.8 ± 2.2	66.7 ± 1.8
TX-100 + SPH	67.0 ± 2.9	73.3 ± 2.6

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would likely increase because of reduced membrane dilution effects.

Sphingosine or SPC ultimately released the same amount of Ca^{2+}. However, the extent of release was only approximately 75% of that mediated by the Ca^{2+} ionophore A23187. This result indicates heterogeneity of accumulated Ca^{2+}; there is a pool apparently resistant to the releasing effects of sphingosine or SPC. There appear to be two discrete pools of Ca^{2+} within cells, one sensitive to IP_3 and the other insensitive (*15–17*). In permeabilized cells, a specific guanosine triphosphate (GTP)–activated process mediates translocation of Ca^{2+} between these two pools via a mechanism independent from IP_3 (*11, 12, 15*). This mechanism probably involves an as yet unidentified monomeric G protein that may regulate Ca^{2+} transfer between separate compartments in intact cells (*16, 17*). SPC or sphingosine induce Ca^{2+} release to an extent (Fig. 2A) identical to the combined actions of IP_3 and GTP (Fig. 2C). Either of the sphingoid bases also caused a further release of Ca^{2+} when added after a supermaximal concentration of IP_3 (10 μM), resulting again in the same extent of release as the combined action of IP_3 and GTP (Fig. 2B). In spite of its lag of action, sphingosine induced exactly comparable results. After maximal release induced by a combination of IP_3 and GTP, sphingosine, or SPC were unable to effect any further release of Ca^{2+}, indicating that the pool of Ca^{2+} unresponsive to IP_3 or GTP is also unresponsive to sphingoid bases (Fig. 2C). In contrast, the Ca^{2+} ionophore A23187 caused complete release of this remaining Ca^{2+} to a value identical to that released if A23187 were added alone, indicating that the Ca^{2+} that cannot be released by SPC and sphingosine remains freely releasable, presumably residing within organelles insensitive to the actions of sphingosine or SPC, as well as GTP and IP_3 (*18*). As a corollary, this indicates that SPC and sphingosine are not acting as Ca^{2+} ionophores. Moreover, the two agents are not having a generalized effect on membrane integrity.

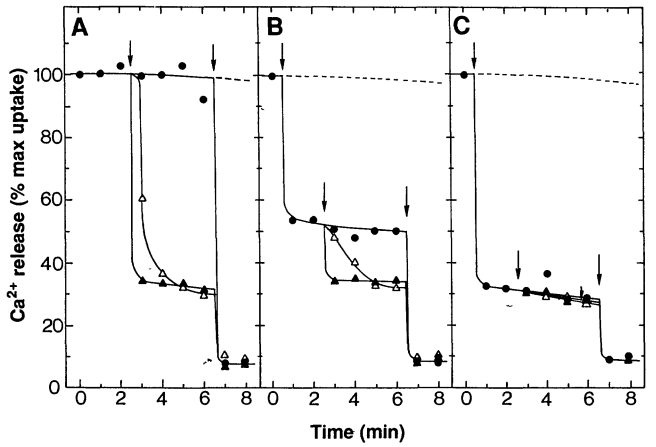

Fig. 2. Characteristics of the Ca^{2+} pool sensitive to sphingoid bases and its relationship to the IP_3- and GTP-sensitive Ca^{2+} pool. Conditions and measurements for Ca^{2+} uptake and release in permeabilized DDT_1MF-2 cells were as in Fig. 1. Ca^{2+} release occurred at the specified times after loading for 6 min. Results from a single experiment are typical of five similar experiments. (**A**) Additions of 30 μM sphingosine (△), 30 μM SPC (▲), or control buffer (●) were made at 2.5 min; all tubes received 5 μM A23187 at 6.5 min. (**B**) Same as in (A) except all tubes received 10 μM IP_3 at 0.5 min. (**C**) Same as in (A) except all tubes received 10 μM IP_3 together with 20 μM GTP at 0.5 min. The arrows denote the additions of the different substances.

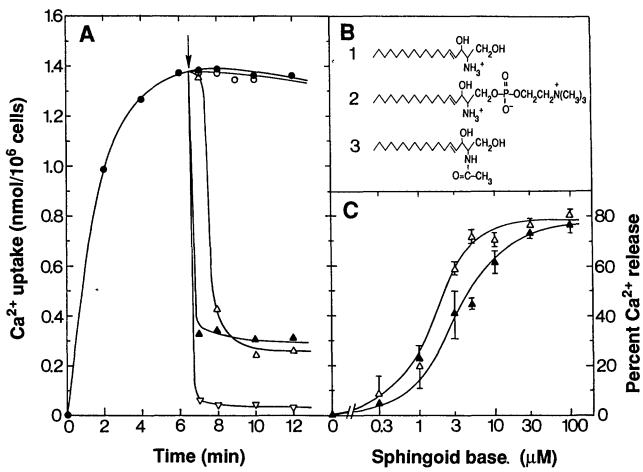

Fig. 1. Ca^{2+} release from permeabilized DDT_1MF-2 cells mediated by sphingosine and derivatives of sphingosine. Cells were cultured in monolayers, detached, and permeabilized in suspension with 0.005% saponin (*11, 12*). After thorough washing, cells were added to stirred Ca^{2+} uptake vials (final concentration, 0.5 × 10^6 cells per milliliter) containing intracellular medium (IM) comprising 140 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Hepes-KOH, pH 7.0, 1 mM ATP, 50 μM $CaCl_2$ (with 150 Ci/mol [^{45}Ca]-$CaCl_2$, buffered to 0.1 μM with EGTA), and 3% polyethylene glycol [the latter to increase cell stability and enhance the rate of GTP-induced translocation (*12*); it has no influence on sphingoid base–mediated Ca^{2+} fluxes]. Accumulation of $^{45}Ca^{2+}$ was measured at 37°C by rapid La^{3+} quench and filtration procedures. Details of these and the incubation conditions are as described (*15–17*). Sphingosine was added from a 2 mM stock in IM with 12% ethanol. (**A**) Ca^{2+} uptake was initiated by addition of cells to uptake medium at zero time, with addition (arrow) at 6.5 min of 30 μM sphingosine (△), 30 μM sphingosylphosphorylcholine (▲), 100 μM *N*-acetyl sphingosine (○), 5 μM A23187 (▽), or buffer control (●). Results from a single experiment were typical of 12 similar experiments. (**B**) Structures of molecules used in (A); 1, sphingosine; 2, SPC; and 3, *N*-acetyl sphingosine. (**C**) Sensitivity curves for the effects of sphingosine (△) and SPC (▲) on Ca^{2+} release; measurements were conducted with 0.5 × 10^6 cells per milliliter, uptake measurements being taken in triplicate from duplicate sets of vials immediately preceding addition (at 6.5 min) and 5 min after addition of sphingoid bases. Results are means ± SD of the six values. EC_{50} values for sphingosine and SPC were 1.6 and 2.8 μM, respectively.

Direct evidence that sphingosine and SPC are not nonspecifically altering the permeability of the organelle from which they effect Ca^{2+} release is provided by examination of their effects on membrane permeability in isolated membrane vesicles. We recently isolated and purified a fraction of rough endoplasmic reticulum (RER) vesicles from DDT_1MF-2 cells, greatly enriched in the IP_3- and GTP-sensitive Ca^{2+} pool (*17, 19*). Significantly, SPC and sphingosine mediate Ca^{2+} release from the RER membrane vesicles almost identically to their effects on permeabilized cells (*20*). A reliable measure of the integrity of the ER (endoplasmic reticulum) membrane is assessment of the latency of hexose-6-phosphatase activity (an intraluminal ER membrane-bound enzyme activity) to mannose-6-phosphate (*21*). The normal substrate, glucose-6-phosphate, gains entry to the ER lumen via the specific glucose-6-phosphate transporter of the ER membrane that does not permit entry of mannose-6-phosphate (*21*). Neither sphingosine or SPC at 50 μM induced any increase in the accessibility of the enzyme to mannose-6-phosphate in the RER vesicles (Table 1). By contrast, Triton X-100 induced mannose-6-phosphatase activity to a level similar to the full glucose-6-phosphatase, indicating complete exposure of the enzyme. Sphingosine or SPC did not have any direct effect on the activity of the enzyme. These results indicate that no increase in the general permeability of the ER membrane results from addition of sphingosine or SPC.

Sphingosine and SPC reversibly interact with the membrane to cause Ca^{2+} release. Thus, after treating the cells with either 60 μM sphingosine or 80 μM SPC for 2 min at 37°C (time sufficient for maximal effects to occur), followed by washing the cells in medium without SPC or sphingosine, up-

take of Ca^{2+} proceeded to an extent approaching that in cells treated identically but without inclusion of the sphingoid bases (Fig. 3, A and B). If SPC or sphingosine were not washed away, uptake proceeded only into the pool from which the sphingoid bases do not effect release. When the cells were pretreated with A23187 (Fig. 3, A and B) and then washed with ionophore-free medium, the ionophore was still completely effective. Thus, the bases, both of which are relatively soluble in aqueous solution, can dissociate from their site of action or, if they remain within the membrane, may be enzymically modified. The majority of SPC was recovered in the medium, as measured by its ability to release Ca^{2+} from fresh cells. Sphingoid base–mediated inhibition of PKC is also reversible (*8*), as is IP_3-mediated Ca^{2+} release (*19*). The results again suggest that nonspecific damage or permeabilization of the membrane is unlikely, since these effects are usually not reversible by simple washing.

The lag in the action of sphingosine suggested that the molecule might require enzymic transformation before functioning. Decreasing the temperature of Ca^{2+} release completely blocked the action of sphingosine without altering that of SPC (Fig. 3, C and D). In these experiments, uptake took place at 37°C, and thereafter cells were either cooled to 4°C (Fig. 3C) or not cooled (Fig. 3D). At the lower temperature, slow efflux of Ca^{2+} occurred because of inhibition of the Ca^{2+} pump. However, sphingosine had no effect at all at this temperature, whereas the effect of SPC was almost as rapid as at the higher temperature. This action is very similar to that of IP_3 on Ca^{2+} release, which is little affected by temperature, consistent with direct IP_3-induced Ca^{2+} channel activation (*11*). The ineffectiveness of sphingosine indicates that sphingosine has no intrinsic activity and must be enzymically converted to something that does. It is known that an enzyme, sphingosine kinase, exists in cells to phosphorylate free sphingosine at the 1-OH position with adenosine triphosphate (ATP) as substrate (*22*). To investigate whether this enzyme was functioning, we eliminated ATP (normally present at 1 mM in the uptake medium to elicit Ca^{2+} pumping) after loading by addition of hexokinase and glucose. Although passive efflux occurred at an increased rate and the effect of SPC was still as in the presence of ATP, the effect of sphingosine was completely eliminated (Fig. 3E). We also investigated whether addition of adenosine diphosphate (ADP) would block the kinase. Indeed, in the continued presence of 1 mM ATP, addition of 100 μM ADP together with 30 μM sphingosine almost completely prevented sphingosine-mediated Ca^{2+} release, whereas the action of SPC was unaffected (Fig. 3F). With sphingosine at 20 μM, the 50% inhibitory concentration (IC_{50}) for ADP was 10 μM. Thus, kinase-mediated conversion of sphingosine is likely required. Since sphingosine effects the same delayed but full effect on Ca^{2+} release from the purified and washed ER vesicles (*20* and above), the enzymic conversion process must occur at the ER membrane itself. Thus, the ER membrane appears to be the target site for both IP_3- and sphingoid base–mediated Ca^{2+} release.

Our results reveal that sphingosine derivatives can mediate Ca^{2+} release from within permeabilized cells, probably from the ER. The sensitivity, molecular specificity, and reversibility of the action of sphingoid bases on Ca^{2+} release are analogous to their effects on PKC inhibition. The one difference is the activity of sphingosine itself, which appears to have no intrinsic Ca^{2+}-releasing action. Phorbol esters do not modify the Ca^{2+}-releasing action of sphingoid bases, which suggests that the effects are independent of PKC activity. At present we know little about the mechanism of sphingoid base–mediated Ca^{2+} translocation. Ionophoric action appears unlikely. General permeability changes are unlikely because of direct measurements on permeability of the target membrane, the high sensitivity of the effect, the reversibility of action, and the specific nature of the active ligands and the target Ca^{2+} pools that are released. Sphingoid base–mediated Ca^{2+} release occurs from the same pool that is released by IP_3; indeed, the rapidity, reversibility, and temperature-independence of sphingoid-base action all closely resemble the Ca^{2+}-releasing effects of IP_3 mediated by direct channel activation (*2*). Heparin, a potent antagonist of the IP_3-activated Ca^{2+} channel (*19*), has no effect on sphingoid base–mediated Ca^{2+} release (*23*); whether sphingoid bases activate or modify this channel without interacting with the heparin-sensitive IP_3-binding site, or alter a distinct membrane channel, remains to be determined.

Bell and colleagues have suggested that sphingosine and sphingosine derivatives may have a signaling role in cells (*5*). Many cellular functions are modified by application of sphingoid bases to cells (*5, 6*). Merrill and colleagues have shown that sphingomyelinase-generated sphingosine is regulated by external effectors (*9*) and that sufficient levels of free sphingosine are generated in cells and tissues (up to 7 nmol per gram of wet tissue) to account for PKC inhibition and other effects believed mediated by

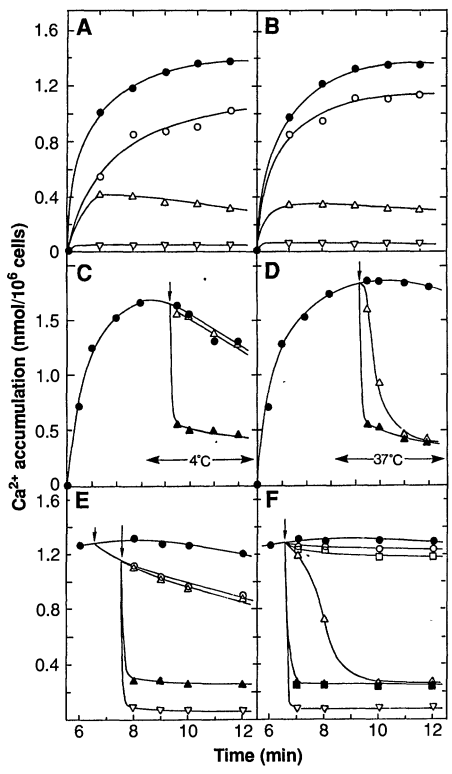

Fig. 3. Reversibility, temperature dependence, ATP requirement, and ADP-induced inhibition of sphingoid base–mediated Ca^{2+} release from permeabilized DDT_1MF-2 cells. Ca^{2+} flux measurements were undertaken as in Fig. 1; in each case, results of experiments are typical of at least three similar experiments. Reversibility of the action of (**A**) sphingosine and (**B**) SPC was assessed by treating permeabilized cells (1×10^6 cells per milliliter) with either 60 μM sphingosine or 80 μM SPC for 2 min at 37°C in IM (Fig. 1); cells were washed three times with 5 ml of sphingoid base–free medium at 4°C, then added to the Ca^{2+} uptake medium at a final concentration of 0.5×10^6 cells per milliliter (○). Alternatively, cells treated identically with sphingosine or SPC were added directly to the uptake medium without washing (△), giving final sphingosine and SPC levels of 30 and 40 μM, respectively. Control cells (●) were treated and washed, but were not exposed to sphingoid bases. Cells were also pretreated with 10 μM A23187, followed by the same three washes before addition to uptake assays (▽). (**C** and **D**) Temperature effects were assessed by loading cells for 6.5 min at 37°C and then either reducing the temperature to 4°C (C) or maintaining 37°C (D). After 2 min of temperature equilibration, 30 μM sphingosine (△), 30 μM SPC (▲), or control buffer (●) were added. (**E**) ATP requirements were assessed by addition of 5 U/ml hexokinase (first arrow) with 4 mM glucose after 6.5 min of ATP-dependent Ca^{2+} uptake, followed after 1 min (sufficient to permit hydrolysis of the 1 mM ATP) with addition (second arrow) of 30 μM sphingosine (△), 30 μM SPC (▲), 5 μM A23187 (▽), or buffer (○). Control (●) received no additions. (**F**) ADP sensitivity was determined by comparing addition of 30 μM sphingosine (△, □), 30 μM SPC (▲, ■) or control buffer (●, ○), either with (□, ■, ○) or without (△, ▲, ■) 100 μM ADP. Additions were all made after 6.5 min of loading. A23187 (5 μM)-mediated Ca^{2+} release (▽) was unaffected by ADP.

sphingoids (*9, 24*). The plasma membrane is a major site of sphingosine localization; it contains sphingomyelinase activity that could generate sphingosine from endogenous substrates (*25*), and it may be the primary site of inhibition of PKC. However, the solubility of sphingosine (>10 μM under intracellular conditions) permits its movement within the cytosol to internal membranes. We propose that at the ER membrane, sphingosine is converted most probably to sphingosine-1-phosphate and there mediates Ca^{2+} release. Analyses of sphingosine-1-kinase in platelets suggest that the enzyme is cytosolic, although with multiple forms (*22, 26*); a microsomal enzyme exists in *Tetrahymena pyriformis* (*27*), a location also suggested by our studies (*20*). Sphingosine-1-phosphate is curiously insoluble in either aqueous or nonaqueous solvents (*28, 29*); thus, if sphingosine-1-phosphate is formed in the ER membrane, it could stay trapped there. Sphingosine-1-phosphate lyase, an enzyme cleaving sphingosine-1-phosphate to palmitaldehyde and phosphoethanolamine, is a known microsomal enzyme (*28*). Thus, the ER appears to have the means to form, retain, and degrade sphingosine-1-phosphate. The regulation of either enzymic step could control Ca^{2+} permeability and hence Ca^{2+} signaling in cells.

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Mediation of Wound-Related Rous Sarcoma Virus Tumorigenesis by TGF-β

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In Rous sarcoma virus (RSV)–infected chickens, wounding leads to tumor formation with nearly 100% frequency in tissues that would otherwise remain tumor-free. Identifying molecular mediators of this phenomenon should yield important clues to the mechanisms involved in RSV tumorigenesis. Immunohistochemical staining showed that TGF-β is present locally shortly after wounding, but not in unwounded controls. In addition, subcutaneous administration of recombinant transforming growth factor–β1 (TGF-β1) could substitute completely for wounding in tumor induction. A treatment protocol of four doses of 800 nanograms of TGF-β resulted in v-*src*–expressing tumors with 100% frequency; four doses of only 10 nanograms still led to tumor formation in 80% of the animals. This effect was specific, as other growth factors with suggested roles in wound healing did not elicit the same response. Epidermal growth factor (EGF) or TGF-α had no effect, and platelet-derived growth factor (PDGF) or insulin-like growth factor–1 (IGF-1) yielded only occasional tumors after longer latency. TGF-β release during the wound-healing response may thus be a critical event that creates a conducive environment for RSV tumorigenesis and may act as a cofactor for transformation in this system.

ROUS SARCOMA VIRUS (RSV) WAS the first RNA tumor virus to be discovered (*1*). RSV rapidly transforms many cell types in culture (*2*), but its ability to induce tumors in vivo is highly dependent on the tissue environment (*3–5*). An example of this is seen in young chicken hatchlings, in which a sarcoma is rapidly formed only at the site of virus injection and at the site of experimentally induced wounds (*5, 6*). In spite of circulating infectious virus, other tissues stay generally free of tumors during the early stages of pathogenesis (*5, 6*). Thus the infliction of a wound and the subsequent healing process appear to confer a state conducive to RSV tumorigenesis. We have used this model system to identify factors that contribute to the generation of such a competent environment.

Growth factors are important in tissue reorganization during the wound-healing process (*7*) and have been implicated in the sustained growth of neoplasms. They may therefore be mediators in the creation of the competent environment. TGF-β has been shown to be one of the most potent effectors of the wound-healing reaction. It enhances

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