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18. A pulse of IP_3 on readdition of extracellular Ca^{2+} to oscillating cells was detected by high-performance liquid chromatography (HPLC) of $[^3H]IP_3$ from cells previously labeled with [^{3}H]inositol. The time course of the IP_3 pulse was similar to that seen by the IP_3 mass assay and peaked 30 s after Ca^{2+} was added back. The IP_3 concentration at that point was 1.8 times more than the preceding period in EGTA. The $[^3H]IP_3$ concentrations were determined with techniques described by S. K. Ambler, B. Thompson, P. A. Solski, J. H. Brown, and P. Taylor [*Mol. Pharmacol.* **32**, 376 (1987)].
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Glycosylphosphatidylinositol: A Candidate System for Interleukin-2 Signal Transduction

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The mechanism of interleukin-2 (IL-2) signal transduction was analyzed by use of an inducible B lymphoma. Like normal antigen-activated B lymphocytes, the lymphoma cells respond to IL-2 by proliferating and differentiating into antibody-secreting cells; both responses are blocked by a second interleukin, IL-4. Analyses of the signaling pathway showed that IL-2 stimulated the rapid hydrolysis of an inositol-containing glycolipid to yield two possible second messengers, a myristylated diacylglycerol and an inositol phosphate-glycan. The myristylated diacylglycerol response exhibited the same IL-2 dose dependence as the growth and differentiative responses, and the generation of both hydrolysis products was inhibited by IL-4. These correlations implicate the glycosyl-phosphatidylinositol system in the intracellular relay of the IL-2 signal.

THE PATHWAYS BY WHICH LYMPHOkine signals are relayed in B and T lymphocytes have yet to be determined. Analyses of IL-2– and IL-4–induced responses indicate that the classical second messenger systems are not involved (*1–3*). Thus, in cells where the lymphokine signals can be distinguished from those initiated at the antigen receptor, the binding of IL-2 and IL-4 to their respective high-affinity receptors does not stimulate calcium mobilization or increase production of phosphatidyl inositol (PI) metabolites. The lymphokine responses cannot be mimicked by ionomycin and/or phorbol ester treatment and are not affected by inhibitors of cyclic adenosine monophosphate (cAMP)– or cyclic guanosine monophosphate (cGMP)–dependent kinases. Finally, none of the lymphokine receptors has a cytoplasmic domain with the characteristic structure of a tyrosine kinase (*4*). In view of these findings, it seems likely that lymphokine signals are transduced by unidentified second messengers and/or by kinases that are associated directly with the lymphokine receptors in the membrane.

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A novel second messenger system has been implicated in insulin (*5*) and nerve growth factor (NGF) signaling (*6*). The precursor is believed to be one of a structurally related set of glycosyl-phosphatidylinositol (Gly-PI) molecules (*7*). Although the structures of the different Gly-PI forms have not been precisely defined, these molecules are known to contain a distinctive hydrophobic domain, usually 1,2-dimyristoylacylglycerol, and a PI that is glycosidically linked to a glycan moiety through glucosamine. When insulin or NGF binds to its receptor, a hormone-sensitive Gly-PI is hydrolyzed, probably through the activation of a specific phospholipase C. The result is the rapid appearance of myristylated diacylglycerol (myr-DAG) in the membrane and the release of inositol phosphate–glycan (IP-glycan) into the cytoplasm. A second messenger function for myr-DAG has yet to be established, but IP-glycan has been shown to mediate some of the effects of insulin on lipid and carbohydrate metabolism (*8*).

To explore the possibility that lymphokine signaling operates through this pathway, we used an IL-2 inducible B cell line. The BCL_1 lymphoma was derived from a cell that had received the early signals in a primary immune response (*9*). Like their normal counterparts (*10*), BCL_1 cells express functional IL-2 receptors and are capable of responding to the lymphokine (*11, 12*). Under standard culture conditions

(10% serum), IL-2 delivers a single differentiative signal (*11*); it induces the cells to transcribe the gene for the pentamer immunoglobulin M (IgM) joining component, the J chain, which is required for the assembly and secretion of IgM antibody. Under low serum conditions, however, IL-2 delivers a second signal (*1*); it induces a dose-dependent proliferation of BCL_1 cells. Previous studies (*1*) of these responses have indicated that the two signals are transduced, at least initially, by a common pathway. The evidence was based on the finding that a second lymphokine, IL-4, blocks both IL-2 responses at a step subsequent to receptor binding. Thus, by identifying early IL-2-induced changes that are blocked by IL-4, the BCL_1 system can be used to define the common steps in the IL-2 signal transduction pathway.

The proliferative response of BCL_1 cells under low serum conditions was measured by incorporation of [^{3}H]thymidine (Fig. 1A). In the absence of IL-2 there was a low amount of [^{3}H]thymidine uptake, which increased fourfold with increasing IL-2. Half-maximal stimulation was achieved with an IL-2 concentration of 10 pM (0.7 U/ml). The inhibition of the response by IL-4 was also dose dependent (Fig. 1B). The addition of 20 U/ml IL-4 decreased [^{3}H]thymidine incorporation by one-half and the addition of 100 U/ml IL-4 completely abrogated the IL-2 response.

The production of myr-DAG was first assessed with a concentration of IL-2 that gave a maximum response in the proliferation assay. BCL_1 cells were labeled with [^{3}H]myristic acid or [^{3}H]arachidonic acid and exposed to 150 pM IL-2 (10 U/ml) for various lengths of time. Analyses of the membrane fractions by thin-layer chromatography (TLC) showed that the IL-2 treatment caused a rapid increase in [^{3}H]myristate-labeled DAG (Fig. 2A). The amount of DAG generated peaked after a 2-min treatment, declined gradually over 30 min, and dropped to base line by 60 min. In contrast, IL-2 treatment did not cause any significant increase in [^{3}H]arachnidonate-labeled DAG, the hydrophobic domain commonly present in inositol phospholipids.

The effect of IL-2 concentration on myr-DAG production was determined by exposing [^{3}H]myristic acid–labeled BCL_1 cells for 2 min to increasing doses of the lymphokine. The pattern obtained (Fig. 2B) resembled the dose response curve of IL-2 induced proliferation (Fig. 1A) and displayed a half-maximal response at a concentration of 10 to 15 pM. Moreover, like the proliferative and J chain responses, the induction of myr-DAG by IL-2 was blocked by IL-4 (Fig. 2C). By itself, IL-4 (200 U/ml) generated little or no myr-DAG production. When the same dose was added simultaneously with IL-2 (750 pM), the IL-2–induced myr-DAG response was reduced by 40%, and when the IL-4 dose was added 5 min before IL-2 addition, the response was completely inhibited.

The generation of IP-glycan was assessed by analyzing aqueous extracts from BCL_1 cells that had been incubated with [^{3}H]glucosamine and exposed to IL-2 (750 pM) for 2 min. High-performance liquid chromatography (HPLC) profiles for the aqueous products showed that IL-2 stimulated the incorporation of [^{3}H]glucosamine into an anionic glycan peak that eluted at 14 min (Fig. 3A). As predicted from the myr-DAG analyses, IL-4 suppressed the IL-2–induced release of anionic glycan (Fig. 3B). In the presence of IL-4 (200 U/ml), a small peak of [^{3}H]glucosamine-labeled glycan was observed that was one-fourth the yield obtained with IL-2 stimulation alone. To obtain evidence that the [^{3}H]glucosamine label was associated with an inositol moiety, the analyses were repeated with [^{3}H]inositol-labeled BCL_1 cells. Addition of IL-2 induced a peak that eluted with the same retention time as the [^{3}H]glucosamine-labeled glycan and distinct from the retention times of other potential inositol phosphate products (Fig. 3C).

The precursor of the IL-2–induced myr-DAG and IP-glycan was characterized by incubating aliquots of BCL_1 cells overnight with the radiolabeled precursors used to ana-

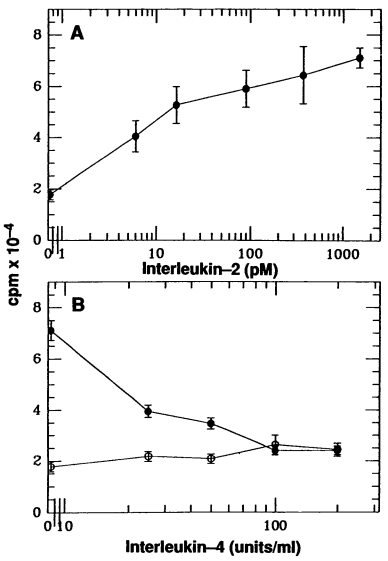

Fig. 1. Regulation of BCL_1 proliferation by IL-2 and IL-4. Triplicate samples of BCL_1 cells (2×10^4) were cultured in microtiter wells with recombinant human IL-2, recombinant mouse IL-4, or both in Roswell Park Memorial Institute 1640 medium that contained 0.5% fetal bovine serum. After 20 hours the cultures were short-term labeled with [^{3}H]thymidine (Amersham) (1 μCi, 4 hours) and harvested on a Ph.D. Cell Harvester. Uptake of thymidine was determined by scintillation counting. (**A**) Proliferation in response to IL-2 (○). (**B**) Proliferation in response to IL-2 (750 pM) and increasing concentrations of IL-4 (●), or IL-4 (200 U/ml) alone (○). The error bars represent the standard errors (SE) from triplicate determinations.

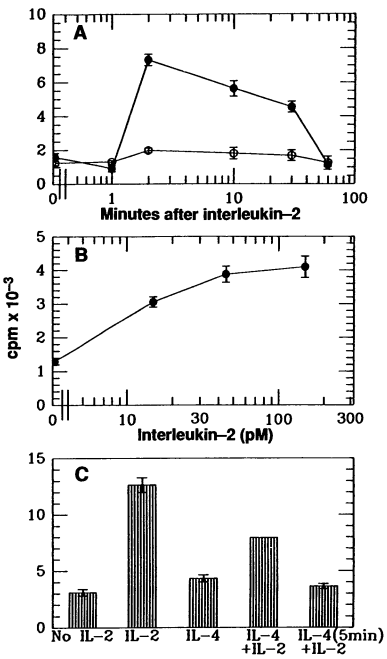

Fig. 2. IL-2 induced production of myr-DAG and its inhibition by IL-4. BCL_1 cells (5×10^5 cells/ml) were cultured overnight with [^{3}H]myristic acid (Amersham) (2 μCi) or with [^{3}H]arachnidonic acid (Amersham) (2 μCi). The cells were washed twice in Hank's balanced salt solution (BSS), and 10^6 cells were treated with lymphokine. The reaction was stopped by addition of chloroform:methanol:1 N HCl (200:100:1) (1 ml) followed by 50 mM formic acid (0.5 ml). The tubes were centrifuged for 5 min at 500 *g*, and the lower organic phase was harvested for analysis of myr-DAG by TLC as described (*5*). Control TLC plates included samples of monomyristoyl glycerol, dimyristoyl glycerol, and myristic acid that migrated 2, 6 to 7, and 10 cm, respectively, from the origin in a 10-cm plate. (**A**) Time course of myristylated (●) and arachnidonylated (○) DAG production by IL-2 (150 pM). (**B**) Myr-DAG production after 2-min treatment with increasing concentrations of IL-2. (**C**) Myr-DAG induced by treatment for 2 min with IL-2 alone, IL-4 alone, IL-2 + IL-4, or IL-4 followed 5 min later by IL-2 [IL-2, 750 pM; IL-4, 200 U/ml; 1 unit of IL-4 is the amount per milliliter that gives one-half the maximum stimulation of the HT-2 line (*21*)]. The values given are the average of duplicate TLC analyses; the bars represent the difference between individual determinations. The measurements of myr-DAG were repeated in several experiments; those of arachnidonylated DAG represent a single experiment. The counts per minute from [^{3}H]myristate that comigrated with the dimyristoyl glycerol standard after IL-2 (2 min) and no IL-2 treatment averaged 5.4% (SE= 1.8%) and 1.2% (SE= 0.5%), respectively, of the number incorporated in the organic phase (142,000; SE = 36,150). The counts per minute from [^{3}H]arachnidonate present in the same fraction after IL-2 (2 min) and no IL-2 treatment represented 0.88 and 0.53%, respectively, of the 134,800 cpm incorporated in the organic phase.

lyze the myr-DAG and IP-glycan responses, [^{3}H]glucosamine, [^{3}H]myristic acid, or [^{3}H]inositol. The lipids were then extracted and resolved by sequential acid-base TLC (7). In the acid solvent plate (Fig. 4A), the three labels appeared together in the region that contained the polar lipids, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIPP). When the material from this region was further purified by TLC in a basic solvent system (Fig. 4B), the three labels comigrated at a position slightly overlapping that of the lyso form of PI (ℓ-PI), but clearly separate from the positions of the other phospholipid standards. This comigration pattern indicated that the 6-cm lipid fraction contained glucosamine, myristic acid, and inositol, known components of Gly-PI. Moreover, the percentage of total radioactivity incorporated in each component of the 6-cm lipid fraction ([^{3}H]myristate, 4.4%; [^{3}H]glucosamine, 6.4%; [^{3}H]inositol, 2.3%) was consistent with the amount of [^{3}H]myristate incorporated in myr-DAG (5.4%) and the amounts of [^{3}H]glucosamine (4.0%) and [^{3}H]inositol (2.1%) incorporated in IP-glycan after IL-2 stimulation.

The presence of Gly-PI was confirmed by demonstrating that the glucosamine was covalently linked to PI. The 6-cm lipid fraction (Fig. 4B) from cells metabolically labeled with [^{3}H]glucosamine was treated with (i) sodium nitrite (pH 3.75), which cleaves glucosamine molecules with free amino groups by acid deamination, or (ii) PI-specific phospholipase C (PI-PLC), which hydrolyzes the phosphodiester linkage of PI. Both treatments released glucosamine into the aqueous phase (Table 1). Similar treatment of [^{3}H]inositol-labeled PI isolated from BCL_1 cells served as a control and, as expected, released only PI to the aqueous phase. These results establish that BCL_1 cells expressed a Gly-PI molecule that could serve as the source of the myr-DAG and IP-glycan generated by IL-2 treatment.

The effect of IL-2 on Gly-PI was measured directly by incubating BCL_1 cells with [^{3}H]glucosamine overnight to achieve steady-state labeling and then exposing the cells to IL-2 (150 pM) for various lengths of time. The TLC analyses of the cellular lipids at each time point showed that IL-2 induced a loss of Gly-PI of 48% within 0.5 min and 70% within 1 min after lymphokine addition (Fig. 4C). Resynthesis of Gly-PI began immediately thereafter and returned to the steady-state value by 5 min. The time course

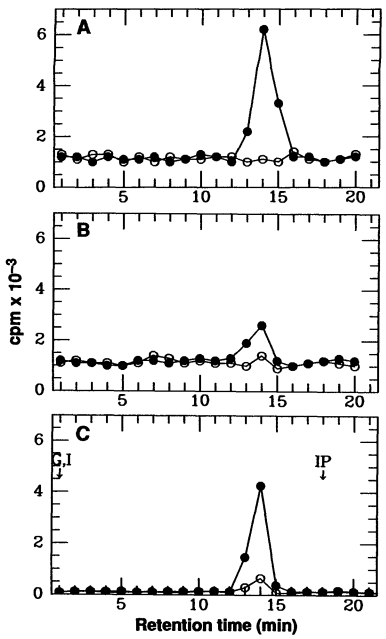

Fig. 3. IL-2 induced production of inositol glycan and its inhibition by IL-4. BCL_1 cells were incubated for 2 hours with [^{3}H]glucosamine or [^{3}H]inositol (3 μCi per 10^6 cells), washed in BSS, and treated for 2 min with the appropriate lymphokine. The reactions were stopped by extraction of cells with chloroform:methanol:HCl as described in the legend to Fig. 2. Samples (5 μl) of the 15-μl aqueous phases were chromatographed in a Sax HPLC column as described (*6*), and the tritium content of the fractions was measured by scintillation counting. (**A**) Inositol glycan production in [^{3}H]glucosamine-labeled BCL_1 cells treated with IL-2 (750 pM) (●) or media alone (○). The total radioactivity incorporated in the aqueous phases of stimulated and unstimulated cells was 483,450 and 478,190 cpm, respectively. (**B**) Inositol glycan production in [^{3}H]glucosamine-labeled BCL_1 cells treated with IL-4 (200 U/ml) followed 5 min later by IL-2 (750 pM) (●) or with IL-4 (200 U/ml) alone (○). The total radioactivity incorporated in the aqueous phases was 481,760 and 473,360 cpm, respectively. (**C**) Inositol glycan production in [^{3}H]inositol-labeled BCL_1 cells treated with IL-2 (750 pM) (●) or media alone (○). The total radioactivity incorporated in the aqueous phases from stimulated and unstimulated cells was 243,800 and 198,300 cpm, respectively. The standards included glucosamine (G), inositol (I), inositol 1-phosphate (IP), inositol 1,4-bisphosphate (IP_2), and inositol 1,4,5-trisphosphate (IP_3). The IP_2 standard eluted 23 min and IP_3 10 min after the elution buffer was increased to 1.2 M triethylamine formate. The measurements of both [^{3}H]glucosamine-labeled and [^{3}H]inositol-labeled glycan were repeated in several experiments.

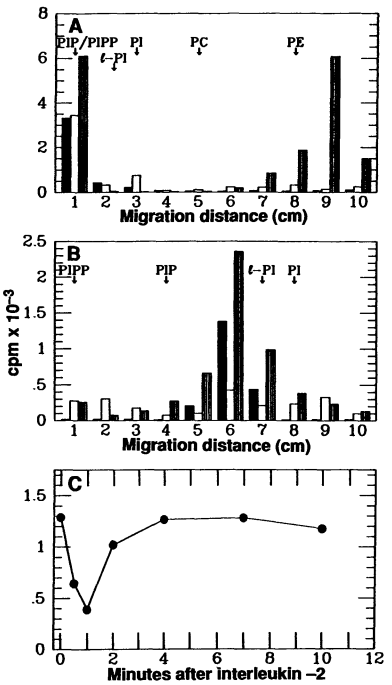

Fig. 4. Analyses of glycosyl-phosphatidylinositol by TLC. (**A**) BCL_1 cells were cultured overnight with [^{3}H]glucosamine, [^{3}H]inositol, or [^{3}H]myristic acid (3 μCi per 10^6 cells); the cellular lipids were extracted and separated by TLC in chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5) as described (7). Solid bars represent [^{3}H]glucosamine; unshaded bars, [^{3}H]inositol; shaded bars, [^{3}H]myristate (in counts per minute). The migration pattern of known phospholipids run in parallel lanes are marked; PC, phosphatidylcholine; PE, phosphatidylethanolamine. The total glucosamine, inositol, and myristic acid radioactivity incorporated in the lipid phase was 21,450, 18,420, and 53,200 cpm, respectively. (**B**) The material in the 1-cm fraction of the acid solvent plate was further purified by TLC with a chloroform:ethanol:NH_4:water (45:45:35:10) solvent system as described (7). The migration pattern of known phospholipids run in parallel lanes are marked. (**C**) Effect of IL-2 on Gly-PI turnover. BCL_1 cells were incubated overnight with [^{3}H]glucosamine (3 μCi per 10^6 cells), washed, and treated with IL-2 (150 pM) for the times indicated. The cellular lipids were extracted (7) and Gly-PI was isolated as described above. The total radioactivity incorporated in the lipid phase was 18,790 cpm. In each TLC analysis the material in 1-cm fractions was scraped off the silica and the radioactivity was determined by scintillation counting. The data given are the results from single experiments.

Table 1. Modification of glycosyl-phosphatidylinositol lipids. Lipids were purified from BCL_1 cells by sequential TLC as described in the legend to Fig. 4. Gly-PI was obtained from cells metabolically labeled with [^{3}H]glucosamine and PI from cells labeled with [^{3}H]inositol. The purified lipids were incubated either sodium nitrite or PI-PLC (Boehringer Mannheim) as described (7). Results are presented as the percentage of the initial label recovered in the aqueous phase after treatment and extraction. Values were corrected for nonspecific aqueous conversion by subtraction of values obtained from control reactions (aqueous conversion in the absence of sodium nitrite = 6%; without PLC = 5%). The limited sensitivity to acid deamination and PI-PLC hydrolysis was similar to that observed for insulin-sensitive Gly-PI (7). In these studies the integrity of the labels was verified by acid hydrolysis, so that the incomplete cleavage was considered to reflect heterogeneity in the Gly-PI population and differences in PI-PLC activities.

Treatment	Percent aqueous conversion	
	Gly-PI	PI
Sodium nitrite (pH 3.75)	35	0
PI-specific phospholipase C*	16	21

*From *Bacillus cereus*.

of Gly-PI degradation correlated with the liberation of myr-DAG, which became detectable after 1 min of IL-2 treatment and reached a maximum after 2 min (Fig. 2A).

These analyses indicate that the hydrolysis of Gly-PI represents an early event in the transduction of the IL-2 signal in B lymphocytes. B cells synthesize a Gly-PI that is sensitive to hydrolysis by PI-PLC and is rapidly degraded in response to IL-2. The products of the hydrolysis, myr-DAG and PI-glycan, are generated within minutes after IL-2 treatment, well before any proliferation or differentiation can be detected. The concentrations of IL-2 that induce these responses indicate that transduction is mediated through high-affinity receptors (*1*), and because the p75 component of the receptor is the signal transmitter (*13*), the observed biochemical response is associated with that protein. Finally, the myr-DAG and IP-glycan responses show the same patterns of IL-2 stimulation and IL-4 inhibition as the proliferative and J chain gene responses.

Although these data were obtained with a B cell model, there is suggestive evidence from studies of IL-2–IL-4 antagonisms that Gly-PI hydrolysis may be involved in IL-2 signal transduction in other lymphoid cells. For example, IL-4 has been shown to inhibit IL-2–induced proliferation of anti-CD3–stimulated T lymphocytes (*14*) and to block both the proliferative and cytotoxic activities of IL-2–stimulated lymphokine-activated killer (LAK) cells (*15*). Definitive evidence for the transducing function of the Gly-PI system will require the demonstration that the hydrolysis products function as second messengers in IL-2 signaling. The next step is to determine whether myr-DAG or IP-glycan activates enzymes identified with IL-2 responses, such as ornithine decarboxylase (*16*), or enzymes associated with other hormone responses, such as insulin-activated cAMP phosphodiesterase (*8*).

This study also indicates that the IL-2 and IL-4 signals are transduced by different pathways that intersect at an early point during the relay of their respective signals. By itself, IL-4 does not stimulate Gly-PI hydrolysis; yet it acts rapidly and efficiently to block the IL-2–induced hydrolysis. This antagonism is unlikely to be mediated by down-regulation of the IL-2 receptors. Studies in several systems have shown that IL-4 decreases the expression of IL-2 receptors (*17, 18*), but the suppression occurs relatively slowly and is only partially effective. The observed 35 to 65% decrease in receptor number over 1 to 4 hours (*18*) cannot account for the rapid inhibition of Gly-PI turnover by IL-4. It is more likely, therefore, that IL-4 exerts its antagonistic action by competing for, or preventing the function of, some component required for the activation of a Gly-PI–specific phospholipase C. One candidate for such a component has been suggested by recent reports (*19*) that a tyrosine kinase is associated with the IL-2 receptor. With these clues, it should be possible to delineate the exact pathway by which the generation of myr-DAG and IP-glycan is stimulated by IL-2 and inhibited by IL-4. This pathway may participate in the immune response, because the relative concentrations of the two lymphokines in lymphoid tissues may determine whether the antigen-activated B cell is driven by IL-2 to pentamer IgM synthesis or driven by IL-4 to switch to the synthesis of monomer IgG_1 and IgE (*20*).

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Whole Animal Cell Sorting of *Drosophila* Embryos

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Use of primary culture cells has been limited by the inability to purify most types of cells, particularly cells from early developmental stages. In whole animal cell sorting (WACS), live cells derived from animals harboring a *lacZ* transgene are purified according to their level of β-galactosidase expression with a fluorogenic β-galactosidase substrate and fluorescence-activated cell sorting. With WACS, incipient posterior compartment cells that express the *engrailed* gene were purified from early *Drosophila* embryos. Neuronal precursor cells were also purified, and they differentiated into neurons with high efficiency in culture. Because there are many *lacZ* strains, it may be possible to purify most types of *Drosophila* cells. The same approach is also applicable to other organisms for which germ-line transformation is possible.

IN MANY ORGANISMS DIFFERENT developmental pathways originate because of differential allocation of egg cytoplasm and regulatory gene products to individual cells; subsequent development of these cells and their descendants is commonly modulated by signals between cells (*1*). Characterization of the biochemical differences among developing cells and a molecular understanding of the interactions between cells might be possible if the different cells of a developing animal could be isolated for analysis and for study in vitro. This has been an important experimental approach in studies of early amphibian development (*2*) and development of the mam-