Interleukin-3-mediated Cell Survival Signals Include Phosphatidylinositol 3-Kinase-dependent Translocation of the Glucose Transporter GLUT1 to the Cell Surface*

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Maintenance of glucose uptake is a key component in the response of hematopoietic cells to survival factors. To investigate the mechanism of this response we employed the interleukin-3 (IL-3)-dependent murine mast cell line IC2.9. In these cells, hexose uptake decreased markedly upon withdrawal of IL-3, whereas its read- diation led to rapid \((t_{1/2} \sim 10 \text{ min})\) stimulation of transport, associated with an \(-4\)-fold increase in \(V_{\text{max}}\) but no change in \(K_m\). Immunocytochemistry and photoaffinity labeling revealed that IL-3 caused translocation of intracellular GLUT1 transporters to the cell surface, whereas a second transporter isoform, GLUT3, remained predominantly intracellular. The inhibitory effects of latrunculin B and jasplakinolide, and of nocodazole and colchicine, respectively, revealed a requirement for both the actin and microtubule cytoskeletons in GLUT1 translocation and transport stimulation. Both IL-3 stimulation of transport and GLUT1 translocation were also prevented by the phosphatidylinositol 3-ki- nase inhibitors wortmannin and LY294002. The time courses for activation of phosphatidylinositol 3-kinase and its downstream target, protein kinase B, by IL-3 were consistent with a role in IL-3-induced transporter translocation and enhanced glucose uptake. We con- clude that one component of the survival mechanisms elicited by IL-3 involves the subcellular redistribution of glucose transporters, thus ensuring the supply of a key metabolic substrate.

Hematopoietic stem and progenitor cells require extracellular stimuli for their survival, and in the absence of such stimuli they undergo apoptosis. By facilitating the rapid elimination of cells after they have served their function, this process plays an important role in maintaining the balance of the hematopoietic population. Recently, several oncogenes associated with human leukemias have also been shown to inhibit apoptosis, and the ability of preleukemic and leukemic cells to survive in the absence of stimuli required by normal cells is believed to be an important feature of this disease. The survival both of normal stem cells and of leukemic cells is thus regulated by the suppression of a latent cell suicide pathway, via signal transduc- tion pathways activated by cytokines and oncogenes, respec- tively (1). Although the involvement of particular signaling molecules depends on the form of stimulation (cytokine or oncogene) and the cellular background in which the pathways are being invoked, certain common components have been identified as having an important role in the resultant cell survival (1–4).

The maintenance of glucose uptake appears to play a key role in the suppression of apoptosis in hematopoietic cells (3). With- drawal of interleukin-3 (IL-3)\(^1\) from such cells results in a steady reduction in glucose uptake in the period preceding irreversible commitment to apoptosis (3, 4). We found that apoptosis induced in this fashion could be suppressed by acti- vation of the oncogene v-\(abl\) in the IL-3-dependent murine mast cell line IC.DP and that this apoptotic suppression was associ- ated with the stimulation of glucose transport (3). More- over, inhibition of glucose uptake by transport inhibitors mark- edly increased the rate of apoptosis in these cells, an effect that could be reversed by the provision of alternative energy sources such as glutamine (3). Additional evidence for a role of glucose transport regulation in the suppression of apoptosis was provided by Berridge et al. (5), who showed that induction of apoptosis by a CD95 receptor-specific antibody in human T- lymphoblastic Jurkat cells caused a decrease in transporter affinity for substrate within minutes of CD95 receptor ligation. More recently, it has been shown that constitutive GLUT1 overexpression significantly retards IL-3 withdrawal-induced apoptosis in FL5.12 cells (6). Hence maintenance of glucose transport by cytokines/oncogenes appears to be an essential feature of the survival response of hematopoietic cells, and

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\(^1\) The abbreviations used are: IL-3, interleukin-3; Bio-LC-ATB-BMPA, 4,4'-O-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoromethyl]benzoyl]amine-1,3-pro- pandiylyl-bis-mannose; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C.
IL-3-stimulated Translocation of GLUT1

Consequently, disruption of glucose transport can have profound effects on cell survival. However, the mechanisms by which acute changes in glucose transport are regulated in hematopoietic cells remain poorly understood.

IL-3 is one of the best characterized cell survival factors. Binding of this cytokine to its receptor leads to the stimulation of multiple signal transduction pathways, including the Jak/STAT pathway, the Ras/Raf/mitogen-activated protein kinase pathway, the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway, and the protein kinase C (PKC) pathway, the last probably involving the phospholipase C-mediated generation of the second messenger sn-1,2-diacylglycerol (7, 8). Activated forms of both PI 3-kinase and PKB are sufficient to prevent apoptosis induced in R–T1 fibroblasts by either serum deprivation or c-myc expression (9). Similarly, numerous reports have linked activation of these enzymes to cell survival in cytokine-dependent cells (10), although some studies have suggested that targets in addition to PI 3-kinase and PKB may be involved in suppression of apoptosis by cytokines in some hematopoietic cells (11, 12).

Alterations in glucose transport in response to cytokines or growth factors have been speculatively attributed to several signaling molecules including PKC, protein-tyrosine kinases and tyrosine phosphatases (4, 13, 14). Nevertheless, the involvement of specific signaling pathways has yet to be demonstrated directly. In the present study we investigated IL-3 stimulation of glucose transport in IL-3-dependent murine hematopoietic cells, with the aims both of establishing the mechanism of transport stimulation and of identifying the signaling pathways involved. We show that IL-3 stimulates GLUT1 translocation from an intracellular compartment to the cell surface, in a process dependent upon both the cytoskeleton and upon activation of PI 3-kinase.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant murine IL-3, LY294002, Gö 6850, 2D698059, Iatruscin B, jasplakinolide, and colchicine were purchased from Calbiochem-Novabiochem. FITC-phallolidin, nocodazole, 2-deoxy-o-glucose, and wortmannin were obtained from Sigma, and 2-deoxy-o-[3H]glucose (specific activity 7.35 Ci/mmol) was purchased from Sigma, and 2-deoxy- D-[3H]glucose (specific activity 7.35 Ci/mmol) was measured over a period of 1 min at 37 °C. Sigma, and 2-deoxy-D-[3H]glucose) was measured over a period of 1 min at 37 °C. Uptake values were therefore considered to represent a true measure of initial rates of hexose transport. Carrier-mediated uptake rates were calculated by subtracting nonspecific uptake, measured in the presence of 20 μM cytochalasin B. Transport was terminated by the addition of ice-cold KRH buffer containing 200 μM phloretin and centrifugation through a layer of mixed oils consisting of Dow Corning 550 silicone fluid (B-D) and dinonylphthalate (Fisher Scientific). The oil layer was washed three times, aspirated, and the resultant cell pellet was solubilized in 100 μl of 1 M NaOH for 1 h. Emulsifier safe liquid scintillant (Packard Bioscience BV, Groningen, The Netherlands) was then added and the radioactivity measured 16 h later. Estimation of the kinetic parameters of transport was performed by nonlinear curve fitting of the data to the Michaelis-Menten equation using the program FigP (Biosoft, Cambridge, UK). Where indicated the two-tailed unpaired Student’s t test was used to determine the statistical significance of results.

Western Blotting—Cells were depleted of cytokine and then treated with or without recombinant IL-3 as described for 2-deoxy-o-glucose transport assays. After washing briefly in phosphate-buffered saline (PBS; 154 mM NaCl, 12.5 mM sodium phosphate, pH 7.4) for the time indicated. Before subsequent transport measurements, incubation with the appropriate concentration of IL-3 and/or inhibitor was performed on Vectabond™-treated (Vector Laboratories) microscope slides using a Jouan CR312 centrifuge (50 × g, 10 min, 4 °C). Cells were then fixed in 4% (w/v) paraformaldehyde and PBS for 20 min, permeabilized in 1% (v/v) Triton X/100 for 1 h, and then incubated with either 1 μg/ml affinity-purified anti-GLUT1 or 120 μg/ml mouse anti-tubulin in 1% (v/v) serum and PBS overnight. Slides were incubated for 1 h with FITC-conjugated goat anti-rabbit/mouse IgG (Sigma) and mounted with Vectashield™ (Vector Laboratories). For visualization of actin filaments, cells were incubated with 1 μg/ml FITC-phallolidin in PBS for 1 h. For imaging, 20 optical sections were captured at 0.2-μm intervals using a DeltaVision® system (Applied Precision, Issaquah, WA), comprising an Olympus IX70 microscope linked to a CCD camera and a work station, and processed using the manufacturer’s deconvolution software. Both camera and deconvolution parameters were kept constant for each image.

Fluorescence Microscopy—Cells were depleted of cytokine and then treated with or without recombinant IL-3 as described for 2-deoxy-o-glucose transport assays. After washing briefly in phosphate-buffered saline (PBS; 154 mM NaCl, 12.5 mM sodium phosphate, pH 7.4) for the time indicated. Before subsequent transport measurements, incubation with the appropriate concentration of IL-3 and/or inhibitor was performed on Vectabond™-treated (Vector Laboratories) microscope slides using a Jouan CR312 centrifuge (50 × g, 10 min, 4 °C). Cells were then fixed in 4% (w/v) paraformaldehyde and PBS for 20 min, permeabilized in 1% (v/v) Triton X/100 for 1 h, and then incubated with either 1 μg/ml affinity-purified anti-GLUT1 or 120 μg/ml mouse anti-tubulin in 1% (v/v) serum and PBS overnight. Slides were incubated for 1 h with FITC-conjugated goat anti-rabbit/mouse IgG (Sigma) and mounted with Vectashield™ (Vector Laboratories). For visualization of actin filaments, cells were incubated with 1 μg/ml FITC-phallolidin in PBS for 1 h. For imaging, 20 optical sections were captured at 0.2-μm intervals using a DeltaVision® system (Applied Precision, Issaquah, WA), comprising an Olympus IX70 microscope linked to a CCD camera and a work station, and processed using the manufacturer’s deconvolution software. Both camera and deconvolution parameters were kept constant for each image.

Photolabeling—Cells (2 × 105) were depleted of IL-3 by washing over a period of 3 h in Fischer’s/HEPES and then incubated for 1 h in Fischer’s/HEPES in either the presence or absence of 10 ng/ml recombinant IL-3 and/or 100 μM LY294002. Photolabeling was then carried out using a modification of the method published by Koumanov et al. (17). Cells were washed in KRH buffer and then resuspended in 200 μl of KRH containing 200 μl Bio-EC-ATB-BMPA. They were then irradiated with a Rayonet RF-100 photochemical reactor (RPB-3000 lamps), washed three times in KRH and then once in TES buffer (10 mM Tris/HCl, 5 mM EDTA, 250 mM sucrose, pH 7.4). The photolabeled cells were homogenized in 1 ml of TES in a Potter homogenizer using 20 strokes, and membranes were isolated by centrifugation for 30 min at 100000 × g. Membranes (500 μg) were solubilized in 1 ml of 1% (w/v) Triton X-100 and then precipitated by continuous mixing with 40 μl of a 50% (w/v) streptavidin-agarose bead slurry (Pierce) overnight at 4 °C. The precipitates were then washed four times in 0.5% (w/v) Triton X-100, four times in 0.1% (w/v) Triton X-100, and finally twice in PBS. The washed pellets were eluted by heating at 95 °C for 20 min in electrophoresis sample buffer containing 2% (w/v) SDS. Samples were removed, denatured at 10% (w/v) SDS, and 0.5% (v/v) glycerol was added. Loading was performed with either affinity-purified anti-GLUT1 or anti-GLUT3 using an Immun-Star goat anti-rabbit IgG detection kit (Bio-Rad). The resultant luminescence was detected by autoradiography and quantified by densitometric scanning using a Fluor-S MultiImager (Bio-Rad) equipped with the Multianalyset program.
RESULTS

Effects of IL-3 on 2-Deoxy-d-glucose Transport in IC2.9 Cells—IL-3 has been reported to stimulate glucose uptake in a number of hematopoietic cell lines (3, 4, 13, 14). In the present study, we investigated the effects of the cytokine on the IL-3-dependent murine mast cell line IC2.9, using uptake of the poorly metabolized analog 2-deoxy-d-glucose as a measure of glucose transport activity. Cells were first deprived of the cytokine for 3 h and then incubated further for 1 h with a range of IL-3 concentrations. The addition of IL-3 to cytokine-deprived cells caused an increase in deoxyglucose uptake in a concentration-dependent manner with maximal stimulation (typically 2.5-fold) occurring between 3 to 10 ng/ml IL-3 (Fig. 1A).

To investigate the time courses of the responses to IL-3 withdrawal and readdition, cells were deprived of IL-3 for 3 h and then incubated for a further 3 h either in the absence or presence of 10 ng/ml IL-3. Withdrawal of IL-3 from cells previously cultured in the presence of cytokine was associated with a significant decrease in glucose transport over the initial 3-h deprivation period and a further decline in transport over the following 3 h (Fig. 1B). Readdition of IL-3 after a 3-h deprivation caused a rapid \((t_{1/2} \sim 10 \text{ min})\) increase in the rate of deoxyglucose transport. After a 1-h treatment, the rate of transport was restored to that measured in cells cultured in the continuous presence of IL-3 and was \(2\)-fold higher than that measured in cells from which IL-3 had been withdrawn for the equivalent time. The effects of IL-3 withdrawal on glucose transport therefore preceded the observed commitment to apoptosis by more than 10 h (16).

Effects of IL-3 on the Kinetics of 2-Deoxy-d-glucose Transport—To elucidate the mechanism of the \(2.5\)-fold increase in transport induced by the readdition of IL-3 to cytokine-deprived cells, we investigated the kinetic properties of transport by measuring 2-deoxy-d-glucose uptake over a range of concentrations \((0.1–10 \text{ mM})\). Fig. 2 shows a representative kinetic plot for IC2.9 cells incubated in the presence and absence of 10 ng/ml recombinant IL-3 for 1 h, after an IL-3 deprivation period of 3 h. The inset shows a Lineweaver-Burk plot of the same data for comparison. Estimates of the kinetic parameters \(K_m\) and \(V_{\text{max}}\) derived from three independent measurements showed that readdition of IL-3 to cytokine-deprived cells was associated with a significant increase (3.7-fold; \(p < 0.05\)) in \(V_{\text{max}}\) (Table I). In contrast, the apparent \(K_m\) calculated in the same experiments was not significantly altered. Thus, the reversible decrease in 2-deoxy-d-glucose uptake rates seen after IL-3 deprivation of IC2.9 cells stemmed from a decrease in the \(V_{\text{max}}\) for the transport process.

Effects of IL-3 on GLUT1 and GLUT3 Expression Levels—A possible explanation for the stimulation of transport in cytokine-deprived cells after readdition of IL-3 was that the cytokine enhanced the cellular expression of glucose transporters. Western blotting using transporter-specific antibodies had shown that IC2.9 cells expressed both the GLUT1 and GLUT3 glucose transporter isoforms but not GLUT2, GLUT4, or GLUT5 (data not shown). The total cellular levels of GLUT1 and GLUT3 were therefore measured in the cells following a 3-h period of IL-3 deprivation and subsequent incubation in the absence or presence of 10 ng/ml IL-3 for periods of up to 3 h. Cell lysates were immunoblotted using isoform-specific affinity-purified antibodies raised against GLUT1 and GLUT3 (Fig. 3). The levels of each immunodetected protein, determined by densitometric scanning, did not differ significantly between cells treated in the absence or presence of IL-3.

Effects of IL-3 on the Subcellular Distributions of GLUT1 and GLUT3—Although the total cellular content of glucose transporters was not affected by IL-3 treatment of cytokine-deprived cells, a second potential explanation for the observed changes in the \(V_{\text{max}}\) for transport was a redistribution of transporters between the cell interior and the surface. Alternatively, the turnover number of cell surface transporters might have been regulated by IL-3. To distinguish between these possibilities, the effects of IL-3 withdrawal and readdition on the subcellular distribution of GLUT transporters were investigated by immunofluorescence microscopy. Examination of cells...
Fig. 2. Kinetic analysis of 2-deoxy-D-glucose transport in IC2.9 cells after the withdrawal and readdition of IL-3. Cells were washed for 3 h at 37 °C to remove IL-3-conditioned culture medium and then incubated for 1 h either in the absence (○) or presence (●) of 10 ng/ml IL-3. Transport of 2-deoxy-D-glucose (0.1–10 mM) was measured at 37 °C for 1 min and corrected for non-carrier-mediated uptake by the addition of 20 μM cytochalasin B. Data were analyzed using nonlinear curve fitting to the Michaelis-Menten equation. Results are the mean ± S.D. of triplicate measurements. The inset shows the same data in the form of a Lineweaver-Burk plot, where v is the corrected uptake data and s is the 2-deoxy-D-glucose concentration.

TABLE I

Kinetic analysis of 2-deoxy-D-glucose transport in IC2.9 cells
Transport was measured over a range of 2-deoxy-D-glucose concentrations (0.1–10 mM) in cells treated as indicated. Uptake data were corrected for non-carrier-mediated transport measured in the presence of 20 μM cytochalasin B before fitting to the Michaelis-Menten equation. Results are the weighted mean ± S.D. calculated as described in Ref. 43 using data from n separate experiments with each measurement performed in triplicate.

| Treatment          | n  | K_m  | V_max |
|--------------------|----|------|-------|
|                    |    | max  | nmol/min/10^6 cells |
| IL-3 (10 ng/ml)    | 7  | 2.75 ± 0.25 | 5.03 ± 0.20 |
| No IL-3            | 3  | 3.15 ± 0.22  | 1.35 ± 0.06  |
| IL-3 (10 ng/ml)    | 4  | 2.28 ± 0.17  | 1.34 ± 0.05  |
| and LYS294002 (100 μM) |   |       |      |

staining for this transporter at the cell surface (Fig. 4b). Similar analysis using a GLUT3-specific antibody showed that this transporter, like GLUT1, was located mainly in intracellular structures close to the nucleus during IL-3 deprivation (Fig. 4c). However, GLUT3 localization did not alter significantly upon readdition of IL-3 (Fig. 4d). Therefore, IL-3 withdrawal was associated with a significant decrease in cell surface levels of GLUT1 but not GLUT3.

Confirmation that readdition of IL-3 resulted in altered distribution of GLUT1 but not of GLUT3 in cytokine-deprived cells and quantification of this redistribution were achieved by using the biotinylated photoaffinity ligand Bio-LC-ATB-BMPA. Upon exposure to UV light, this membrane-impermeant reagent becomes covalently linked to the exofacial substrate-binding site of members of the GLUT family of glucose transporters, including GLUT1 and GLUT3, only when these are present at the cell surface (17). Precipitation of biotinylated proteins followed by quantitative Western blotting for individual GLUT isoforms revealed that cell surface levels of GLUT1 present in IL-3-treated cells were 3-fold higher than those in IL-3-deprived cells (310% ± 77% compared with 100% ± 25%; mean ± S.E.; n = 3) (Fig. 5). In contrast, the cell surface labeling of GLUT3 did not alter significantly after IL-3 deprivation or readdition (data not shown). The specificity of GLUT labeling...
was demonstrated by the profound inhibition caused by the presence of 500 mM glucose (illustrated for GLUT1 in Fig. 5). The specificity of this method for detecting cell surface glucose transporters was confirmed by the negligible signal obtained for samples UV irradiated in the absence of Bio-LC-ATB-BMPA (Fig. 5).

Involvement of the Actin Cytoskeleton in IL-3-induced GLUT1 Translocation—IL-3-induced trafficking of GLUT1 from an intracellular, perinuclear location to the cell surface resembled the insulin-induced translocation of the homologous glucose transporter isofrom GLUT4 from the cell interior to the surface in adipocytes and skeletal muscle cells (22). In these cells, efficient GLUT4 translocation requires an intact cortical actin network (23–25). To examine the possible involvement of actin filaments in IL-3-induced translocation of GLUT1 in IC2.9 cells, we used the actin monomer-binding toxin latrunculin B to destabilize actin filaments. This agent, at concentrations of 2 μM and above, completely abolished the stimulation of 2-deoxy-d-glucose uptake by IL-3 in cytokine-depleted cells (Fig. 6A). In contrast, when used at a concentration of 2 μM, it had no significant effect on the basal rate of hexose uptake by the cells. Staining of the cells using FITC-conjugated phalloidin revealed the presence of a cortical actin layer in the IC2.9 cells which was most pronounced after treatment of the cytokine-depleted cells with IL-3 (Fig. 6D, a and c). Incubation of cells with latrunculin B at a concentration, 2 μM, which prevented stimulation of hexose uptake by IL-3, led to essentially complete loss of cortical actin staining (Fig. 6D, b and d). Similarly, treatment of cells with this concentration of latrunculin B prevented the IL-3-induced translocation of GLUT1 seen in the absence of inhibitor (Fig. 6C).

In adipocytes, jasplakinolide, a cell-permeable agent that promotes the formation and/or stabilization of actin filaments, also inhibits GLUT4 translocation and the stimulation of glucose transport by insulin (26). It has therefore been concluded that dynamic cortical actin rearrangements rather than stable cortical actin structures are required for the response to this hormone. To assess the possibility that dynamic actin structures were similarly required for the response to IL-3, we examined the effect of jasplakinolide on IC2.9 cells. Because jasplakinolide competes for the phalloidin binding site on F-actin, we were not able directly to assess its effect on the actin cytoskeleton per se. However, at concentrations of 400 nM and above, jasplakinolide drastically inhibited the stimulation of 2-deoxyglucose uptake by IL-3, with only a minor effect on the basal rate of hexose uptake (Fig. 6B). Moreover, it completely prevented the IL-3-induced translocation of GLUT1 (Fig. 6C). These data suggested that dynamic remodeling of actin is required for IL-3-induced GLUT1 translocation.

Involvement of the Microtubule Cytoskeleton in IL-3-induced GLUT1 Translocation—In contrast to the requirement for the actin cytoskeleton, microtubules are not required for insulin-stimulated appearance of GLUT4 in the plasma membrane in 3T3-L1 adipocytes, although they appear to play a part in the perinuclear location of this transporter (27, 28). To examine the potential role of microtubules in the response of IC2.9 cells to IL-3, the effects of microtubule depolymerizing agents were next examined. Nocodazole acted as a potent inhibitor, a concentration of 2 μM completely preventing the stimulation of hexose uptake by IL-3 without inhibiting the basal rate of uptake (Fig. 7A). A second agent, colchicine, similarly inhibited the effect of IL-3, almost complete inhibition being evident at a colchicine concentration of 10 μM, with only a slight inhibition of the basal rate of uptake (Fig. 7B). To investigate the origin of these effects, the organization of microtubules in IC2.9 cells was examined by staining with anti-tubulin antibodies (Fig. 7D). In the absence of inhibitors, both cytokine-depleted and IL-3-treated cells showed a network of microtubules radiating from the microtubule-organizing center to the cell periphery, where a cortical network of microtubules was also evident. Treatment of the cells with 2 μM nocodazole drastically diminished the staining of the microtubule-organizing center and radiating network and led to a restriction in area of the cortical network. Similar changes were seen in cells treated with 10 μM colchicine. Treatment of cytokine-depleted cells with nocodazole and colchicine also prevented the IL-3-induced translocation of GLUT1 to the cell surface seen in the absence of inhibitors and led to dispersion of intracellular GLUT1 from the vicinity of the microtubule-organizing center (Fig. 7C). Disruption of the Cytoskeleton Reduces Cell Survival in IC2.9 Cells—The involvement of the cytoskeleton in IL-3-mediated regulation of glucose transport, described above, is interesting in the light of previous findings that hexose transport regulation plays a part in suppression of apoptosis in hematopoietic cells (3). To ascertain whether cytoskeleton-mediated transporter translocation was involved in IL-3-mediated cell survival, the effects of cytoskeleton-disrupting agents on the number of apoptotic cells present in cultures was assayed using annexin V staining (a marker for commitment to apoptosis) and flow cytometry. The proportion of annexin V-positive cells present in cultures deprived of IL-3 for 24 h was increased from 33 ± 2% (mean of duplicate observations, ± range) to 72 ± 2% in the presence of 2 μM LB and to 86 ± 3% in the presence of 2 μM nocodazole. After 24 h the effect of these inhibitors on cells in the presence of IL-3 was only slight. However, after a 48-h culture in the presence of 2 μM nocodazole the proportion of annexin V-positive cells was almost doubled (48.4 ± 4.9%, mean of three observations ± S.E.) compared with the control population at 25.4 ± 3.3%. After a 48-h culture in the presence of 2 μM LB and IL-3, the proportion of apoptotic cells was also
FIG. 6. Involvement of the actin cytoskeleton in IL-3-induced stimulation of glucose transport and GLUT1 translocation. IL-3-depleted cells were incubated for 1 h with or without 10 ng/ml IL-3 in the presence or absence of latrunculin B or jasplakinolide at the concentrations indicated and then assessed by assay of glucose transport and by immunocytochemistry. A and B, 2-deoxy-D-glucose uptake. Results shown are the mean ± S.D. of triplicate measurements and are representative of three independent experiments. C and D, fluorescence micrographs of cells labeled with affinity-purified anti-GLUT1 and FITC-conjugated phalloidin, respectively. Each image corresponds to one representative deconvolved optical section. Scale bars, 10 μm. Results shown are representative of three independent experiments. Asterisks (*) indicate significant reduction in the extent of IL-3 stimulation of 2-deoxy-D-glucose transport (p < 0.05).
increased, to 29.9 ± 1.5%, although the magnitude of the difference from the control did not attain statistical significance at the \( p < 0.05 \) level. The data from these assays are compatible with cytoskeleton-mediated translocation of GLUT1 playing a part in the maintenance of cellular viability in the IC2.9 cell line.

**Fig. 7.** Involvement of the microtubule cytoskeleton in IL-3-induced stimulation of glucose transport and GLUT1 translocation. IL-3-depleted cells were incubated for 1 h with or without 10 ng/ml IL-3 in the presence or absence of nocodazole or colchicine at the concentrations indicated and then assessed by assay of glucose transport and by immunocytochemistry. A and B, 2-deoxy-D-glucose uptake. Results shown are the mean ± S.D. of triplicate measurements and are representative of three independent experiments. C and D, fluorescence micrographs of cells labeled with affinity-purified anti-GLUT1 and anti-tubulin antibodies, respectively. Each image corresponds to one representative deconvolved optical section. Scale bars, 10 μm. Results shown are representative of three independent experiments. Asterisks (*) indicate significant reduction in extent of IL-3-stimulation of 2-deoxy-D-glucose transport \( (p < 0.05) \).
Effects of Lipid and Protein Kinase Inhibitors on IL-3-stimulated 2-Deoxy-D-glucose Transport

Multiple signaling pathways are activated by the IL-3 receptor. To determine which pathway(s) were involved in the glucose transport response elicited by IL-3 the effects of several kinase inhibitors on the stimulation of transport by IL-3 were investigated. Inhibitors were incubated with IL-3-deprived IC2.9 cells for 15 min prior to stimulation with 10 ng/ml IL-3 for a further 1 h. No significant inhibition of either basal transport rates or IL-3 stimulation of transport was observed with the mitogen-activated protein kinase kinase inhibitor PD98059 at concentrations up to 100 \( \mu \text{M} \), well above the reported IC\(_{50} \) values of 2–7 \( \mu \text{M} \) (data not shown) (29). The bisindolylmaleimide compound Gö 6850 (GF 109203X; bisindolylmaleimide I), which inhibits both conventional and novel PKC isoforms, the latter with IC\(_{50} \) values \( \leq 250 \text{ nM} \) (30, 31), inhibited both basal and IL-3-stimulated transport in parallel up to a concentration of 20 \( \mu \text{M} \). The -fold stimulation of transport by IL-3 was therefore unaffected (data not shown). The bisindolylmaleimide Gö 6983, which inhibits conventional, novel, and atypical PKC isoforms with IC\(_{50} \) values in the range of 7–60 nM (32), was likewise without effect up to a concentration of 60 \( \mu \text{M} \) (data not shown).

In contrast to the lack of effect of mitogen-activated protein kinase kinase and PKC inhibitors, the PI 3-kinase inhibitors

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**Fig. 8. Involvement of PI 3-kinase in IL-3-induced stimulation of glucose transport and GLUT1 translocation.** IL-3-depleted cells were incubated for 1 h with or without 10 ng/ml IL-3 in the presence or absence of wortmannin or LY294002 at the concentrations indicated and then assessed by assay of glucose transport and by immunocytochemistry. A, 2-deoxy-D-glucose uptake. Results shown are the mean ± S.D. of triplicate measurements and are representative of three independent experiments. B, fluorescence micrographs of cells labeled with affinity-purified anti-GLUT1. Each image corresponds to one representative deconvolved optical section. Scale bar, 10 \( \mu \text{m} \). Results shown are representative of three independent experiments. Asterisks (*) indicate significant reduction in the extent of IL-3 stimulation of 2-deoxy-D-glucose transport (\( p < 0.05 \)).
wortmannin and LY294002 significantly inhibited the stimulation of transport by IL-3, in a concentration-dependent manner (Fig. 8A). In the case of wortmannin, ~50% inhibition was produced by an inhibitor concentration of 20 nM, and complete inhibition was achieved using concentrations ≥100 nM, whereas the basal rate of 2-deoxyglucose uptake was inhibited by only ~50%. The less potent PI 3-kinase inhibitor LY294002 decreased the stimulation of transport by IL-3 to ~50% when used at a concentration of 1 μM and completely inhibited transport stimulation at a concentration of 100 μM. To investigate the mechanism of the LY294002 effect, a detailed examination of the kinetics of 2-deoxy-D-glucose transport was made on cytokine-deprived IC2.9 cells treated for 1 h with 10 ng/ml recombinant IL-3 in the presence or absence of 100 μM LY294002. Transport parameters estimated from four independent experiments showed that the presence of 100 μM LY294002 during IL-3 stimulation resulted in a 3.7-fold decrease in V_{max} compared with cells that had been treated with IL-3 alone for the equivalent time (Table 1). In contrast, the apparent K_s for 2-deoxy-D-glucose transport, estimated from the same experiments, was not altered significantly.

**Inhibition of PI 3-Kinase Prevents GLUT1 Translocation in Response to IL-3**—To determine whether the inhibition of transport stimulation by IL-3 seen in the presence of PI 3-kinase inhibitors reflected an inhibition of cytokine-regulated transporter translocation, the subcellular distribution of GLUT1 in inhibitor-treated cells was examined next. Immunofluorescence microscopy revealed that the subcellular location of GLUT1 in cells treated with IL-3 in the presence of either 100 nM wortmannin or 100 μM LY294002 resembled that of cytokine-deprived cells, with most of the GLUT1 staining being associated with intracellular, cytoplasmic structures (Fig. 4B). Photolabeling experiments with Bio-LC-ATB-BMPA confirmed this finding. The cell surface level of GLUT1 in cells treated with IL-3 and 100 μM LY294002 was reduced drastically compared with that in cells treated with IL-3 alone (Fig. 5). In the case of GLUT3, the proportion of transporter molecules at the cell surface was low and was unaffected by LY294002 treatment (data not shown).

**IL-3 Activation of PI 3-Kinase and PKB Precedes Glucose Transport Stimulation**—The effects of wortmannin and LY294002 suggested that PI 3-kinase was involved in IL-3-stimulated regulation of glucose transport. However, to support this hypothesis it was important to demonstrate that PI 3-kinase was activated by IL-3 prior to increases in glucose transport. To this end, we compared the time courses for activation of IL-3 3-kinase, of one of its downstream effectors, PKB, and of 2-deoxy-D-glucose transport. Fig. 9 shows that both PI 3-kinase and PKB were activated by IL-3 before glucose transport stimulation occurred; PI 3-kinase was activated maximally after 2 min and PKB activity peaked after 10 min, whereas glucose transport approached maximal levels at 30 min. These data strongly supported the hypothesis that IL-3-mediated GLUT1 translocation was regulated by PI 3-kinase, with the possible downstream involvement of PKB.

**Inhibition of PI 3-Kinase Reduces Cell Survival in IC2.9 Cells**—The apparent involvement of PI 3-kinase in glucose transport regulation, described above, is interesting in the light of previous findings that both transport regulation and PI 3-kinase activity play a part in suppression of apoptosis in other hematopoietic cells (3, 10). To establish that the PI 3-kinase pathway did in fact play a role in the maintenance of viability in IC2.9 cells, the effects of LY294002 on [3H]thymidine incorporation and cell growth in a range of IL-3 concentrations were determined (Fig. 10). Addition of IL-3 to cells caused a concentration-dependent effect on [3H]thymidine incorporation and cell viability. Small increases in [3H]thymidine incorporation and viability were observed at 0.1 ng/ml IL-3 compared with cytokine-deprived cells (Fig. 10, A and B(ii)). However, the presence of 10 ng/ml IL-3 led to a substantial increase in [3H]thymidine incorporation (Fig. 10A) and cell viability (Fig. 10B(iii)). Addition of LY294002 significantly reduced the incorporation of [3H]thymidine and the viability of cells in the presence of both 0.1 and 10 ng/ml IL-3 (Fig. 10, A and B) and inhibited IL-3-stimulated activation of PKB (Fig. 10C). These observations indicated that, as reported previously for other hematopoietic cells, PI 3-kinase played a key role in the survival of IC2.9 cells. Further confirmation that PI 3-kinase is involved in suppression of apoptosis came from experiments in which treatment of cultures with LY294002 was followed by assay of the number of apoptotic cells present using annexin V staining and flow cytometry. The proportion of annexin V-positive cells present in control cultures grown in the presence of IL-3 was 10 ± 1.7% (mean of five observations, ± S.E.). The addition of 20 μM LY294002 for a period of 24 h induced a substantial inhibition of DNA synthesis (see Fig. 10), but the relative level of apoptosis remained similar to that seen for control cells, in that the proportion of annexin V-positive cells was barely elevated compared with the control population at 14.4 ± 1.5%. The addition of a greater concentration of LY294002, 100 μM, led to apoptotic cell death for the majority of the population (69.2 ± 10.8% annexin V-positive, mean of five observations, ± S.E.). The data from these assays argue that the activation of PI 3-kinase is therefore pivotal not only in stimulating glucose transport but also in the maintenance of cellular viability in the IC2.9 cell line.

**DISCUSSION**

A decrease in the rate of cellular glucose metabolism can induce apoptosis, and an early change associated with induction of apoptosis by withdrawal of growth factors such as IL-3 from hematopoietic cells is a reduction in the capacity to take up this key nutrient (3, 6). Although IL-3 withdrawal for extended periods, associated with apoptotic commitment, has recently been shown to result in down-regulation of a number of nutrient inhibitors.
transporters (33), the rapid loss of glucose transport activity upon withdrawal of IL-3 appears to be of particular importance, in that the provision of alternative substrates such as glutamine and pyruvate can suppress apoptosis even in the absence of IL-3 (3). Decreased glucose transport precedes any commitment of cells to apoptosis. Moreover, we have shown that specific inhibition of glucose uptake by competitive inhibitors such as maltose can inhibit the suppression of apoptosis caused by activation of v-abl in IL-3-deprived cells (3). To probe the mechanism of this phenomenon, in the present study we employed the IL-3-dependent cell line IC2.9. IL-3 withdrawal from these cells resulted in impairment in the ability to take up 2-deoxy-d-glucose and subsequent loss of viability. This impairment could be reversed rapidly by readdition of IL-3 to cytokine-deprived cells.

The increase in transport activity seen in cytokine-deprived cells after the IL-3 readdition stemmed from an increase in the $V_{\text{max}}$ for transport without significant change in the apparent affinity for substrate. Although no change in the total cellular content of the transporter occurred, the increase in $V_{\text{max}}$ correlated qualitatively and quantitatively with an increase in the cell surface level of GLUT1, as revealed by immunofluorescence microscopy and cell surface photoaffinity labeling, respectively. Similar effects of IL-3 in maintaining cell surface protein levels have recently been reported for transporters of other nutrients, including amino acids, iron, and low density lipoproteins, although in these experiments FL5.12 cells were deprived of IL-3 for a much longer period (24 h), and changes in the total cellular content of transporters were apparent (33). The effects of IL-3 on hexose transport and GLUT1 location in IC2.9 cells paralleled those of the Abelson protein-tyrosine kinase in IC.DP cells, as reported previously by our laboratory (3), suggesting commonalities in the mechanisms by which growth factors and oncogenes suppress apoptosis in hematopoietic cells.
The translocation of GLUT1 in IC2.9 cells in response to IL-3 paralleled that seen in insulin-responsive cells where GLUT4 and, to a lesser extent, GLUT1, are moved rapidly to the cell surface from an intracellular pool in response to insulin (34). An additional similarity was that GLUT1 translocation in IC2.9 cells, like that of GLUT4 induced by insulin in adipocytes, was dependent on a dynamic actin cytoskeleton. Insulin has recently been shown to regulate actin dynamics in adipocytes via a PI 3-kinase independent pathway that involves Cbl, the adaptor protein CrkII, the guanine nucleotide exchange factor C3G, and the Rho family GTPase TC10 (35). No information is yet available on the possible involvement of a similar pathway in the response of hematopoietic cells to IL-3. However, C3G is known to be recruited to activated IL-3 receptors and appears to play a part in the rapid activation of Rac in hematopoietic cells (36). This Rho family GTPase is a key regulator of cytoskeletal dynamics, and its expression in constitutively active form in murine IL-3-dependent BAF3 cells renders them resistant to apoptosis upon IL-3 withdrawal (37).

In adipocytes, the perinuclear location of GLUT4 glucose transporters is disrupted by microtubule-depolymerizing agents, and expression of the microtubule-binding protein hTau40, which impairs kinesin motors, delayed the appearance of GLUT4 at the plasma membrane in response to insulin (38). However, although microtubules may be involved in the long range trafficking of GLUT4 vesicles, they do not appear to be essential for insulin-stimulated glucose transport or GLUT4 translocation (28). In contrast, in the present study microtubule-depolymerizing agents were found not only to disperse the intracellular GLUT1-containing compartment from its perinuclear location in IC2.9 cells, but also to prevent the stimulation by IL-3 of hexose transport and of GLUT1 translocation to the cell surface. At the concentrations of these agents which maximally inhibited transport stimulation, there was little effect on the basal rate of transport. It is therefore unlikely that they acted directly upon the transporters, although nocodazole is known to inhibit GLUT4 when used at higher concentrations (28). Instead, it can be concluded that stimulation of glucose uptake by IL-3 is likely to require long range trafficking of GLUT1-containing vesicles assisted by the microtubule cytoskeleton.

To identify the signal transduction pathway(s) involved in the regulation of such trafficking by IL-3, we used inhibitors of several kinases which have been implicated in IL-3 signaling. The results of these investigations singularly showed that IL-3 stimulation of transport was prevented by PI 3-kinase inhibitors (wortmannin and LY294002), suggesting that this lipid kinase plays a key role in the corresponding signal transduction pathway. The effect of LY294002 was attributable to a decrease in the V_{max} for transport, to the levels seen in IL-3-deprived cells. Furthermore, the effects of both inhibitors paralleled that of cytokine deprivation in that they decreased the levels of cell surface GLUT1 in IL-3-treated cells to that seen in IL-3-deprived cells. Additional support for the involvement of PI 3-kinase in that activation of this enzyme by IL-3 preceded the stimulation of glucose transport.

Interestingly, translocation of GLUT4 and GLUT1 to the adipocyte plasma membrane also stems from stimulation of Class 1a PI 3-kinase activity by insulin (35, 39). The relevant downstream effectors of PI 3-kinase in adipocytes remain uncertain but probably include both PKB and the atypical protein kinase C isotypes PKCγ and PKCα (35, 40). We observed no effect of G6 6983 on glucose transport in IC2.9 cells, suggesting that atypical PKCs are not involved in the regulation of GLUT1 by IL-3. However, the finding that PI 3-kinase-dependent activation of PKB preceded stimulation of transport was consistent with a role for this enzyme. Importantly, it has recently been reported that overexpression of constitutively active PKB protects IL-3-dependent FL5.12 cells from growth factor withdrawal-induced apoptosis (41). In these cells, the action of PKB to prevent long term down-regulation of cell surface uptake systems for amino acids, iron, and lipoproteins in response to IL-3 withdrawal appears to involve mTOR (33). However, we found that rapamycin, a specific inhibitor of this kinase, had no effect on stimulation of hexose transport by IL-3 in IC2.9 cells (data not shown). The downstream effectors of PI 3-kinase/PKB involved in regulation of GLUT1 trafficking therefore remain unclear.

In the present study we have shown that a key response of IL-3 cells to IL-3 is the maintenance of glucose transporter levels at the cell surface: removal of the cytokine was associated with a rapid decline in glucose transport capability and subsequent induction of apoptosis. It is likely that the maintenance, in the absence of IL-3, of glucose uptake in cells harboring oncogenes such as v-abl (3) and bcr-abl (42) involves similar mechanisms and signaling components. Given the importance of this phenomenon in the suppression of apoptosis by these oncogenes, the contribution of such suppression to this process of leukemogenesis, further elucidation of the molecular mechanisms involved is now an important goal.
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