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The α subunit of the human granulocyte–macrophage colony-stimulating factor receptor signals for glucose transport via a phosphorylation-independent pathway

(signal transduction/*Xenopus laevis* oocytes/staurosporin/human neutrophils/HL-60 cells)

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ABSTRACT **The receptor for granulocyte–macrophage colony-stimulating factor (GM-CSF) is composed of an α and β subunit, which together form the high-affinity receptor. The α subunit by itself binds ligand at low affinity, whereas the isolated β subunit does not bind GM-CSF. It is generally believed that the high-affinity receptor is responsible for the multiple functions of GM-CSF and that the isolated α subunit (GMRα) does not transduce a signal. *Xenopus laevis* oocytes injected with RNA encoding human GMRα expressed up to 10^{10} low-affinity sites for GM-CSF (K_d = 6 nM). GM-CSF binding to the α subunit expressed in *Xenopus* oocytes caused activation of 2-deoxyglucose transport through endogenous glucose transporters. 2-Deoxyglucose transport was stimulated by similar low concentrations of GM-CSF in HL-60 leukemia cells as well as normal human neutrophils and *Xenopus* oocytes expressing GMRα. Engagement of the isolated α subunit in oocytes did not lead to protein phosphorylation or tyrosine phosphorylation of mitogen-activated protein kinase (MAP kinase). Staurosporin and genistein inhibited GM-CSF-induced tyrosine phosphorylation of MAP kinase in human neutrophils and HL-60 cells without affecting GM-CSF-stimulated uptake of 2-deoxyglucose. These results provide direct evidence that the isolated α subunit signals for hexose transport and can do so without engagement of the kinase cascade. Our data also indicate that signaling for hexose uptake may occur in a phosphorylation-independent manner in cells expressing the high-affinity GM-CSF receptor.**

The receptor for granulocyte–macrophage colony-stimulating factor (GM-CSF) is composed of an α and β subunit, and the α subunit (GMRα) by itself binds ligand at low affinity (K_d = 3–7 nM) (1–6). The isolated β subunit (GMRβ) does not bind GM-CSF; however, it associates with GMRα to form a high-affinity receptor (K_d = 20–40 pM) (3–6). GM-CSF receptors are present on myeloid progenitors and mature neutrophils, eosinophils, and mononuclear phagocytes (7–13). GMRα is also present in some nonhematopoietic cells, such as placenta and melanoma cells (1, 14, 15), while the GMRβ is mainly restricted to hematopoietic cells (3–5, 12). It is generally believed that the biological functions exerted by GM-CSF are mediated by the high-affinity receptor and that the isolated GMRα does not transduce a signal (3–6, 16, 17). GMRα, however, binds GM-CSF and in association with GMRβ transduces signals involving protein phosphorylation (2, 18–20). To examine the functional capability of the isolated α subunit, we expressed it in *Xenopus laevis* oocytes and discovered that it could signal for glucose transport without involving the phosphorylation of mitogen-activated protein kinase (MAP kinase).

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MATERIALS AND METHODS

Complementary DNAs for the human GMRα (21) and the human glucose transporter 4 (GLUT4) (22) cloned in pBluescript (Stratagene) were linearized by digestion with restriction enzymes, and the sense or antisense RNA was transcribed *in vitro* (23). Stage V and VI oocytes (24) were harvested from the ovaries of adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) and cultured in OR_2 medium as described (23). After 16–24 hr of culture, 20 ng of capped mRNA in 30 nl of DEPC-H_2O (23) was microinjected into the vegetal pole of the oocytes. Assays were performed after 2 days' culture in OR_2 medium.

For binding studies, oocytes (20 oocytes per group) were incubated with a fixed amount of ^{125}I-labeled human GM-CSF (DuPont, 0.9 μCi/mmol; 1 μCi = 37 kBq) and increasing concentrations of unlabeled human GM-CSF (Amgen Biologicals, rhGM-CSF from *Escherichia coli*, 0–30 nM) in 0.5 ml of OR_2 containing bovine serum albumin (BSA) at 1 mg/ml for 4 hr at 4°C. Oocytes were then washed with 5 ml of ice-cold phosphate-buffered saline (PBS) three times, and radioactivity was measured in a γ spectrometer to quantify the bound GM-CSF.

The uptake of 2-deoxyglucose by *Xenopus* oocytes was measured as previously described (23). Oocytes (30 per group) were incubated with rhGM-CSF or insulin in 1 ml of OR_2 containing BSA at 1 mg/ml at room temperature for 1 hr, followed by the addition of 2-deoxy-D-[1,2-^{3}H]glucose and 2-deoxy-D-glucose to give final concentrations of 0.1–0.5 μCi/ml and 0.2 mM, respectively (23).

Uptake of 2-deoxyglucose was also measured in HL-60 cells and human neutrophils. HL-60 cells were cultured in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and antibiotics. Leukopaks from normal donor blood were provided by the New York Blood Center and neutrophils were purified by Ficoll–Hypaque density centrifugation. Cells in suspension were incubated in a serum-free, glucose-free buffer (15 mM Hepes/135 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/0.8 mM $MgCl_2$, pH 7.4) for 3 hr, followed by incubation with rhGM-CSF for 1 hr. The uptake assay was carried out in the same way as for *Xenopus* oocytes. Freshly purified neutrophils or HL-60 cells were incubated in the

Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; GMRα, GM-CSF receptor α subunit; MAP kinase, mitogen-activated protein kinase; BSA, bovine serum albumin.

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above salt buffer containing 0.2% BSA and 30 μM genistein (Biomol) at room temperature for 30 min. Cells were then washed and incubated with GM-CSF with or without genistein for 60 min at 37°C and 2-deoxyglucose uptake was measured. HL-60 cells were incubated in IMDM containing 0.3% BSA with or without 1 μM staurosporin (Biomol, Plymouth Meeting, PA) at 37°C for 30 min before incubation with 0.3 nM GM-CSF, and uptake of 2-deoxy-D-glucose was assessed.

Analysis of protein-tyrosine phosphorylation was carried out by Western blotting using an anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology). MAP kinase (p42) was detected by anti-MAP kinase polyclonal antibody (erk1-CT, Upstate Biotechnology). *Xenopus* oocytes were incubated with 0.01–10 nM rhGM-CSF and lysed at intervals in kinase buffer (25) and immunoblotted using the ECL protocol (Amersham) (25). As a positive control (26), uninjected oocytes were treated with progesterone at 10 μg/ml in OR_2 for 18 hr. Human neutrophils were incubated in IMDM containing 0.2% BSA and 100 μM genistein for 30 min at 37°C. Cells were washed by centrifugation and incubated with GM-CSF in the presence or in the absence of 100 μM genistein at 37°C for 10 min. The cells were then washed with PBS at 4°C, lysed, and immunoblotted. HL-60 cells serum starved for 16 hr in 0.3% BSA/IMDM were incubated with or without different concentrations of staurosporin at 37°C for 30 min. GM-CSF was then added to the incubation and cells were lysed at intervals and immunoblotted.

For analysis of total protein phosphorylation, oocytes were labeled with [^{32}P]orthophosphate (NEN/DuPont) at 0.3 mCi/ml in phosphate-free OR_2 medium for 3 hr and then incubated with 0.3 nM GM-CSF from 1 to 60 min. Oocytes were lysed in kinase buffer and 30 μg of protein was separated by SDS/10% PAGE and autoradiographed. Serum-starved HL-60 cells were preincubated with phosphate-free IMDM for 2 hr and labeled with [^{32}P]orthophosphate at 1 mCi/ml for 2 hr at 37°C. The labeled cells were then incubated in IMDM with or without 1 μM staurosporin at 37°C before treatment with 0.3 nM GM-CSF. The cells were lysed at intervals and subjected to SDS-PAGE and autoradiography.

RESULTS

Human GMRα Expressed in *Xenopus* Oocytes Is a Low-Affinity GM-CSF Receptor. Oocytes injected with human GMRα RNA bound human GM-CSF at low affinity (Fig. 1).

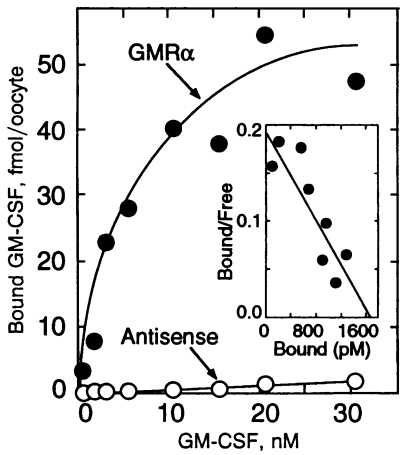

FIG. 1. Expression of the low-affinity GM-CSF receptor in *Xenopus laevis* oocytes. Oocytes were injected with *in vitro* synthesized mRNA encoding human GMRα or with antisense RNA. After 2 days, binding of ^{125}I-GM-CSF to oocytes was measured and the data were subjected to Scatchard transformation (*Inset*).

GM-CSF binding increased in a dose-dependent manner and saturated when the ligand concentration reached 30 nM. In contrast, uninjected oocytes (data not shown) or oocytes microinjected with antisense RNA (Fig. 1) did not bind GM-CSF even at high concentrations. Scatchard transformation (Fig. 1 *Inset*) showed a single binding site with a K_d of 6 nM and 10^{10} receptors per oocyte. Therefore, the α subunit was expressed in oocytes with high efficiency and bound ligand at a K_d characteristic of the low-affinity GM-CSF receptor (1). Immunoblotting with a polyclonal antibody that we developed against a bacterial fusion protein comprising the C terminus of GMRα indicated the presence of an immunoreactive band of $M_r \approx 80{,}000$ in cell membranes prepared from oocytes injected with RNA encoding GMRα but not in oocytes injected with the corresponding antisense RNA (data not shown). The apparent M_r of 80,000 is consistent with the expected size of the glycosylated form of the GMRα, as *in vitro* translation using rabbit reticulocyte lysates yielded a polypeptide of M_r 45,000 (data not shown).

GMRα Signals for Hexose Transport. GM-CSF activates glucose uptake in hematopoietic cells (27). To determine whether GMRα alone could signal for glucose transport, we measured 2-deoxyglucose uptake in oocytes expressing the human GMRα. Fig. 2 shows that insulin activated 2-deoxyglucose transport in uninjected oocytes and GM-CSF did not. This result indicates no endogenous responsiveness of oocytes to GM-CSF, an observation consistent with the lack of binding sites for GM-CSF in uninjected oocytes. The activation of glucose transport by insulin is probably mediated by the amphibian insulin growth factor 1 receptor (23, 28). GM-CSF, however, activated 2-deoxyglucose transport in oocytes microinjected with mRNA encoding the human GMRα, while no response to GM-CSF was observed in oocytes injected with antisense RNA (Fig. 2). To control for possible nonspecific effects related to expression of a foreign protein on the oocyte surface, we injected RNA encoding the glucose transporter GLUT4. In the presence of GLUT4 and absence of the GMRα, GM-CSF did not enhance glucose uptake, while insulin did (Fig. 2).

In oocytes expressing the human GMRα, GM-CSF increased glucose uptake with maximum responsiveness at 1 nM (Fig. 3*A*). A half-maximal effect was seen at 100 pM. There was no increase in 2-deoxyglucose uptake in oocytes injected with antisense RNA (Fig. 3*A*). These results indicate

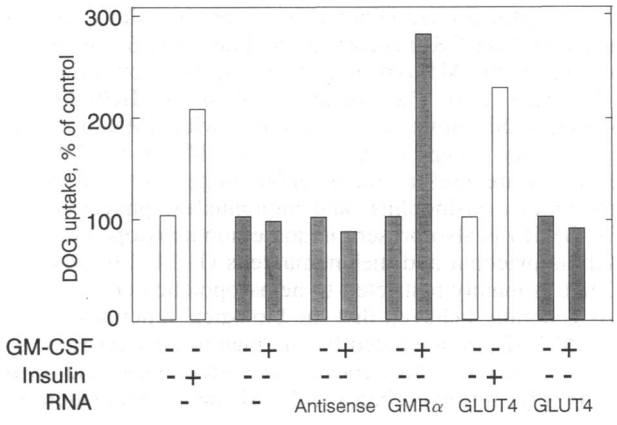

FIG. 2. Uptake of 2-deoxyglucose by *Xenopus* oocytes. Oocytes were injected with antisense RNA or with mRNA encoding the human GMRα or the hexose transport protein GLUT4. Two days after injection, oocytes were incubated for 60 min in medium in the absence or the presence of 1 μM insulin or 0.5 nM GM-CSF before uptake of 2-deoxyglucose (DOG) was measured. Uptake in insulin- or GM-CSF-treated oocytes is expressed as percentage of the basal uptake of unstimulated oocytes.

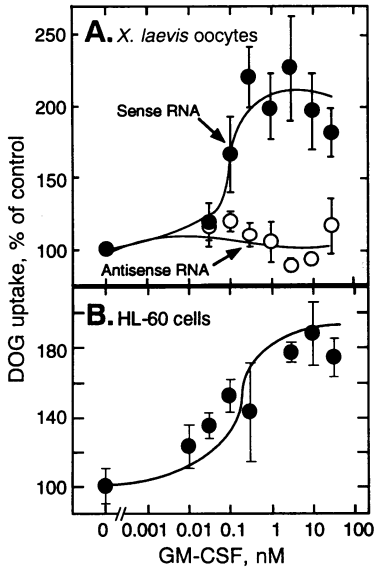

FIG. 3. (*A*) Effect of GM-CSF on the uptake of 2-deoxyglucose by oocytes expressing the GMRα. Oocytes were injected with antisense RNA or with sense RNA encoding the GMRα. After 2 days, oocytes were incubated for 60 min in the presence of increasing concentrations of GM-CSF before uptake of 2-deoxyglucose (DOG) was measured. Data represent the mean ± SEM of four independent experiments. (*B*) Effect of GM-CSF on the uptake of 2-deoxyglucose by human HL-60 cells. Cells were treated with GM-CSF as above and the uptake of 2-deoxyglucose was measured afterwards. Data represent the mean ± SD of three independent experiments.

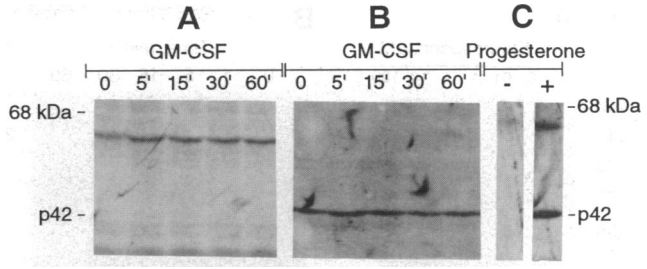

FIG. 4. Protein phosphorylation in *Xenopus* oocytes. (*A*) Effect of GM-CSF. Oocytes were injected with mRNA encoding the GMRα. After 2 days, oocytes were treated with 1 nM GM-CSF for different periods of time at room temperature, lysed, and analyzed by immunoblotting with a monoclonal antibody to phosphotyrosine. Similar results were obtained when the oocytes were treated with concentrations of GM-CSF as high as 30 nM or incubated with GM-CSF for up to 24 hr. (*B*) The position of MAP kinase in the gel (p42) was assessed by reprobing the blots with an anti-MAP kinase antibody. (*C*) Alternatively, oocytes were incubated in buffer alone (−) or in buffer containing progesterone at 10 μg/ml (+) for 18 hr before analysis by immunoblotting.

that signaling through the α subunit can occur at picomolar concentrations of ligand.

We also measured 2-deoxyglucose uptake in a human leukemia cell line, HL-60, which expresses both GMRα and GMRβ (11, 13). GM-CSF enhanced 2-deoxyglucose uptake in HL-60 cells (Fig. 3*B*), with maximal effect at 1 nM. Half-maximal stimulation of glucose transport was observed at 30–100 pM GM-CSF. A comparison of the GM-CSF dose–response curves in *Xenopus* oocytes and HL-60 cells (Fig. 3) revealed close similarity, although the oocyte expressed only the α subunit and the HL-60 cells have a high-affinity receptor composed of the $\alpha\beta$ complex. The magnitudes of the GM-CSF-mediated increase in glucose transport stimulated in both cell types were also comparable (Fig. 3). GM-CSF induced a similar increase in the capability of human neutrophils to take up 2-deoxyglucose (data not shown).

GMRα-Mediated Transport Activation Does Not Involve Protein Phosphorylation. Protein phosphorylation is an important step in GM-CSF signal transduction (18, 25, 29–31). To evaluate its role in GMRα-mediated glucose transport activation, we examined protein-tyrosine phosphorylation in oocytes expressing the human GMRα in response to GM-CSF. Western analysis showed that GM-CSF treatment of oocytes microinjected with human GMRα mRNA did not lead to significant changes in the level of tyrosine-phosphorylated proteins during short (5–60 min) (Fig. 4*A*) or long (24 hr) (data not shown) incubations. Since tyrosine phosphorylation of MAP kinase is an important intermediate step in GMR signal transduction, we specifically determined if MAP kinase was present in these blots. When the blot shown in Fig. 4*A* was probed with a rabbit anti-MAP kinase antiserum, MAP kinase (p42) was found to be present at a constant level at a position in the blot where no tyrosine phosphorylation was detected (Fig. 4*B*). This result indicates that MAP kinase was present but was not tyrosine phosphorylated by GM-CSF although the ligand bound to the isolated α subunit. Total protein phosphorylation was also measured by labeling the oocytes with [^{32}P]orthophosphate. No detectable changes in protein phosphorylation were observed in oocytes injected with GMRα RNA and treated with GM-CSF for up to 60 min. As a positive control, progesterone, which is known to induce MAP kinase phosphorylation in *Xenopus* oocytes (26), caused prominent phosphorylation of the 42-kDa protein as assessed by Western analysis (Fig. 4*C*) and [^{32}P]orthophosphate labeling (data not shown).

Protein Phosphorylation Is Not Essential for Activation of Hexose Uptake in Cells Expressing the High-Affinity GM-CSF Receptor. Treatment of human neutrophils with genistein, a specific inhibitor of protein-tyrosine kinases (32), produced a prominent decrease in the tyrosine phosphorylation of MAP kinase, with a minor effect on other phosphorylated proteins. MAP kinase was identified by reprobing the blot with anti-MAP kinase antiserum (data not shown). GM-CSF was still capable of inducing an increase in 2-deoxyglucose uptake in human neutrophils and HL-60 cells in the presence of genistein (data not shown). Low concentrations of staurosporin, a general protein kinase inhibitor, induced an overall decrease in the tyrosine phosphorylation of protein induced by treatment with GM-CSF, and it abolished tyrosine phosphorylation at 1 μM (Fig. 5*A*). Treatment of HL-60 cells with 0.3 nM GM-CSF induced a transient increase in the phosphorylation of a number of cellular proteins, including the p42 MAP kinase, that peaked at 5 min and was undetectable after 60 min (Fig. 5*B*). This effect on protein-tyrosine phosphorylation was abolished in the presence of 1 μM staurosporin (Fig. 5*C*). Similarly, [^{32}P]orthophosphate labeling experiments indicated that staurosporin abolished the transient increased phosphorylation observed in the presence of GM-CSF (data not shown). The maximal effect of GM-CSF on 2-deoxyglucose uptake was observed after 60 min of incubation (Fig. 5*D*). GM-CSF was still capable of inducing an increase in 2-deoxyglucose uptake by the HL-60 cells in the presence of staurosporin (Fig. 5*D*). These results indicate that the GM-CSF-induced protein phosphorylation is not essential for signaling for glucose uptake in cells expressing the high-affinity GM-CSF receptor.

DISCUSSION

While the isolated α subunit provides the site for ligand binding, association with the β subunit is required to form the high-affinity receptor (3–6). Previous studies suggested that the spectrum of metabolic, functional, and proliferative effects of GM-CSF were seen only in cells expressing a β

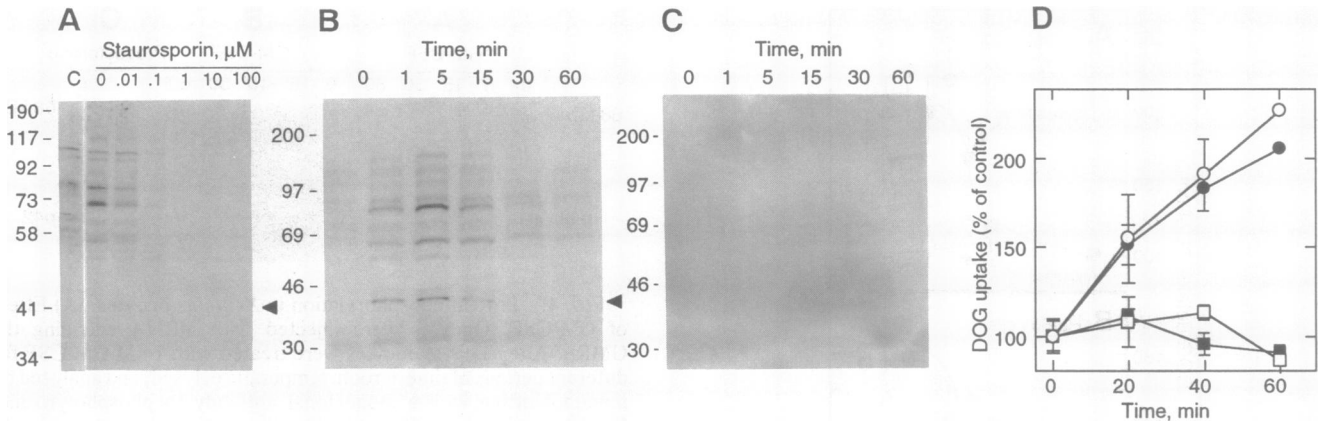

FIG. 5. (*A*) Effect of staurosporin on GM-CSF-induced tyrosine phosphorylation in HL-60 cells. Cells were treated with different concentrations of staurosporin followed by treatment with 0.3 nM GM-CSF. Cell extracts were analyzed by immunoblotting with a monoclonal antibody to phosphotyrosine. (*B*) Time course of GM-CSF-induced tyrosine phosphorylation in HL-60 cells. Cells were incubated with 0.3 nM GM-CSF for different periods of time, lysed, and analyzed by immunoblotting with a monoclonal antibody to phosphotyrosine. (*C*) Effect of staurosporin on the time course of GM-CSF-induced tyrosine phosphorylation in HL-60 cells. Cells were incubated for 30 min with 1 μM staurosporin and then processed as in *B*. Positions of molecular weight standards ($\times 10^{-3}$) and of the p42 MAP kinase (arrowheads) are indicated in *A–C*. (*D*) Effect of staurosporin on the GM-CSF-induced increased uptake of 2-deoxyglucose by HL-60 cells. Cells were treated with (●, ■) or without (○, □) 1 μM staurosporine, and the time course of GM-CSF- (●, ○) induced activation in the uptake of 2-deoxyglucose was measured afterwards. Controls (■, □) were incubated in medium lacking GM-CSF.

subunit (3–6, 16, 17, 29–31). Experiments in fibroblasts transduced with a functional α subunit have shown no response to GM-CSF as measured by protein-tyrosine phosphorylation and cell growth (16). Recent studies, however, have shown that the α subunit is required for inducing intracellular phosphorylation in concert with the β subunit (18). To determine whether the α subunit has inherent signaling capacity, we examined the function of the isolated α subunit and discovered that it can transduce a signal for activating glucose transport without engaging the protein kinase cascade.

We expressed the GMRα subunit in *Xenopus* oocytes and demonstrated ligand binding to a low-affinity receptor (K_d = 6 nM) with about 10^{10} sites per oocyte. GM-CSF treatment of oocytes expressing the α subunit resulted in activation of hexose transport as measured by 2-deoxyglucose uptake. At least with regard to the GLUT4 member of the glucose transporter family, insulin causes intracellular vesicles containing the GLUT4 transporter to come to the cell surface (22, 33). Since the insulin receptor has an intrinsic tyrosine kinase domain, it is believed that tyrosine phosphorylation is essential for signaling for glucose transporter translocation, although this is not known with certainty (34). Studies using insulin receptors lacking an intrinsic kinase activity (35–37) and anti-insulin receptor antibodies that mimic the effect of insulin without activating the autophosphorylation of the receptor (37, 38) have revealed a dissociation between autophosphorylation and activation of hexose uptake. Analysis of the primary structure of the α and β subunits of the GM-CSF receptor has failed to reveal homology to any known tyrosine or serine-threonine kinase, indicating that the GM-CSF receptor lacks an intrinsic kinase domain. We have shown that the GM-CSF treatment of oocytes expressing the α subunit alone does not result in MAP kinase phosphorylation, demonstrating the functional capability of the isolated α subunit in terms of activating hexose transport in a phosphorylation-independent fashion.

Treatment of HL-60 cells and neutrophils with GM-CSF led to activation of glucose transport. In these cells, expressing the αβ complex, several proteins were phosphorylated in response to GM-CSF stimulation (25, 29–31). Our data, however, indicate that the kinase cascade is not essential in GM-CSF signaling for glucose uptake in cells expressing a high-affinity receptor, as HL-60 cells were able to respond to GM-CSF with an increased uptake of 2-deoxyglucose in the presence of 1 μM staurosporin. No protein phosphorylation was observed under those conditions.

Low concentrations of GM-CSF were able to activate glucose transport in oocytes expressing the isolated α subunit, and similar concentrations were effective in HL-60 cells expressing the high-affinity αβ complex. Half-maximal effects in both systems were seen at concentrations of GM-CSF of about 100 pM. A possible explanation for the activity of low concentrations of GM-CSF in the oocyte system could relate to the density of receptors expressed in the oocyte. Binding studies allowed us to estimate 5×10^8 to 1×10^{10} sites per oocyte, which corresponds to about 1000 to 20,000 sites per human cell, based on an average cell-surface area calculation (24, 39). The dose–response curve for GM-CSF was, however, not related to the number of expressed receptors. Oocytes expressing about 5×10^8 low-affinity sites are similar in terms of density of receptors to HL-60 cells having a low number of high-affinity sites (several hundred per cell). The description of low-affinity sites on HL-60 cells (10) suggests there may be α subunits in excess of β subunits, and such an excess could potentially explain our observations of a maximal effect of GM-CSF in HL-60 cells at 1 nM. These findings can be interpreted as suggesting that signaling for increased glucose uptake may be transduced via the α subunit in cells expressing a high-affinity receptor for GM-CSF. Responsiveness to ligand at concentrations well below the K_d of the receptor has been documented also for other members of the family of cytokine receptors lacking an intrinsic kinase activity, and it does not appear to be related only to the GM-CSF receptor or to be specific for the receptor expressed in oocytes (17, 18, 40).

Our results indicate that GM-CSF signals for glucose transport in hematopoietic cells and that the α subunit of the GM-CSF receptor in isolation is capable of transducing that signal without activation of the protein kinase cascade. We propose the existence of multiple intracellular signaling pathways activated through engagement of the GM-CSF receptor, with specific events modulated by the α subunit alone. We have identified one signaling pathway activated by the isolated α subunit, signaling for hexose uptake. Our results, however, do not rule out the participation of the β subunit in this pathway. Others have described an essential role for the β subunit in signaling for phosphorylation in concert with the

α subunit (18), an observation that is consistent with our data indicating lack of phosphorylation mediated by the isolated α subunit. The importance of the β subunit is emphasized by the fact that it is shared by three different receptors with clear specificities for their respective ligands, GM-CSF, interleukin 3, and interleukin 5. These receptors possess overlapping functional characteristics, and more than one receptor may be expressed simultaneously in a given cell type (19, 40–48). The α subunit of each receptor may play a role in defining the specificity of the cellular response triggered by the binding of the respective ligand. Our data suggest that the α subunit not only participates as the binding component of the $\alpha\beta$ complex but also is an active element of the transport signaling machinery. Since glucose is central to cellular metabolic activity, it is possible that the glucose transport functional component relates to prolonged cell survival induced by GM-CSF delaying programmed (apoptotic) cell death (49, 50).

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