Regulation of Colony-Stimulating Factor 1–Induced Proliferation by Heterotrimeric $G_{12}$ Proteins

By Isabelle Corre and Sylvie Hermouet

Receptors for hematopoietic cytokines possess intrinsic tyrosine-kinases or are associated with tyrosine-kinases; interactions between metabolic pathways activated by tyrosine-kinases and heterotrimeric G proteins are suspected, but not yet proven. To investigate whether alteration of G protein function affects signal transduction of hematopoietic cytokines, we expressed mutant $G_{12}$ proteins in BAC 1.2F5 cells, a murine macrophage cell line that is dependent on monoocyte-macrophage colony-stimulating factor (CSF 1) for its proliferation. Mutations made in $\alpha$ subunits constitutively activate ($\alpha_{G205L}$) or inactivate ($\alpha_{G204A}$) $G_{12}$ heterotrimers. We show that expression of $\alpha_{G205L}$ in BAC 1.2F5 cells does not induce independence from CSF 1, but reduces the cells' requirement in CSF 1, shortens the length of the $G_1$ phase and the cell doubling time in response to CSF 1, and protects cells from death by apoptosis induced by CSF 1 withdrawal, exposure to H$_2$O$_2$ or heat shock, but not mitoxantrone. More importantly, expression of $\alpha_{G204A}$, a dominant negative mutant, inhibits BAC 1.2F5 cell proliferation in response to CSF 1, increases the length of the $G_1$ phase and the cell doubling time, and accelerates apoptotic cell death after withdrawal of CSF 1, exposure to H$_2$O$_2$ or heat shock. We conclude that the metabolic pathways regulated by $G_{12}$ proteins and CSF 1 tyrosine-kinase receptors converge on a common effector necessary for the regulation of macrophage survival and proliferation.

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trimer to the membrane, and also appear to modulate effector function.\textsuperscript{26,30} \(\alpha\) subunits possess an intrinsic GTPase activity and the reaction stops when GTP is hydrolyzed into GDP, allowing reassociation of \(\alpha\) and \(\beta\gamma\) subunits and formation of an inactive, heterotrimeric G protein. No activating mutation of \(\beta\) or \(\gamma\) subunits has yet been described, so we can modulate G\textsubscript{3}s activity only through its \(\alpha\) subunit. The domains involved in binding GTP are highly conserved among G proteins; several mutations in one of these domains, initially described in \(\alpha\) subunits of G proteins, constitutively activate or inactivate G\textsubscript{3}. The Q227L mutation decreases the activation of Gi2.\textsuperscript{25,35} We stably expressed aiz-Q205L and ai2-G204A mutants in BAC 1.2F5 cells and studied the growth characteristics of aiz-transfected cells in response to CSF 1.

MATERIALS AND METHODS

Cell cultures. CSF 1-dependent BAC 1.2F5 macrophages were obtained from Dr E. Richard Stanley (Albert Einstein College of Medicine, Bronx, NY).\textsuperscript{19,36} The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), or RPMI 1640, supplemented with 10% fetal bovine serum and 10% L929 cell-conditioned medium (L929 CM) as a source of murine CSF 1.\textsuperscript{37} L929 CM was prepared as follows: L929 cells were grown to confluency in DMEM (L929 CM), or RPMI 1640, supplemented with 10% serum, without CSF 1. Medium and L929 CM were replaced twice a week. Counts of viable cells (excluding Trypan blue) were determined in triplicate every 2 days after addition of CSF 1.

Protein analysis. Both G418-resistant cell pools and clones were screened by Western blotting for aiz\textsubscript{2} expression. Confluent cultures were scraped, pelleted and washed three times in 10 mL of phosphate-buffered saline (PBS), pH 7.5. Cell pellets were frozen (<70°C), thawed once, and crude membranes were prepared, as previously described.\textsuperscript{34} Protein concentration was determined by the bicinchoninic Acid (BCA) method (Pierce) with bovine serum albumin (BSA) used as a standard.\textsuperscript{34} A total of 5 \(\mu\)g of membrane proteins were resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and immunoblotted with the affinity-purified antiserum AS 7, specific for aiz.\textsuperscript{39} Detection of the antibody-antigen complex was by enhanced chemiluminescence procedure (ECL kit; Boehringer-Mannheim, Mannheim, Germany). Blots were then exposed to X-Omat Kodak films (Eastman Kodak, Rochester, NY) for 1 minute to visualize immunoreactive bands.

mRNA analysis. Cells were grown to half confluence, scraped, pelleted, washed three times with sterile PBS and kept frozen at −70°C. PolyA+ mRNAs were prepared using Micro-FastTrack mRNA Isolation Kits (Invitrogen, San Diego, CA) according to manufacturer’s instructions. After addition of glyoxal/dimethylsulfoxide, polyA+ mRNAs were separated on 1% agarose gels (3 \(\mu\)g/lane), and transferred to nylon membranes (Hybond-N; Amersham, Les Ulis, France). Membans were prehybridized for 4 hours at 42°C in 10 mL of 50 mmol/L TRIS pH 7.0 containing 10X Denhardt’s solution, 50% formamide, 1% SDS, 1 mL NaCl, and 100 \(\mu\)g/mL salmon sperm DNA. Hybridization was performed overnight at 42°C with the random-primed \(^{32}\)P-labeled aiz\textsubscript{2} cDNA probe in 10 mL of the same solution used for prehybridization. The probe used was the entire coding region of rat aiz\textsubscript{2} cDNA. After hybridization, the membranes were washed twice in 2X SSC for 15 minutes at room temperature, once in 0.5X SSC + 0.1% SDS for 30 minutes at 65°C, and once in 0.2X SSC + 0.1% SDS for 30 minutes at 65°C. Blots were exposed to X-Omat Kodak films. Sizes of mRNA were determined by comparison with the migration of a 0.25- to 9.5-kb RNA ladder standard (BRL, Life Technologies, Eragny, France).

Growth Studies in Monolayer Cultures

Doubling time studies. Cells were plated at a density of 30,000 cells per 30 mm culture dish and grown in RPMI 1640 supplemented with 10% serum and 1% or 5% of L929 CM as a source of CSF 1. Medium and L929 CM were replaced twice a week. Counts of viable cells (excluding Trypan blue) were determined in triplicate every day from day 1 to day 10. The results obtained were analyzed for exponential doubling time.

Cell survival in the absence of CSF 1. Cells were plated at a density of 10\textsuperscript{5} cells per 30 mm culture dish, grown in the presence of 10% L929 CM for 24 hours, then washed three times with warm PBS to remove any trace of CSF 1, and 2 mL of fresh RPMI 1640 supplemented with 10% serum (no CSF 1) were added. Cells were fed thereafter every other day with RPMI 1640 supplemented with 10% serum, without CSF 1. Counts of viable cells were assessed by Trypan blue exclusion in triplicate every other day from day 1 to day 12.

Colony assays in collagen. Suspensions of 1,000 cells were mixed in 1 mL of a growth medium containing 25% rat collagen (Jacques Boy, Reims, France), 25% Iscove × 2, 10% serum, and up to 40% Iscove × 1 including, or not, variable amounts of CSF 1 (purified human recombinant CSF 1 or L929 CM), as previously described.\textsuperscript{40} These semisolid cultures were carried out in triplicate in 30 mm culture dishes. Colonies were scored after a 7-day incubation. In some experiments, cells were grown in collagen in the absence of CSF 1 for up to 9 days; CSF 1 was added to the cultures in 150 mL. Iscove × 1 at indicated times. Colonies were scored 7 days after addition of CSF 1. All colony assays were repeated at least three times.

Apoptosis studies. Apoptotic cells are found in culture supernatants by virtue of their reduced adherence; only a small percentage of adherent cells show DNA fragmentation. Thus, DNA fragmentation studies can be made only on cells found in culture supernatants:
unfortunately, once detached, BAC 1.2F5 cells die very rapidly and only very small amounts of DNA, often totally degraded, can be extracted. Because of these technical difficulties, apoptosis studies were performed with colony assays, not DNA fragmentation studies, after checking that heat shock, mitoxantrone, H2O2, and CSF 1 withdrawal, indeed, induced apoptotic death in BAC 1.2F5 cells. Heat-shock studies were performed as follows: suspensions of 1,000 cells in Iscove x 1 were incubated in triplicate at 42°C for 15 minutes and plated in collagen mixture in the presence of 10% L929 CM. For the other apoptosis studies, cells were incubated for 90 minutes at 37°C in the presence of 10% of L929 CM, then washed twice to remove CSF 1 and incubated for 24 hours without CSF. Expression of α22 mutants did not induce CSF 1 independence in BAC 1.2F5 cells: none of the α22-infected clones was able to proliferate in the absence of CSF 1.

**RESULTS**

**Expression of α22 mutants in BAC 1.2F5 cells.** G418-resistant BAC 1.2F5 cells were obtained for each α22 construct. Cell morphology was slightly different for BAC 1.2F5 cells expressing α22-G204A; these cells were rounded and apparently less adherent than control cells. The level of membrane expression of α22 proteins in macrophages is high; in pools of α22-Q205L and α22-G204A-infected cells, levels of α22 expression were roughly similar to the level expressed in vector-infected cells (Fig 1A). α22 mRNA expression in infected BAC 1.2F5 cells was studied: the sizes of mRNAs detected by the α22 probe were approximately 2.4 to 2.6 kb for endogenous α22 and 5.0 to 5.5 kb, and 10 kb for transfected α22-Q205L and α22-G204A (Fig 1B). Therefore, in infected BAC 1.2F5 cells, mutant α22 subunits are expressed, but the total amount of α22 subunits is nearly unchanged.

**CSF 1-dependence.** After G418 selection, clones of α22-infected BAC 1.2F5 cells were grown in the presence of serum, but without CSF 1. Expression of α22 mutants did not induce CSF 1 independence in BAC 1.2F5 cells: none of the α22-infected clones was able to proliferate in the absence of CSF 1.

**Growth characteristics of α22-infected BAC 1.2F5 cells in response to CSF 1.** When BAC 1.2F5 cells were grown in monolayers in the presence of 5% L929 CM (Fig 2), cells expressing the activated form of α22 grew faster than vector-infected cells. To the contrary, cells expressing the dominant negative mutant of α22 grew very slowly. Doubling times were respectively: 52 hours (vector-infected cells), 46 hours (α22-Q205L-infected cells), and 65 hours (α22-G204A-infected cells). Similar results were observed in the presence of 1% or 10% L929 CM (data not shown). To explain the difference in proliferation rate and doubling time, we studied the percentage of infected BAC 1.2F5 cells in G1 phase in the presence of 5% L929 CM (Table 1). We found that the percentage of cells in G1 phase was 57.4% for control cells, 50.8% for α22-Q205L-expressing cells, and 59.6% for α22-G204A-expressing cells. The percentage of cells in S phase

![Fig 1. Expression of α22-mutants in infected BAC 1.2F5 cells.](image-url)
GTP-BINDING G? PROTEINS AND CSF 1 SIGNALING

was 30.9% for control cells and 39.4% for aiz-Q205L-expressing cells. Therefore, the duration of G1 phase was 25.4 hours for control cells, 19.1 hours for aiz-Q205L-expressing cells, and 33.1 hours for aiz-G204A expressing cells. The duration of S + G2 + M phases was also longer for aiz-G204A expressing cells (31.9) hours than for control cells and aiz-Q205L-expressing cells (26.6 hours and 26.9 hours, respectively).

Clonogenic assays were performed in the presence of variable concentrations of purified human recombinant CSF 1 (Table 2). No colony formation was observed in the absence of CSF 1, no matter which aiz mutation was expressed in BAC 1.2F5 cells. BAC 1.2F5 cells expressing aiz-Q205L formed almost twice as many colonies as control cells at low concentrations of CSF 1, confirming that aiz-Q205L expressing BAC 1.2F5 cells require less CSF 1 for their proliferation. The size of the colonies obtained with aiz-Q205L-expressing cells was also significantly larger. When high doses of CSF 1 were used, clonogenicity of aiz-Q205L cells remained the highest, but the difference with control cells narrowed. To the contrary, clonogenicity and size of colonies were reduced with all concentrations of CSF 1 for aiz-G204A cells; at high concentrations (2,000 u/mL), the number of colonies formed by aiz-G204A-expressing cells was still about half the number obtained with control cells.

Table 1. Effects of Mutated aiz Expression on the Cell Cycle of BAC 1.2F5 Cells in the Presence of 5% L929 CM

<table>
<thead>
<tr>
<th>Phase</th>
<th>Vector Alone</th>
<th>aiz-Q205L</th>
<th>aiz-G204A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time (h)</td>
<td>52</td>
<td>45</td>
<td>65</td>
</tr>
<tr>
<td>Percent of cells in phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>57.4 ± 1.6</td>
<td>50.8 ± 2.3*</td>
<td>58.6 ± 1.5*</td>
</tr>
<tr>
<td>S</td>
<td>30.1 ± 3.4</td>
<td>39.4 ± 2.4*</td>
<td>30.0 ± 1.6</td>
</tr>
<tr>
<td>G2/M</td>
<td>11.7 ± 3.5</td>
<td>9.8 ± 1.4</td>
<td>10.4 ± 1.8</td>
</tr>
<tr>
<td>Phase length (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>25.4</td>
<td>19.1</td>
<td>33.1</td>
</tr>
<tr>
<td>S + G2 + M</td>
<td>26.6</td>
<td>26.9</td>
<td>31.9</td>
</tr>
</tbody>
</table>

Clonogenicity of aiz-Q205L cells infected with vector alone or aiz mutants were plated in collagen in triplicate and incubated for 7 days with variable doses of purified human recombinant CSF 1. Colonies of more than 30 cells were scored after 7 days. Results are the mean ± SEM.

Table 2. Colony Formation of BAC 1.2F5 Cells Expressing aiz-Q205L or aiz-G204A in Response to CSF 1

<table>
<thead>
<tr>
<th>CSF 1 (u/mL)</th>
<th>Vector Alone</th>
<th>aiz-Q205L</th>
<th>aiz-G204A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment no. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>156 ± 15 (1.0)</td>
<td>291 ± 12 (1.86)</td>
<td>75 ± 13* (0.48)</td>
</tr>
<tr>
<td>500</td>
<td>250 ± 22 (1.0)</td>
<td>384 ± 31 (1.54)</td>
<td>147 ± 22 (0.59)</td>
</tr>
<tr>
<td>1,000</td>
<td>403 ± 13 (1.0)</td>
<td>533 ± 12 (1.32)</td>
<td>195 ± 21 (0.48)</td>
</tr>
<tr>
<td>Experiment no. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>116 ± 8 (1.0)</td>
<td>75 ± 3 (0.65)</td>
<td></td>
</tr>
<tr>
<td>1,000</td>
<td>243 ± 5 (1.0)</td>
<td>161 ± 7 (0.66)</td>
<td></td>
</tr>
<tr>
<td>2,000</td>
<td>243 ± 7 (1.0)</td>
<td>142 ± 5 (0.58)</td>
<td></td>
</tr>
</tbody>
</table>

BAC 1.2F5 cells infected with vector alone or aiz mutants were plated in collagen in triplicate and incubated for 7 days with variable doses of purified human recombinant CSF 1. Colonies of more than 30 cells were scored after 7 days. Results are the mean ± SEM.

* Clusters of less than 20 cells; values in parentheses indicate fold increase over control (vector alone) in clonogenicity.

1.2F5 cells. BAC 1.2F5 cells expressing aiz-Q205L formed almost twice as many colonies as control cells at low concentrations of CSF 1, confirming that aiz-Q205L expressing BAC 1.2F5 cells require less CSF 1 for their proliferation. The size of the colonies obtained with aiz-Q205L-expressing cells was also significantly larger. When high doses of CSF 1 were used, clonogenicity of aiz-Q205L cells remained the highest, but the difference with control cells narrowed. To the contrary, clonogenicity and size of colonies were reduced with all concentrations of CSF 1 for aiz-G204A cells; at high concentrations (2,000 u/mL), the number of colonies formed by aiz-G204A-expressing cells was still about half the number obtained with control cells.

BAC 1.2F5 cell survival in the absence of CSF 1. After infection and G418 selection, several clones expressing aiz-Q205L and aiz-G204A were maintained without CSF 1. Five clones infected with vector alone and 10 clones expressing aiz-Q205L were tested (Table 3, part A). None of the clones infected with vector alone remained viable after day 5. All clones expressing aiz-Q205L remained viable until day 10; eight of these clones survived until day 35, and three until day 55. These cells survived without dividing. Clonogenic assays were performed on cell pools with delayed addition of purified CSF 1 (Table 3, part B). In these assays, cells were maintained for 3, 6, or 10 days without CSF 1, then 500 u/mL of CSF 1 was added, and colonies were scored for 7 days after CSF 1 addition. After 3 days of CSF 1 withdrawal, clonogenicity was reduced by 40% for vector-infected cells, and only 12% for aiz-Q205L-expressing cells, and by 53% for cells expressing aiz-G204A. Six days after CSF 1 withdrawal, aiz-Q205L cells' clonogenicity (14.6%) was almost five times the clonogenicity of control cells (3.0%); clonogenicity of aiz-G204A cells was only 1.6%. In other experiments, cells were grown in monolayers to semiconfluency in the presence of CSF 1, then washed and maintained with RPMI and serum only. A typical experiment is shown on Fig. 3. BAC 1.2F5 cells infected with vector alone or aiz-G204A died rapidly: 70% of cell viability was lost at day
2. To the contrary, most BAC 1.2F5 cells expressing α2-Q205L remained viable for more than 7 days; 50% of cell viability was lost only after day 7.

Protection from apoptosis. BAC 1.2F5 cells, like other growth factor-dependent cells, die by apoptosis when their growth factor is withdrawn (Fig 4). Mutated α2 subunits have opposite effects on BAC 1.2F5 cell survival in the absence of CSF 1, i.e., on apoptosis induced by withdrawal of CSF 1. We investigated if expression of mutant α2 proteins could also interfere with apoptosis induced by other methods. Response to apoptotic agents was studied using clonogenic assays of BAC 1.2F5 cells pretreated with H2O2, mitoxantrone, or submitted to heat-shock (see Materials and Methods). Results are shown in Table 4. Cells expressing α2-Q205L resisted to apoptosis induced in G1 phase (heat-shock or H2O2 treatment) better than control cells; loss of viability and clonogenicity in these conditions was more important in cells expressing α2-G204A than in control cells. However, expression of α2-Q205L did not protect BAC 1.2F5 cells from apoptosis after exposure to mitoxantrone, a drug-inducing cell death in S phase or after, while α2-G204A–expressing cells appeared protected.

DISCUSSION

In this study, we show for the first time that heterotrimeric G proteins modulate signal transduction of hemopoietic cytokines: Gαi protein can up or downregulate proliferation and survival signals of CSF 1, a cytokine with tyrosine-kinase receptors. Expression of Gαi proteins is upregulated during differentiation of myeloid cells into macrophages. Thus, macrophages have very high levels of Gαi proteins, making overexpression of Gαi mutants difficult in these cells: in transfected BAC 1.2F5 cells, mutant Gαi expression represented probably no more than 50% of all Gαi proteins. Nonetheless, as observed in other cells, this level of expression was sufficient to significantly alter Gαi’s function in cell growth regulation. The α2-G204A mutant, once coupled to βγ subunits liberated by endogenous α2, does not dissociate. In stable transfections, this means that endogenous Gαi is progressively and constantly replaced by inactive G204A-Gαi; replacement of 40% or 50% of endogenous Gαi by G204A-Gαi is sufficient to inhibit significantly Gαi’s metabolic pathways, i.e., to reproduce PT effects on macrophage proliferation.

CSF 1 stimulates cell proliferation by making cells go into G1 phase, then stimulate G1/S transition and G1 exit; CSF 1 also regulates the duration of S phase. When they express a constitutively activated form of Gαi, BAC 1.2F5 cells remain strictly dependent from CSF 1 for their survival and proliferation, but the length of G1 phase is reduced and the cells become responsive to very low concentrations of CSF 1. To the opposite, inhibition of Gαi function in BAC 1.2F5 cells results in partial loss of adherence, reduced proliferation potential, lengthened G1 and S phases, and doubling time, which cannot be overcome by high concentrations of CSF 1. Besides cell proliferation, hemopoietic cytokines promote cell survival, or protection from apoptosis. BAC 1.2F5 cells expressing α2-Q205L survive longer in the absence of CSF 1, and this ability to delay programmed cell death protects them from apoptosis induced by other methods, such as heat shock or H2O2. Apoptosis

Table 3. BAC 1.2F5 Cells Survival and Clonogenicity in the Absence of CSF 1

<table>
<thead>
<tr>
<th>Part A</th>
<th>No. of Days Without CSF 1</th>
<th>No. of Viable Clones</th>
<th>Part B</th>
<th>Colony Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector Alone α2-Q205L</td>
<td></td>
<td></td>
<td>Vector Alone α2-Q205L α2-G204A</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>3</td>
<td></td>
<td>20.2 ± 4.3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>3</td>
<td></td>
<td>19.2 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>2</td>
<td></td>
<td>18.3 ± 0.5</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>1</td>
<td></td>
<td>17.4 ± 0.0</td>
</tr>
<tr>
<td>55</td>
<td>0</td>
<td>1</td>
<td></td>
<td>16.5 ± 0.9</td>
</tr>
</tbody>
</table>

Part A: Immediately after G418-selection, α2-Q205L expression was verified by Western and Northern blotting and BAC 1.2F5 clones were maintained without CSF 1 in 100-mm Petri culture dishes with medium replaced twice a week. Part B: 1,000 BAC 1.2F5 cells expressing mutated α2 were plated in collagen in triplicate without CSF 1. A total of 500 μl of purified human recombinant CSF 1 was added immediately (0), or 3, 5, or 10 days later. Colonies of more than 30 cells were scored 7 days after addition of CSF 1. Results are the mean ± SEM.

Fig 3. Viability of BAC 1.2F5 cells expressing α2 mutants in the absence of CSF 1. BAC 1.2F5 cells were infected with vector alone (●), α2-Q205L (■) or α2-G204A (▲). A total of 10⁶ cells were maintained in the absence of CSF 1 in 30-mm Petri dishes. Viable cells (excluding Trypan blue) were counted in triplicate every day; standard deviation was less than 10%. For BAC 1.2F5 cells expressing α2-Q205L, at least 20% of cells remained viable until day 10. Two other experiments gave comparable results.

DISCUSSION

In this study, we show for the first time that heterotrimeric G proteins modulate signal transduction of hemopoietic cytokines: Gαi proteins can up or downregulate proliferation and survival signals of CSF 1, a cytokine with tyrosine-kinase receptors. Expression of Gαi proteins is upregulated during differentiation of myeloid cells into macrophages. Thus, macrophages have very high levels of Gαi proteins, making overexpression of Gαi mutants difficult in these cells: in transfected BAC 1.2F5 cells, mutant Gαi expression represented probably no more than 50% of all Gαi proteins. Nonetheless, as observed in other cells, this level of expression was sufficient to significantly alter Gαi’s function in cell growth regulation. The α2-G204A mutant, once coupled to βγ subunits liberated by endogenous α2, does not dissociate. In stable transfections, this means that endogenous Gαi is progressively and constantly replaced by inactive G204A-Gαi; replacement of 40% or 50% of endogenous Gαi by G204A-Gαi is sufficient to inhibit significantly Gαi’s metabolic pathways, i.e., to reproduce PT effects on macrophage proliferation.

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induced by growth factor deprivation, heat shock or H₂O₂, occurs after arrest of the cells in G1 phase.⁴² Apparently, in α₂Q205L-expressing BAC 1.2F5 cells, G1 arrest does occur, because cells survive without dividing in the absence of CSF 1, and eventually die if deprived of CSF 1 for more than 7 days. However, activation of G₁₂ does not protect cells from apoptosis induced by mitoxantrone, a drug that induces cell death in S phase or after.⁴² Expression of α₂Q205L probably makes cells more sensitive to mitoxantrone because of the short G₁ phase. On the contrary, cells expressing α₂G204A have less ability to resist to apoptosis induced in G₁ phase (by CSF 1 deprivation, heat shock, or H₂O₂) and their long G₁ phase probably explains their resistance to a short exposure to mitoxantrone. We suggest that G₁₂ exerts its protective effect on G₁ phase-induced apoptosis by regulating G₁ arrest: expression of α₂Q205L seems to delay G₁ arrest, while expression of α₂G204A seems to accelerate G₁ arrest. However, once cell arrest in G₁ or G2/M phase occurred, rapid activation of apoptosis is still possible in all α₂Q205L-infected BAC 1.2F5 cells.

Koyasu et al⁶ showed that PT inhibition of IL-3-induced proliferation was associated with increased G₁ phase and cell doubling time. Miller et al,¹³ described PT inhibition of Epo signal transduction and suggested that G₁₂ or G₁₃ might be involved; Kesselring et al¹⁴ recently reported selective changes in G₁₂ and G₁₃ expression associated with erythroid differentiation. Our data provide the proof that G₁₂ is the G protein responsible for the effects of PT on CSF 1-stimulated cell proliferation, and strongly suggest that G₁₂ is also the G protein responsible for PT effects on signal transduction of other hematopoietic cytokines. More importantly, we show that inhibition of G₁₂ function inhibits CSF 1-signal transduction, even at the highest CSF 1 concentrations: therefore, it is likely that G₁₂ regulates an effector that is also activated by the CSF 1 receptor and essential for its signal transduction. Again, because PT inhibits signal transduction of IL-3, IL-4, GM-CSF, and Epo receptors,⁵,¹³ it is probable that G₁₂'s effector plays an important role in the signal transduction of these hematopoietic cytokines.

The effector regulated in BAC 1.2F5 cells by G₁₂ and CSF 1 receptors, remains to be identified. Like most hematopoietic cytokine receptors, signal transduction of CSF 1 receptors includes p21ras, raf kinase, mitogen activated protein (MAP)-kinase, and at least one phosphotyrosine phosphatase.⁸⁻¹⁵ From studies concerning G₁₂'s role in the regulation of fibroblast proliferation, we know that PT inhibits, and that βγ subunits stimulate, MAP-kinase activity; hence, G₁₂'s action on cell growth could result from modulation of MAP-kinase activity. In fibroblasts, p21ras GTP-loading is inhibited by PT,⁸ indicating that G₁₂'s effector acts upstream of p21ras, and not on MAP-kinase itself. Tyrosine phosphoryla-

Table 4. Response of BAC 1.2F5 Cells Expressing α₂Q205L or α₂G204A to Apoptosis-Inducing Treatments

<table>
<thead>
<tr>
<th>No. of Colonies/1,000 Cells</th>
<th>Vector Alone</th>
<th>α₂Q205L</th>
<th>α₂G204A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment no. 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C, 15 min (control)</td>
<td>190 ± 10</td>
<td>325 ± 39</td>
<td>102 ± 14</td>
</tr>
<tr>
<td>42°C, 15 min (heat shock)</td>
<td>66 ± 14</td>
<td>212 ± 5</td>
<td>25 ± 16</td>
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<tr>
<td><strong>Experiment no. 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47 ± 10</td>
<td>94 ± 22</td>
<td>51 ± 1*</td>
</tr>
<tr>
<td>H₂O₂ 0.50 mmol/L</td>
<td>5 ± 4</td>
<td>48 ± 4</td>
<td>3 ± 2*</td>
</tr>
<tr>
<td>0.25 mmol/L</td>
<td>22 ± 1</td>
<td>96 ± 11</td>
<td>11 ± 5*</td>
</tr>
<tr>
<td>Mitoxantrone 10⁻⁴ mmol/L, 90 min</td>
<td>27 ± 5</td>
<td>29 ± 9</td>
<td>54 ± 15*</td>
</tr>
<tr>
<td>Mitoxantrone 10⁻⁵ mmol/L, 90 min</td>
<td>27 ± 5</td>
<td>54 ± 6</td>
<td>63 ± 4*</td>
</tr>
</tbody>
</table>

BAC 1.2F5 cells infected with vector alone or mutated α₂ were exposed to heat shock for 15 minutes or mitoxantrone for 90 minutes at 37°C, then washed once, and plated in triplicate in collagen. For H₂O₂ treatment, H₂O₂ was included in the collagen mixture. Cells were incubated for 7 days in the presence of 10% L929 CM.

* Only colonies of more than 30 cells were scored, except for α₂G204A cells, which sometimes formed only clusters of less than 30 cells. Results are the mean ± SEM.
tation, or dephosphorylation, also occurs after stimulation of receptors coupled to PT-sensitive G proteins; in fibroblasts and pancreatic cells, phosphatase activity appears to be part of PT-sensitive metabolic pathways. These observations suggest that candidate effectors common to CSF 1 and CSF 1 receptors (and other cytokine receptors?) may be tyrosine-kinases or phosphotyrosine-phosphatases acting upstream of p21ras.

Another important point to elucidate is how G_{i2}'s metabolic pathway is regulated in macrophages, and in hematopoietic cells, in general. For macrophages, as for most cells, the metabolic pathway is regulated in macrophages, and in hematopoietic cells remain to be identified, and BAC 1.2F5 cell lines expressing mutant effectors in hematopoietic cells remain to be identified, and BAC 1.2F5 cell lines expressing mutant G_{i2} proteins should be useful tools in these studies.

ACKNOWLEDGMENT

We are grateful to Drs Allen Spiegel and Silvio Gutkind for their help and support during this work, Dr Laurie Lischer (Genetics Institute) for the gift of purified human recombinant CSF 1, Drs Michel Lanotte and Vincent Praloran for helpful discussions, and Marie-Paule Mellerin and Nelly Robillard for expert technical help.

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Regulation of colony-stimulating factor 1-induced proliferation by heterotrimeric Gi2 proteins

I Corre and S Hermouet