Biochemical identification of glycosylation inhibiting factor

(phospholipase inhibitory protein/lipocortin/suppressor T-cell factor)

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ABSTRACT Rat monoclonal antibody against mouse glycosylation inhibiting factors was obtained, and radiolabeled glycosylation inhibiting factors from the mouse T-cell hybridoma, 231F1 cells, were purified by using the monoclonal antibody and antilipomodulin antibody. Analysis of the affinity-purified lymphokine by PAGE demonstrated two proteins of 14.4 kDa and 41 kDa. Both proteins migrated similarly under the reduced and unreduced conditions, indicating that each of the two species consist of a single polypeptide chain. Biologic activity of the lymphokine was recovered by extraction of the proteins from gel slices followed by renaturation. Evidence was obtained that suggested the 14.4-kDa peptide was a degradation product of the 41-kDa molecules. The 14.4-kDa peptide was also recovered by extraction of the surface-labeled 231F1 cells with Ca^{2+} chelator, indicating that the glycosylation inhibiting factor is associated with the cell surface.

In the course of our previous studies on regulation of the IgE antibody response, we described a T-cell factor that inhibits the N-glycosylation of IgE-binding factors (IgE-BFs) during their biosynthesis and renders the latter factor(s) to selectively suppress the IgE synthesis (1). The glycosylation inhibiting factor (GIF) bound to monoclonal antibodies (mAbs) against rat lipomodulin or rabbit lipomodulin (2, 3). The lymphokine exerted phospholipase inhibitory activity after treatment with alkaline phosphatase (2), suggesting that GIF is a phosphorylated derivative of a phospholipase inhibitory protein. Subsequent studies provided evidence that GIF has immunosuppressive activities. Repeated injections of affinity-purified GIF into antigen-primed mice suppressed both IgE and IgG antibody responses to a priming antigen and facilitated the generation of antigen-specific suppressor T cells *in vivo* (4). We have established suppressor T-cell hybridomas specific for ovalbumin (Ova). The hybridomas constitutively secreted GIF that had no affinity for Ova. However, upon stimulation with Ova-pulsed syngeneic macrophages, the same cells formed GIF with affinity for Ova-coupled Sepharose (5). Ova-binding GIF suppressed the *in vivo* antihapten antibody response of BDF1 mice to dinitrophenyl derivatives of Ova in a carrier-specific manner (5) and appeared to be composed of antigen (Ova)-binding chain and nonspecific GIF chain (6). Thus, Ova-binding GIF seems identical to effector-type suppressor factor (Ts_e F) described by other investigators (7–9). A possible relationship between GIF and antigen-specific suppressor T-cell factors was supported by the fact that representative suppressor factors from hapten-specific suppressor T-cell hybridomas bound to antilipomodulin-Sepharose and were recovered by elution of the immunosorbent at acid pH (10).

The present experiments were undertaken to biochemically identify GIF in SDS/PAGE. Rat mAb specific for mouse GIF was prepared, and GIF was isolated by affinity

chromatography on both anti-GIF and antilipomodulin-coupled Sepharose. The results show that the molecular mass of GIF is 14.4 kDa and suggest that this protein is a degradation product of 41-kDa molecules.

MATERIALS AND METHODS

Antibodies, Immunoglobulins, and Lipocortin. Purified monoclonal rat IgE from immunocytoma IR-162 and monoclonal mouse IgE from the hybridoma H-1-DNP- ϵ -26 were the same preparations as those described (11). A goat antiserum against rat IgM and a rabbit antiserum against rat IgA were obtained by immunization with monoclonal rat IgM (IR-202) and rat IgA (IR-22), respectively, included in complete Freund's adjuvant. The monoclonal rat IgM and IgA were supplied by H. Bazin, University of Louvain, Belgium. Specifically purified goat anti-rat immunoglobulin and alkaline phosphatase-coupled goat anti-rat immunoglobulin were purchased from Jackson Immunoresearch. The monoclonal antilipomodulin antibody (141-B9) has been described (11). The monoclonal antilipomodulin, the γ globulin fraction of anti-rat IgM and anti-rat IgA sera, human IgG (HGG, Miles), mouse IgG1 (MOPC21, Sigma), and crystalline Ova (Sigma) were coupled to Sepharose CL-4B. About 5 mg of protein was coupled to 1 ml of Sepharose. Recombinant human lipocortin 1 was supplied by Jeff Browning and R. B. Pepinsky (Biogen).

Hybridomas. The mouse T-cell hybridoma 231F1 cells (12) and 12H5 cells (13) have been described. Both hybridomas were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) previously described (13). To obtain GIF, the 231F1 cells (1×10^6 cells per ml) were cultured for 24 hr in the DMEM supplemented with 10% Nu-Serum (Collaborative Research).

Construction of B-Cell Hybridomas That Produce Monoclonal Anti-GIF. GIF in culture supernatants of the 231F1 cells was partially purified by using anti-lipomodulin (141-B9)-Sepharose (12). The preparation could switch the 12H5 cells for the formation of IgE-suppressive factor at the dilution of 1:100 (see below). Lewis strain rats were immunized by five i.p. injections of the purified GIF included in complete Freund's adjuvant. One week after the last booster immunization, their spleen cells were obtained and fused with hypoxanthine phosphoribosyltransferase-deficient SP2/O-Ag 14 cells (14).

The hybridomas were selected by ELISA assay described by Steele *et al.* (10) with slight modifications. Briefly, Immulon I plates (Dynatech) were coated overnight with 100 μl of an affinity-purified GIF diluted in 0.1 M carbonate coating buffer, pH 9.6. Plates were washed 3 times with phosphate-buffered saline (PBS)/0.05% Tween 20 between each of the following steps. Plates were blocked with 1% bovine serum

albumin (BSA) in PBS for 6–9 hr. One hundred microliters of culture supernatant of each hybridoma was then added to the wells, and plates were kept overnight at 4°C. Binding of rat immunoglobulin to the plate was detected by using alkaline phosphatase-coupled anti-rat immunoglobulin and alkaline phosphatase substrate (Sigma). ELISA signal was read in a microplate reader MR 700 (Dynatech Lab) with a 405-nm filter 2 hr after addition of the substrate. Isotype of mAbs was determined with ELISA assay by using an isotyping kit for rat mAbs (Zymed Laboratories). A method described by Beaman and Cohn (15) was used to obtain ascitic fluids of irradiated BDF1 mice that contained monoclonal anti-GIF.

Bioassay for the Detection of GIF. GIF was detected by its ability to switch the mouse T-cell hybridoma 12H5 cells from the formation of glycosylated IgE-BF to the formation of unglycosylated IgE-BF (11). Briefly, a suspension of the hybridoma cells was cultured for 24 hr with an equal volume of a sample to be tested in the presence of 10 µg of mouse IgE per ml. Culture supernatants were filtered through CF 50A membranes, and filtrates containing IgE-BF were fractionated on lentil lectin-Sepharose (Pharmacia). Both the effluent (unbound) and eluate (bound) fractions were assessed for the presence of IgE-BF by rosette inhibition between $Fc_\gamma R^+$ lymphocytes and IgE-coated erythrocytes (16). Mesenteric lymph node cells of rats infected with *Nippostrongylus brasiliensis* were used as a source of $Fc_\gamma R^+$ lymphocytes. The proportion of rosette-forming cells in 300 lymphocytes was determined in triplicate and was expressed as the average $\pm$ SD. When the 12H5 cells were cultured with IgE alone, essentially all IgE-BF formed by the cells bound to lentil lectin-Sepharose and were recovered by elution with α -methylmannoside (14). If a sufficient amount of GIF was added to the culture of the 12H5 cells, the majority of IgE-BF formed by the cells lacked affinity for lentil lectin and were recovered in the effluent fraction (11).

Radiolabeling of GIF. The 231F1 cells (2×10^6 cells per ml) were cultured for 24 hr in complete DMEM supplemented with 5% Nu-Serum, and culture supernatants (≈ 500 ml) were concentrated 70-fold in the presence of 0.2 mM phenylmethylsulfonyl fluoride (Sigma) and 10 mM sodium azide. The supernatants were preabsorbed with 1 ml of a mixture of HGG-Sepharose and Ova-coupled Sepharose. GIF in the supernatant was then absorbed with 1 ml of 141-B9-Sepharose overnight at 4°C and recovered by elution with 0.1 M glycine hydrochloride buffer, pH 3.0. The eluate was dialyzed against 50 mM borate buffer, pH 8.5/0.15 M NaCl, concentrated to 0.5 ml, and then labeled with 1 mCi (1 Ci = 37 GBq) of ^{125}I by using Bolton Hunter reagent (Amersham). Further absorption of radiolabeled GIF with either 141-B9-Sepharose or anti-GIF Sepharose (see below) was done by mixing the sample with the immunosorbent overnight at 4°C in the presence of bovine serum albumin (BSA) at 5 mg/ml. The immunosorbent was washed with 40 column volumes of PBS before elution with 0.1 M glycine hydrochloride buffer, pH 3.0.

GIF was biosynthetically labeled with [^{35}S]methionine (Amersham). The 231F1 cells were washed with RPMI 1640 medium containing no methionine (GIBCO), supplemented with 5% fetal calf serum, 10 mM Hepes, and 2 mM glutamine and resuspended in the medium at 2×10^6 cells per ml. The cells were cultured for 24 hr in the presence of [^{35}S]methionine (25 µCi/ml) and 25 mM unlabeled methionine. Culture supernatant (120 ml) was concentrated 20-fold, preabsorbed with HGG-Sepharose and Ova-Sepharose, and then mixed with 1 ml of 141-B9-Sepharose overnight at 4°C. Immunosorbent was washed with 40 ml of PBS or 10 ml of Tris-buffered saline, containing 0.1% SDS, 0.5% Nonidet P-40, and BSA at 1 mg/ml; 10 ml of PBS containing 0.5% Nonidet P-40; and 20 ml of PBS; the immunosorbent was then eluted with 50 mM glycine hydrochloride buffer, pH 3.0/0.15 M

NaCl. Eluates were neutralized, protease inhibitors and BSA (5 mg/ml) were added, and fractionation was continued on an appropriate immunosorbent.

For cell-surface labeling, 10^8 231F1 cells were suspended in Dulbecco's phosphate-buffered saline (DPBS) and were radiolabeled with 2 mCi of ^{125}I (Amersham) by using the lactoperoxidase system (17). Cells were passed through a layer of fetal calf serum and washed 3 times. The cells were resuspended in DPBS/5 mM EGTA/0.03% BSA at 5×10^6 cells per ml, and the suspension was gently shaken for 30 min at room temperature. Supernatants were treated with protease inhibitors and then fractionated on an appropriate immunosorbent.

Detection of Radiolabeled GIF by SDS/PAGE. Affinity-purified, radiolabeled GIF was dialyzed against distilled water in the presence of 0.1% Nonidet P-40 and then lyophilized in a Speedvac (Savant Instruments, Hicksville, NY). Nonidet P-40 in the samples was removed by acetone extraction with 4 µg of cytochrome *c* as a carrier protein (18), and the samples were analyzed by SDS/gel electrophoresis in 15% polyacrylamide slab gels by using the Laemmli system (19). After proteins were biosynthetically labeled with [^{35}S]methionine, gels were treated with EN ^{3} HANCE (New England Nuclear). Autoradiography was performed at -70°C by using X-Omat AR film (Kodak) and DuPont Lightning Plus intensifying screens (DuPont).

Extraction of GIF from SDS Gels and Renaturation. The method described by Hager and Burgess (20) was used. Protein markers (Pharmacia) in SDS gel were visualized by soaking at 4°C in 0.25 M KCl/2 mM dithiothreitol. The appropriate regions were excised, and the gel slices were soaked in 2 mM dithiothreitol for 10 min. Elution buffer (1.3 ml) (0.1% SDS/0.2 M Tris hydrochloride, pH 7.5/5 mM EDTA/5 mM dithiothreitol/BSA at 0.1 mg/ml) was added to the slices, and the gels were homogenized. After 2 hr at 25°C, the suspensions were centrifuged to recover the supernatants. SDS was removed from the extracts by protein precipitation with -20°C acetone. The precipitates were collected by centrifugation ($13,000 \times g$) for 10 min at 0°C and rinsed with ice-cold 80% (vol/vol) acetone/40 mM Tris hydrochloride, pH 7.5/1 mM dithiothreitol. The precipitates were dried under vacuum, and the dried pellet was dissolved in 20 µl of 6 M guanidine hydrochloride (Sigma)/0.2 M Tris-hydrochloride, pH 7.9/2 mM dithiothreitol/2.5 mM EDTA/bovine serum albumin at 1 mg/ml. After 20 min, 1 ml of renaturation buffer (160 mM Tris-hydrochloride, pH 7.9/16 mM dithiothreitol/2 mM EDTA/bovine serum albumin at 0.8 mg/ml/20% (vol/vol) glycerol) was added. The solution was kept overnight at 4°C, and samples were dialyzed against DPBS, followed by RPMI 1640 medium.

RESULTS

Preparation of Monoclonal Anti-Mouse GIF. Anti-GIF-producing hybridomas were selected from 49 rat immunoglobulin-producing hybrid clones by ELISA assay with GIF-coated plates. Eleven hybridoma clones, which gave a significant ELISA signal, were subcloned, and a representative subclone of each hybridoma was tested for the production of anti-GIF. One hundred microliters of purified GIF (titer, 1:100) was mixed with 300 µl of culture supernatants from each clone. After overnight incubation at 4°C, the mixtures were filtered through CF 50A Diaflo membranes, and the filtrates were assessed for the presence of GIF by using the 12H5 cells. The results showed that culture supernatants of five hybridoma clones bound GIF activity. Four of the five clones produced IgM, whereas one clone produced IgA. mAb from each of the five clones was purified from 300 to 600 ml of culture supernatant with anti-IgM or anti-IgA immunosorbent. Affinity-purified mAbs were then assessed for the

ability to absorb GIF by the same procedures described above. The results, shown in Table 1, indicated that mAb from 31C3, 32B10, and 32C2 clones completely absorbed GIF at the concentration of 1 μ g/ml, whereas the antibody from 31A5 clone absorbed GIF at 3 μ g/ml.

Ascitic fluids were obtained from BDF1 mice that received the hybridoma 31C3. IgM in the ascitic fluids were precipitated at between 30% and 50% saturation of ammonium sulfate, and the proteins in the fraction were coupled to Sepharose CL-4B. Fractionation of culture supernatants of 231F1 cells on the immunosorbent showed that GIF activity was absorbed by the immunosorbent and recovered by elution at acid pH. The yield of GIF activity in the eluate fraction was >66% of that present in the original culture supernatant. However, neither the anti-GIF (31C3)-Sepharose nor antilipomodulin (141-B9)-Sepharose bound recombinant human lipocortin I, which also had GIF activity (21).

Identification of GIF. GIF in culture supernatant of the 231F1 cells was purified by using 141-B9-Sepharose and labeled with ^{125} I. The preparation was mixed with 0.4 ml of mAb 31C3-Sepharose overnight. After washing with DPBS, proteins bound to the immunosorbent were eluted at an acid pH. To eliminate mouse IgG, which might be released from the 141-B9-Sepharose, the eluate fraction was absorbed with anti-mouse immunoglobulin-Sepharose. Each of three aliquots of the preparation was then mixed with mAb 141-B9-Sepharose, mAb 31C3-Sepharose, or mouse IgG1-Sepharose for 7 hr, and proteins bound to the beads were eluted at acid pH. Analysis of the proteins in the eluates by SDS/PAGE under reduced conditions is shown in Fig. 1A. The eluates from both the 141-B9-Sepharose and the 31C3-Sepharose gave the major band of 14.4 kDa and a faint band of 41 kDa, whereas neither band was detected in the eluate from IgG1-Sepharose.

Experiments of the same design were done with biosynthetically labeled materials. GIF in culture supernatants of the 231F1 cells in the presence of [^{35} S]methionine was purified by using 141-B9-Sepharose. One-fourth of the aliquots of the partially purified GIF was refractionated on IgG1-, 141-B9- or 31C3-Sepharose. The eluate fractions as well as the partially purified GIF before refractionation were analyzed by SDS/PAGE. The results (Fig. 1B) confirmed that the molecular masses of the major bands are 41 kDa and 14.4 kDa. Densitometric analysis of the autoradiographs

indicated that the intensity of the 41-kDa band markedly diminished after rechromatography on either 141-B9-Sepharose or 31C3-Sepharose, whereas the intensity of the 14.4-kDa band increased after rechromatography. To confirm this finding, gel strips corresponding to each band were cut off,

FIG. 1. SDS/PAGE of radiolabeled GIF. (A) GIF was labeled with ^{125} I, absorbed with 141-B9-Sepharose, and recovered by acid elution. Aliquots of the eluate fraction were absorbed with IgG1-Sepharose (lane 1), 141-B9-Sepharose (lane 2), and 31C3-Sepharose (lane 3); proteins eluted from the columns were then analyzed. (B) Biosynthetically labeled GIF was purified with 141-B9-Sepharose and divided into four equal portions. One aliquot was analyzed without refractionation (lane 1). The same purified GIF was refractionated on IgG1-Sepharose (lane 2), 141-B9-Sepharose (lane 3), and 31C3-Sepharose (lane 4); proteins recovered from the columns were then analyzed. Radioactivities of the 41-kDa and 14.4-kDa bands in lane 1 were 1177 and 4388 cpm, respectively, whereas those in lane 3 were 467 and 4968 cpm. Radioactivities of the two bands in lane 4 were 438 and 5720 cpm, respectively. (C) Biosynthetically labeled GIF was analyzed under reduced (lane 1) and unreduced conditions (lane 2). (D) The 231F1 cells were surface-labeled with ^{125} I. EGTA extracts of the cells were fractionated on IgG1-Sepharose (lane 1) or 141-B9-Sepharose (lane 2), and acid eluates were analyzed. The effluent from the IgG1-Sepharose was absorbed with 31C3-Sepharose, and the acid eluate from the latter immunosorbent was analyzed (lane 3). K, kDa.

Table 1. Adsorption of GIF activity by mAb

mAb from hybridoma	Isotype	Immunoglobulin added, mg/ml	GIF activity in filtrate*
31A5.1	μ κ	1.0	13/36 (±)
		3.0	0/35 (—)
31C3.19	μ κ	1.0	0/39 (—)
		3.0	2/36 (—)
31D6.2	μ κ	3.0	15/22 (±)
32B10.3	α κ	1.0	6/32 (—)
		3.0	3/34 (—)
32C2.12	μ κ	1.0	1/42 (—)
		3.0	0/36 (—)
None†	—	0	40/3 (+)
Medium control		0	0/43 (—)

A constant amount of GIF was incubated with mAb, and the mixture was filtered through CF 50A membranes. GIF activity in the filtrates was assessed by using the 12H5 cells.

*Numbers in the column represent the percent rosette inhibition by the effluent/eluate fractions from lentil lectin-Sepharose. GIF in the filtrate was considered negative (—) when the ratio was <0.2. The proportion of IgE-rosette forming cells in the absence of IgE-BF was $25.6 \pm 1.9\%$.

†GIF was filtered through a CF 50A membrane, and the 12H5 cells were cultured with the filtrate and IgE. Essentially all IgE-BF formed by the cells failed to bind to lentil lectin-Sepharose.

and their radioactivities were measured in a scintillation spectrometer. The results (see legend of Fig. 1) clearly showed that the 14.4-kDa band increased after rechromatography, with concomitant decrease in the 41-kDa band, suggesting that the 14.4-kDa protein is derived from the 41-kDa protein.

To test the possibility that GIF may be composed of multiple polypeptide chains, ^{35}S -labeled GIF was analyzed by SDS/PAGE under reduced and unreduced conditions. Fig. 1C shows that purified GIF clearly gave both the 41-kDa and 14.4-kDa bands, even under unreduced conditions, indicating that both proteins are composed of a single polypeptide chain.

Attempts were made to recover the 14.4-kDa and 41-kDa protein from SDS gel. Culture supernatants of 231F1 cells were concentrated and absorbed with a mixture of HGG-Sepharose and Ova-Sepharose; the GIF in the preparation was then affinity-purified by using 141-B9-Sepharose followed by 31C3-Sepharose. The final preparation could completely switch the 12H5 cells from the formation of glycosylated IgE-BF to that of unglycosylated IgE-BF at a dilution of 1:200. The sample was diluted with an equal volume of twice-concentrated sample buffer and applied to SDS gel. As control, a mixture of sample buffer and DPBS was applied to the same gel. After electrophoresis, the 41-kDa and 14- to 15-kDa regions were excised. Proteins in the gel were extracted and renatured by the described procedures. As control, purified GIF preparation was mixed with the elution buffer and treated with acetone; proteins were renatured at the same time. All preparations were dialyzed against RPMI 1640 medium and assessed for GIF activity. Table 2 shows that extracts of the 14- to 15-kDa region, as well as the original GIF preparation, switched the 12H5 cells to form exclusively unglycosylated IgE-BF, whereas extracts of the corresponding region of control gel failed to change the nature of IgE-BF. Extracts of the 40- to 42-kDa region partially changed the nature of IgE-BF formed.

Association of GIF with the Plasma Membrane. As will be published in a separate paper, we have evidence that GIF is associated with the plasma membrane of GIF-producing hybridomas and can be extracted with Hanks' balanced salt solution (BSS)/5 mM EGTA. Extracts of the same cells with Hanks' BSS alone did not contain GIF activity. Therefore, attempts were made to detect membrane-associated GIF. The 231F1 cells were surface-labeled with ^{125}I , and the cells were extracted with PBS/5 mM EGTA. Extracts were treated with CaCl_2 , adjusted to pH 7.4, and concentrated 7-fold. After preabsorption with HGG-Sepharose and Ova-

Sepharose, a portion of the sample was mixed with 141-B9-Sepharose, and the remainder was mixed with IgG1-Sepharose for 10 hr. Effluent fraction from the IgG1-Sepharose was absorbed with 31C3-Sepharose. Acid eluates from each immunosorbent were analyzed by SDS/PAGE, and the results are shown in Fig. 1D. The major radioactive peptide recovered from the 141-B9-Sepharose was 14.4 kDa, a peptide that was not detected in the eluates from IgG1-Sepharose. Furthermore, the same peptide was recovered from the effluent (unbound) fraction from IgG1-Sepharose by absorption with the mAb 31C3-Sepharose. Both the eluate fraction from the 141-B9-Sepharose and 31C3-Sepharose gave additional faint bands of 41 kDa and 52 kDa, the latter of which had not been detected in the supernatant of the 231F1 cells (Fig. 1C). However, the 52-kDa band was consistently detected when the 231F1 cells were biosynthetically labeled with ^{35}S methionine and the EGTA extracts of the cells were fractionated on 141-B9-Sepharose or 31C3-Sepharose (data not shown).

DISCUSSION

Monoclonal anti-mouse GIF obtained in the present experiments appears to be specific for the lymphokine. Although the results are not shown, the mAb (31C3)-coupled Sepharose absorbed none of the murine IL-2, IL-4, or glycosylation enhancing factor. Neither the monoclonal anti-GIF (31C3) nor monoclonal antilipomodulin (141-B9) were found to bind recombinant human lipocortin I. Using the two mAbs, we identified mouse GIF. Both mAbs bound two molecular species of 14.4 kDa and 41 kDa. Because the two species migrated similarly under reduced and unreduced conditions, both proteins apparently consist of a single polypeptide chain (Fig. 1C). In some experiments, an additional faint band of 30 kDa was detected in the acid eluates of 141-B9- or 31C3-Sepharose. However, the presence of this protein in the eluates was inconsistent. Critical evidence for the association of GIF activity with the 14.4-kDa and 41-kDa peptides is that the activity was recovered by extraction of the peptides from SDS-gel, after which renaturation occurred.

The molecular size of GIF shown by SDS/PAGE corresponded to those sizes estimated by gel filtration. When the 3-day culture supernatants of the 231F1 cells were fractionated on either a Sephadex G-75 column or TSK G3000 SWG column, the GIF activity was detected only in the fractions corresponding to 13 kDa–15 kDa (5). When the 24-hr-culture supernatants of another T-cell hybridoma 71B4 cells were fractionated, GIF activity was demonstrated not only in the 14- to 15-kDa fraction but also in the 45- to 50-kDa fraction (11). Similar results were obtained with a 24-hr-culture supernatant of the 231F1 cells. It appears that the 45- to 50-kDa GIF obtained by gel filtration corresponded to the 41-kDa molecule detected on SDS/PAGE. We speculate that the 14.4-kDa peptide is a degradation product of the 41-kDa molecules. Rechromatography of a partially purified GIF on 141-B9-Sepharose resulted in an increase of the 14.4-kDa molecule and a decrease of the 41-kDa molecule (Fig. 1B). Exposure of the partially purified GIF to room temperature for 12–24 hr also caused substantial decrease of the 41-kDa molecule (data not shown). Our experiments also showed that GIF is associated with the plasma membrane of the 231F1 cells and can be extracted with EGTA. In addition to the 14.4-kDa GIF, 52-kDa protein was detected in the extract. Although the yield of this protein in the extract was much less than that of the 14.4-kDa peptide, the 52-kDa protein could represent the original GIF formed by the cells. Further studies are required to determine the relationship between the 52-kDa protein and the 14.4-kDa GIF.

Previous experiments provided evidence that GIF is a derivative of phospholipase inhibitory protein (2). Because

Table 2. Detection of GIF in the extracts of SDS gel

Sample subjected to SDS/PAGE	Region of gel strip	GIF activity*, effluent/eluent
Purified GIF	No fractionation†	23/3 (+)
	14–15 kDa	22/1 (+)
	40–42 kDa	27/10 (+)
Buffer	14–15 kDa	0/23 (–)
Medium control		0/23

Fifty microliters of purified GIF was applied to two lanes. The same volume of DPBS was applied to the next 2 lanes. After electrophoresis, gel strips of the two lanes were mixed and extracted with 1.3 ml of elution buffer.

*Renatured protein in the extracts was concentrated to 1.5 ml and dialyzed against RPMI medium; 1 ml of the sample was mixed with an equal volume of a suspension of the 12H5 cells for GIF assay. GIF activity was considered positive (+) when the ratio of percent rosette inhibition between the effluent/eluate fractions was >2.0 . No GIF activity was detected when a 1:3 dilution of each sample was used.

†One hundred microliters of the purified GIF was mixed with elution buffer. Proteins were precipitated with acetone and then renatured.

the lymphokine binds to antilipomodulin mAbs, GIF was suspected to be a lipomodulin-like molecule. However, GIF does not appear identical to lipocortin 1 or lipocortin 2. Neither the monoclonal anti-GIF (31C3) nor antilipomodulin (141-B9) bound recombinant human lipocortin 1, which has 90% identity with rat lipocortin in amino acid sequences and reacted with anti-rat lipocortin (22, 23). Neither the rat anti-lipocortin 1 nor anti-lipocortin 2, which were provided by J. Browning and B. Pepinsky, were found to bind GIF from the 231F1 cells. Using the same antibodies, these workers showed that neither lipocortin 1 nor lipocortin 2 was detected in the lysate of the 231F1 cells by immunologic blot. Furthermore, B. F. Wallner (Biogen) has shown that the 231F1 cells did not contain mRNA that hybridizes with a cDNA clone encoding lipocortin 1 (personal communication). In contrast, lipocortin mRNA was detected in various rat and mouse cells by using the same cDNA clone (24), and lipocortin 2 was detected in mouse thymocytes, spleen cells, and some T-cell clones by immunoblot (25). These findings collectively indicate that murine GIF is neither lipocortin 1 nor lipocortin 2. Pepinsky *et al.* (26) described five distinct phospholipase inhibitory proteins that share identity with lipocortin 1. It is still possible that GIF belongs to the family of lipocortins. Elucidation of the relationship between GIF and the lipocortin family requires further structural studies.

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