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Requirement of posttranslational modifications for the generation of biologic activity of glycosylation-inhibiting factor

(suppressor T cells/macrophage-migration inhibitory factor/endoplasmic reticulum)

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ABSTRACT **Secretion of bioactive glycosylation-inhibiting factor (GIF) appears to be restricted to suppressor T (T_s) cells, although various human and murine cell line cells secrete the 13-kDa peptide that reacts with anti-GIF. Nucleotide sequences of GIF cDNA from the T_s and non-T_s cells are identical, indicating that bioactive GIF and inactive GIF have an identical amino acid sequence. A stable transfectant of human GIF (hGIF) cDNA in BMT10 cells secretes inactive GIF peptide, whereas transfection of a chimeric cDNA encoding a fusion protein consisting of the N-terminal region of pro-calcitonin precursor and hGIF into the same cells results in secretion of bioactive GIF. Evidence was obtained that the fusion protein goes into the endoplasmic reticulum and is cleaved for the secretion of mature GIF peptide, whereas the inactive 13-kDa peptide synthesized by the former transfectant does not go through the endoplasmic reticulum. However, a stable transfectant of hGIF cDNA in mouse T_s hybridoma contains inactive GIF in the cytosol and secretes bioactive hGIF without participation of the endoplasmic reticulum–Golgi system. Heterogeneity of the 13-kDa hGIF from the transfectant was detected in two-dimensional electrophoresis. The results suggested that T_s cells have a machinery that converts a portion of inactive cytosolic GIF peptide to bioactive GIF during secretion.**

In our previous studies, we reported that suppressor T-cell (T_s) hybridomas produce glycosylation-inhibiting factor (GIF), a 13-kDa cytokine involved in selective formation of IgE-suppressive factors (1). GIF inhibits N-glycosylation of IgE-binding factors (IgE-BF), and the unglycosylated IgE-BF then selectively suppress IgE synthesis. Further, GIF facilitates the generation of antigen-specific T_s cells (2, 3) and appears to be a subunit of antigen-specific suppressor T-cell factors (4, 5). We speculated that formation of the 13-kDa GIF may be specific for T_s. However, molecular cloning of GIF cDNA provided several unexpected findings (6). Although polyclonal antibodies against recombinant 13-kDa peptide bound GIF from T_s hybridomas and transfection of the cDNA into COS-1 cells resulted in the secretion of 13-kDa peptide, the recombinant peptide had substantially less bioactivity than the hybridoma-derived GIF. Because the peptide does not have signal peptide sequence, one may predict that the recombinant 13-kDa peptide synthesized in this system does not go through the endoplasmic reticulum (ER). Indeed, expression of a chimeric cDNA encoding a fusion protein consisting of the N-terminal pro region of calcitonin precursor (pro-CT) and human GIF (hGIF) and cotransfection with furin cDNA to allow intracellular cleavage of the fusion protein resulted in secretion of a 13-kDa peptide that was comparable to hybridoma-derived GIF in its bioactivity (6). Another unexpected finding during the molecular cloning was that essentially all mouse and human cell line cells tested contained a 0.6-kb mRNA that hybridized with GIF cDNA. Furthermore, the predicted amino acid sequence of mouse GIF was exactly the same as the product of growth factor-induced delayed-early-response gene (7) and the 13-kDa peptide formed by mouse pituitary cells (8). The nucleotide sequence of hGIF cDNA was identical to that of human cDNA-encoding macrophage-migration inhibitory factor (MIF), except for one base (6, 9). These findings suggested that synthesis of the peptide is not unique for T_s cells and that a certain posttranslational modification of the peptide is essential for the generation of GIF bioactivity. The present experiments were undertaken to test this possibility. Our results indicate that T_s hybridomas have machinery for posttranslational modification of the peptide, which is required for the generation of GIF bioactivity.

MATERIALS AND METHODS

Cell Line Cells. Murine T-cell hybridoma 231F1 (4), 12H5 (10), human T-cell hybridoma AC5 (11), 31E9 (12), and hypoxanthine/aminopterin/thymidine-sensitive human T-cell line BUC (11) were described. Suppressor T-cell hybridoma CKB-Ts3-3.11 (13) was supplied by Martin Dorf, Harvard Medical School. A transformed monkey kidney cell line, BMT10, was from Cold Spring Harbor Laboratory. The murine B-cell line A20.3 and mouse pituitary cell line AtT-20 were from the American Type Culture Collection. Unless otherwise specified, these cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with either 5% fetal calf serum or 10% Nu-serum to recover culture supernatants.

Subcellular Fractionation. Cells were suspended in 10 mM Hepes, pH 7.4/42 mM KCl/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride at 1×10^7 cells per ml. After 15 min at 4°C, cells were disrupted by a Dounce homogenizer. The postnuclear fraction was centrifuged for 30 min at 100,000 × *g* to obtain the cytosolic supernatant and crude particulate fraction. The particulate fraction was resuspended in lysis buffer (pH 7.5) containing 10 mM Tris·HCl, 0.5% Nonidet P-40, and 0.5% Triton X-100 for further analysis.

PCR and DNA Sequencing. Poly(A) mRNA was isolated from an appropriate cell line by using a Fast Track mRNA isolation kit (Invitrogen) and then reverse-transcribed into cDNA as template for PCR. On the basis of the nucleotide sequence of mouse GIF cDNA (6), two primers, 5′-ATG CCT ATG TTC ATC GTG AAC-3′ and 5′-TCA AGC GAA GGT GGA ACC GTT-3′; were used to set up PCR to amplify cDNA fragment. If GIF cDNA is present, DNA fragment amplified in PCR should span 348 bp, including an entire open reading frame of 345 nt. As a control, two glyceraldehyde-

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Abbreviations: GIF, glycosylation-inhibiting factor; hGIF, human GIF; IgE-BF, IgE-binding factors; ER, endoplasmic reticulum; pro-CT, proregion of calcitonin precursor; MIF, macrophage-migration inhibitory factor.

3-phosphate dehydrogenase (GAPDH) primers—i.e., 5'-GTG AAG GTC GGT GTC AAC GG-3' and 5'-GAA GAC GCC AGT AGA CTC-3'—were used. DNA fragment amplified in PCR should span a 297-bp cDNA fragment (14). PCR was done for 35 cycles, and the product was analyzed by agarose gel electrophoresis. The GIF cDNA fragment from each of the cell lines was isolated and inserted into the TA cloning vector pCRII (Invitrogen). DNA sequencing was done by using a Sequenase sequencing kit (United States Biochemical) with forward and reverse primers.

Construction of Expression Vector and Stable Transfection. Mammalian expression vector pEFneo was constructed by replacing the SRα promoter of pME18S (15) with the EF promoter of pEF-BOS (16) and then by inserting a neo-resistant unit from pRc/CMV (Invitrogen) for stable transfection. hGIF cDNA was inserted at *Eco*RI and *Xba* I sites. The construct of pro-CT and GIF chimeric (pro-CT–hGIF) cDNA was essentially the same as that described in ref. 6, except that the carboxyl terminus of pro-CT encodes two basic residues at positions −4 and −6 to facilitate cleavage at the fusion junction by endogenous processing enzyme (17). The nucleotide sequence of the chimeric cDNA is shown in Fig. 1. The pro-CT–hGIF cDNA was also inserted at *Eco*RI and *Xba* I sites of pEFneo. Gene transfection was by electroporation (Bio-Rad) at 250 V, 960 μF for BMT 10 cells and at 200 V, 960 μF for 231F1 cells. Stable transfectants were selected by G418 resistance (0.5 mg/ml). The expression of GIF protein by the transfectants was evaluated by immunoblotting, as described below, and a clone with the highest GIF expression was selected. Stable transfectants were maintained in the presence of G418 at 0.5 mg/ml. The transfectants (2–4 × 10^6 per ml) were cultured overnight to recover supernatants.

Purification of GIF. Mouse GIF in culture supernatants or cytosolic fraction of an appropriate cell line cells was purified by using Affi-Gel 10 (Bio-Rad) coupled to the IgG fraction of a rabbit antiserum against recombinant mouse GIF (anti-GIF) (6). hGIF from an appropriate cell line was purified by using anti-hGIF monoclonal antibody 388F1 (11) coupled to Affi-Gel 10. Preparation of the immunosorbents and procedures for the fractionation have been described (6). Proteins retained in the column were recovered by elution with 0.1 M glycine·HCl buffer, pH 3.0.

SDS/PAGE and Immunoblotting. Affinity-purified GIF preparations were analyzed by SDS/PAGE in a 15% polyacrylamide slab gel under reducing conditions (18), and proteins in the gel were visualized by silver staining. An aliquot of a purified GIF preparation was analyzed along with serial 2-fold dilutions of *Escherichia coli*-derived recombinant hGIF (6) of known concentrations, and the concentration of the 13-kDa peptide in the sample was estimated by the intensity of the band in silver staining. The *E. coli*-derived recombinant hGIF was supplied by T. Mikayama from Kirin Brewery Co., Maebashi, Japan. Immunoblotting was done with enhanced chemiluminescence, immunoblot detection system (Amersham), using the IgG fraction of polyclonal rabbit anti-GIF described above.

Modified Two-Dimensional Electrophoresis. Culture supernatant concentrated 10-fold, cytosolic fraction, or affinity-purified GIF was first subjected to isoelectric focusing under nondenaturing conditions for 4 hr at 400 V using ampholyte (pH 5.0–8.3) from Bio-Rad and then to second-dimensional SDS/PAGE under nonreducing conditions (19). Proteins in the gel were detected either by silver staining or by immunoblotting with polyclonal anti-GIF.

Detection of GIF Bioactivity. The activity 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. Detailed procedures for the assay have been described (3). Briefly, the 12H5 cells were cultured with mouse IgE (10 μg/ml) in the presence or absence of a test sample, and IgE-BF in culture filtrates were fractionated on lentil lectin-Sepharose. 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 methyl α-D-mannoside. When a sufficient amount of GIF was added to the 12H5 cell culture, most of the IgE-BF formed by the cells was not retained in the lentil lectin-Sepharose column and was recovered in the flow-through fraction.

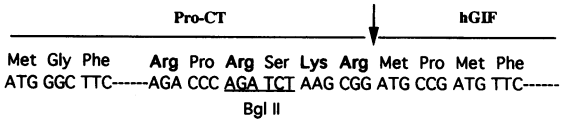

FIG. 1. Nucleotide sequence of the insert encoding a fusion protein of pro-CT with hGIF. Deduced amino acid sequence is shown above the nucleotide sequence. The recognition motif for a furin-like endoproteinase is Arg-Xaa-Arg-Xaa-Lys-Arg. The cleavage site is shown by an arrow.

RESULTS

Bioactivity of 13-kDa GIF from Various Cell Line Cells. Because our preliminary experiments showed that several murine cell line cells—i.e., 231F1, CKB Ts3-3.11, 12H5, A20.3, and AtT-20 cells—contained 0.6-kb mRNA that hybridized mouse GIF cDNA in Northern blotting, we determined whether the cells secrete GIF peptide. As expected, GIF bioactivity was detected in culture supernatants of the two T_s hybridomas—i.e., 231F1 and CKB-Ts3-3.11 but was not detected in those of the other cell line cells (compare in Table 1). However, analysis of the same preparations by SDS/PAGE and immunoblotting revealed that all of the culture supernatants contained the 13-kDa peptide that bound anti-GIF. Thus, the peptide in the culture supernatants was affinity-purified using anti-GIF-coupled Affi-Gel. All of the affinity-purified preparations gave a single band of 13 kDa in SDS/PAGE. The peptide from T_s hybridomas could switch the 12H5 cells for the formation of unglycosylated IgE-BF at the level of 5–15 ng/ml, whereas the peptide from the other cell line cells failed to do so (Table 1). Similar experiments were done with GIF-producing human T-cell hybridomas and a parent cell line used for the construction of hybridomas—i.e., BUC cells. GIF in culture supernatants was purified using 388F1 Affi-Gel. The results showed that 10 ng of the 13-kDa peptide from the human hybridomas per ml was sufficient for detection of GIF bioactivity, whereas 1 μg of the peptide from BUC cells per ml was required (Table 1). The results indicated that the bioactivity of the 13-kDa peptide differs, depending on the cell source of the peptide.

We considered the possibility that the 13-kDa peptide from various cell sources differed in amino acid sequences. To test this possibility, poly(A) mRNA was isolated from the 231F1, CKB-Ts3-31, 12H5, and A20.3 cells and reverse transcribed to cDNA. DNA fragment was then amplified by reverse transcription-PCR using two primers described in *Material and Methods*. A 348-bp fragment spanning the entire coding region was detected in the cells producing either active GIF (231F1 and CKB-Ts3-31) or inactive 13-kDa peptide (12H5 and A20.3) (data not shown). With the glyceraldehyde-3-phosphate dehydrogenase cDNA fragment as a control, no quantitative difference could be detected for transcription of GIF among the four cell lines. Furthermore, nucleotide sequences of the cDNA from the four cell lines were exactly the same as that described for mouse GIF cDNA (6), suggesting that amino acid sequences of the 13-kDa peptide formed by the four cell lines are identical.

Table 1. GIF bioactivity of purified 13-kDa peptide from murine and human cell line cells

Species	Cell source of GIF	GIF activity in culture supernatant*	13-kDa peptide for bioactivity,† ng/ml
Murine	231F1	+	5
	CKB-Ts3	+	15
	12H5	−	>100‡
	A20.3	−	>250‡
	AtT-20	−	>500‡
Human	AC5	+	10
	31E9	+	10
	BUC	−	1000

*Cells were cultured for 24 hr. Culture supernatants were concentrated 10-fold and assessed for GIF bioactivity.
†The 13-kDa peptide was affinity purified. The purity and concentration of the peptide were determined by SDS/PAGE. Numbers show the minimum peptide concentration required for detection of bioactivity.
‡Secretion of 13-kDa peptide was limited. The GIF activity was not detectable at the maximum concentration tested.

Biochemical Properties and Bioactivity of 13-kDa hGIF from Stable Transfectants. To determine that bioactivity of the 13-kDa peptide depends on posttranslational modification of the peptide, stable transfectants of GIF cDNA were established in BMT10 cells. Based on our previous observations in transient expression of the cDNA in COS-1 cells (6), a chimeric cDNA consisting of pro-CT and hGIF cDNA (Fig. 1) was transfected to establish a stable transfection clone, BTCH. Another stable transfectant—i.e., BTH—was established by transfection of hGIF cDNA. As shown in Fig. 2*A*, the original BMT10 cells secreted only a minute amount of 13-kDa peptide, and the formation of the peptide markedly increased by transfection. The 13-kDa GIF in culture supernatant of the transfectants was affinity-purified, and the purified GIF preparations, which gave a single 13-kDa band in SDS/PAGE, were titrated for GIF bioactivity. Table 2 shows that the peptide from BTCH clone had a high GIF bioactivity, whereas the same peptide from BTH clone failed to show activity at the 1 μg/ml level.

One may predict that recombinant pro-CT–hGIF fusion protein synthesized in BTCH clone will go through the ER and be cleaved by a furin-like endoproteinase in the Golgi system for the formation of mature 13-kDa peptide (6). To test this possibility, cytosolic supernatant and crude particulate fraction from BTH and BTCH clones were subjected to SDS/PAGE, followed by immunoblotting with anti-GIF. In BTH cells, GIF was detected in the cytosolic fraction but was not detected in the particulate fractions that contain organelles for classical secretion. When BTCH cells were fractionated, fusion protein of pro-CT and hGIF could be detected in the particulate fraction but could not be detected in the cytosol, indicating that the fusion protein was transported through the classical secretory network (Fig. 2*B*). Because a furin-like endoproteinase is absent in cytosol, the 13-kDa peptide in the cytosol fraction of BTCH cells would be endogenous GIF. The 13-kDa peptide in the cytosolic fraction of BMT10, BTH, and BTCH cells was affinity-purified using 388F1-Affi-Gel, and the purified peptide was tested for bioactivity. Table 2 shows that the cytosolic 13-kDa peptide from these cells failed to switch the nature of IgE-BF at 1 μg/ml. The results strongly suggest that bioactive GIF secreted by the BTCH cells was derived from the fusion protein that went through the classical secretory pathway.

Secretion of bioactive GIF by T_s hybridomas, together with lack of signal peptide sequence in GIF cDNA from the 231F1 cells (6), suggests that the hybridoma has some machinery to convert the inactive 13-kDa GIF peptide to bioactive GIF. If so, one may expect that transfection of hGIF cDNA into the hybridoma may result in the formation of bioactive hGIF. Thus, hGIF cDNA in pEFneo was transfected into 231F1 cells to establish a stable transfectant 2FH2 clone. Analysis of culture supernatant of this clone by immunoblotting with anti-GIF showed that the concentration of the 13-kDa GIF peptide in the supernatant of 2FH2 cells was 10- to ≈20-fold higher than that in the supernatant of 231F1 cells (Fig. 3*A*). Indeed, ≈90–95% of GIF bioactivity in the supernatant bound to 388F1 Affi-Gel and was recovered by acid elution, whereas the remaining ≈5–10% of the activity in the flow-through fraction bound to anti-GIF Affi-Gel. In view of previous findings that mouse GIF could not be retained in 388F1-Affi-Gel (11), acid eluate fraction from the immunosorbent should represent hGIF. Table 2 shows that ≈10 ng of the peptide per ml in the eluate was sufficient for detection of bioactivity, indicating that 2FH2 cells secrete bioactive hGIF.

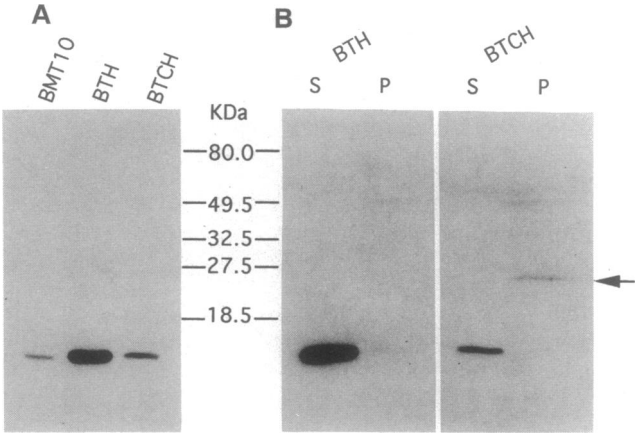

FIG. 2. Formation of the 13-kDa peptide by stable transfectants BTH and BTCH. (*A*) Immunoblotting of culture supernatants of BMT10, BTH, and BTCH with anti-GIF, indicating that the secretion of the 13-kDa peptide markedly increased by transfection. (*B*) Cytosolic fraction (S) and particulate fraction (P) from BTH and BTCH were analyzed. Arrow indicates the fusion protein consisting of pro-CT and hGIF.

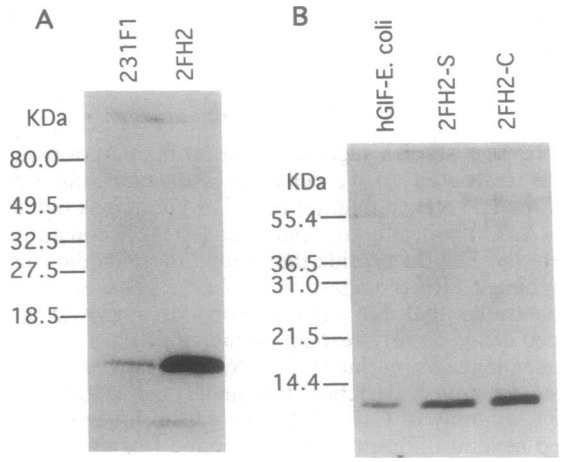

FIG. 3. The 13-kDa peptide by a stable transfectant 2FH2. (*A*) Analysis of culture supernatants of 231F1 cells and 2FH2 cells by immunoblotting with anti-GIF, indicating a marked increase in peptide formation by transfection. (*B*) Comparisons among *E. coli*-derived recombinant hGIF and 13-kDa peptide from culture supernatant (S) and cytosol fraction (C) of 2FH2 cells. Peptides from the 2FH2 cells were purified using 388F1 Affi-Gel, and the purified materials were analyzed by SDS/PAGE and silver staining. Biologic activity of preparations is shown in Table 2.

Table 2. Bioactivity of the 13-kDa peptide formed by stable transfectants

Source	cDNA transfected	GIF in supernatant		GIF in cytosol	
		Yield,* μg	Activity,† ng/ml	Yield,* μg	Activity,† ng/ml
BTH	hGIF	3.5	>1000	25.0	>1200
BCTH	pro-CT–hGIF	2.1	12–25	4.5	>500
2FH2	hGIF	6.0	10	80.0	1000
231F1	None	0.3	5	5.0	1000

*13-kDa peptide was affinity-purified from culture supernatant or cytosol fraction. Yield represents micrograms of peptide obtained from 100 ml of culture supernatant or cytosol of cells recovered from 100-ml culture. Data represent averages of two experiments.
†Concentration of 13-kDa peptide required for detection of GIF bioactivity.

We realized that lysates of the 2FH2 cells contained a large quantity of the 13-kDa GIF peptide. Fractionation of post-nuclear fraction showed that essentially all of the intracellular peptide is present in the cytosolic fraction. Crude particulate fraction did not contain a detectable amount of the peptide, as determined by immunoblot with anti-GIF. No difference could be detected by SDS/PAGE between the 13-kDa peptide in the cytosol and that present in culture supernatant (Fig. 3*B*). However, bioactivity of the cytosolic GIF peptide was 100-fold less than that recovered from culture supernatant (Table 2). We wondered whether the same principle applies to T_s hybridomas. Thus, we purified GIF peptide from culture supernatant and cytosolic fraction of the 231F1 cells by using anti-GIF-coupled Affi-Gel and compared their bioactivities. The results showed that 5 ng of 13-kDa murine GIF per ml of culture supernatant was sufficient for detection of GIF bioactivity, whereas 1 μg of the peptide per ml of cytosol was required for detecting activity (Table 2).

Attempts were made to detect biochemical differences between the inactive GIF in cytosol and the active GIF in culture supernatant of 2FH2 cells. Because no difference was detected by SDS/PAGE under reducing conditions (Fig. 3*B*), we used a modified two-dimensional electrophoresis. Fig. 4 shows that two spots of GIF peptides were detected in cytosol, but three spots of GIF could be detected in culture supernatant. The same results were obtained when GIF was affinity-purified from cytosol and culture supernatant by using 388F1-Affi-Gel and was analyzed by the same method, followed by silver staining. These findings indicate that GIFs in cytosol and culture supernatant are heterogeneous and that GIF in the supernatant contains a species that is lacking in the cytosol.

DISCUSSION

Our previous studies suggested that production of bioactive GIF is restricted to T_s cells (1). However, the present experiments showed that not only T_s hybridomas but also B cells (A20.3) and pituitary cell lines (AtT-20) constitutively secrete the 13-kDa peptide that bound to antibodies against recombinant GIF (compare in Table 1). Lanakan *et al.* (7) demonstrated that the cDNA probe of a growth factor-induced delayed-early-response gene, which has exactly the same sequence as murine GIF cDNA, hybridized with many murine genomic restriction fragments and suggested that there is a family of GIF (MIF)-like genes. Indeed, we have cloned several different mouse genomic fragments and detected some mutations in the GIF-coding sequence (data not shown). These findings suggested the possibility that GIF mRNA expressed in various tissues may not be identical in nucleotide sequences. However, the present findings showed that GIF cDNAs from four different lymphocyte cell line cells had identical sequences, although the GIF peptide from A20.3 and 12H5 cells was inactive. The sequence was also identical to a MIF-like gene from AtT-20 pituitary cells (8), indicating that an identical gene is expressed in various mouse cells. In the human genome, only a single functional MIF-like gene, with an identical sequence to our GIF gene, was identified (20). These findings collectively indicate that both inactive GIF and bioactive GIF have identical amino acid sequences and suggested that bioactivity of the peptide depends on posttranslational modification of the peptide. This idea is supported by the fact that BMT10 cells transfected with hGIF cDNA secreted inactive GIF, whereas transfection of a chimeric cDNA encoding a pro-CT–hGIF fusion protein into the same cells resulted in bioactive-GIF production. In the BTCH clone, the fusion protein was detected in the particulate fraction but was not detected in the cytosol (Fig. 2*B*), indicating that the chimeric molecule is transported through the ER–Golgi network. In our previous publication, in which a chimeric cDNA encoding pro-CT–IgG Fc fragment was expressed in COS-1 cells (17), almost all of the fusion protein with Arg-Pro-Arg-Ser-Lys-Arg sequence at the C terminus of pro-CT was cleaved by endoproteinase in the Golgi apparatus for secretion of the Fc fragment, of which N-terminal amino acid sequence was consistent with

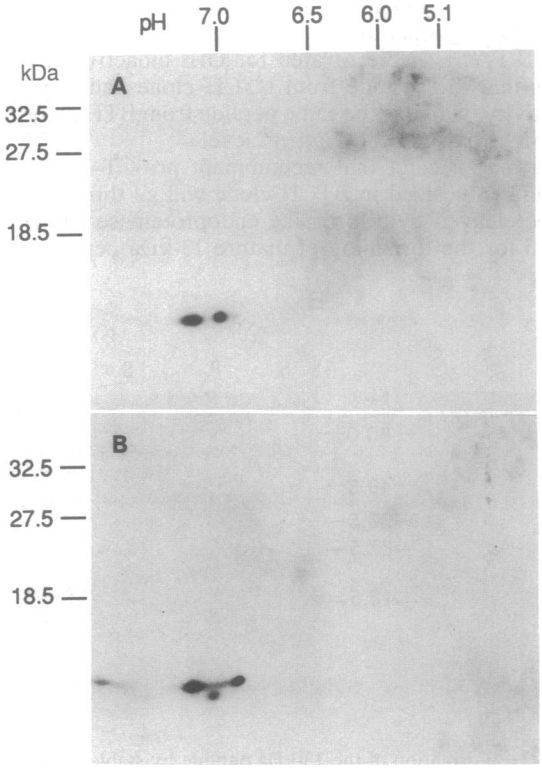

FIG. 4. Two-dimensional electrophoresis analysis of the GIF peptide in cytosol (*A*) and culture supernatant (*B*) of 2FH2 cells. The peptide was identified by immunoblotting with anti-GIF. Distributions of two spots in *A* and three spots in *B* were reproduced in three separate experiments.

the sequence encoded by the Fc cDNA in the fusion plasmid. It is reasonable to expect that the amino acid sequence of mature 13-kDa peptide from BTCH clone is the same as the peptide from BTH clone. It is not known how GIF peptide could be secreted from BTH clone. In this clone, a large amount of GIF peptide is present in cytosol, but it could not be detected in the particulate fraction, indicating that the peptide did not go through a classical secretory pathway. Accumulating evidence indicates that proteins without a signal peptide sequence, such as interleukin 1 (21) and fibroblast growth factor (22), are secreted independently of the ER–Golgi apparatus pathway through unknown mechanisms (23). It is reasonable to speculate that a 100-fold difference in bioactivity between the BTCH-derived GIF peptide and the BTH-derived peptide is ascribed to post-translational modification that may occur in the ER/Golgi system.

An important finding from the present experiments is that transfection of hGIF cDNA into mouse T_s hybridoma resulted in hGIF secretion, almost comparable in bioactivity to that produced by human T_s hybridomas (compare in Tables 1 and 2). It should be noted that an identical plasmid was used to establish 2FH2 and BTH clones, the latter of which secreted inactive GIF. It is conceivable that T_s cells have a transporter protein for the transfer of GIF peptide through ER, which converts inactive GIF peptide to bioactive GIF. However, this possibility is unlikely because the 2FH2 cells contained a substantial quantity of GIF peptide in the cytosol but did not contain any in the particulate fraction. Apparently GIF is secreted from both T_s cells and non-T_s cells without association with the ER–Golgi system. The GIF peptide in the cytosol of 2FH2 and 231F1 cells is 100-fold less active than that secreted from the cells. One might speculate that T_s cells have an unknown machinery that converts a portion of inactive cytosolic GIF peptide to bioactive GIF during the secretory process.

An important problem remaining to be solved is the definition of biochemical differences between bioactive and inactive GIFs. Our experiments have shown that both cytosolic GIF from 2FH2 cells and active GIF in culture supernatant are heterogeneous in two-dimensional electrophoresis. It is unlikely that one of the three species in Fig. 4*B* represents murine GIF because murine GIF from the 231F1 cells has a pI of 5.5 (24). The results suggested that bioactive GIF is structurally distinct from inactive GIF. However, no difference was detected in SDS/PAGE (under reducing conditions) among GIF in culture supernatant, inactive GIF in cytosol, and *E. coli*-derived recombinant hGIF. Recent experiments on MS analysis of the *E. coli*-derived GIF have shown that the molecular weight of the peptide corresponded to the theoretical value calculated from its amino acid sequence (Mikayama and Iwamatsu, personal communication). It is quite unlikely that N-glycosylation is responsible for the heterogeneity. We also found that GIF in the 2FH2 culture supernatant could not be metabolically labeled with [^{32}P]phosphate and failed to bind anti-phosphotyrosine or anti-phosphoserine antibody in immunoblotting, suggesting that the bioactive GIF is not phosphorylated (data not shown). At present, the best explanation for the heterogeneity of GIF is conformational transition of the peptide. The importance of a proper conformation for the protein function is well documented in the study on the scrapie-causing agent prion (25, 26). The hypothesis could possibly explain the secretion of bioactive GIF from BTCH cells. Deduced amino acid sequence of GIF include three cysteine residues (6). Protein chaperones such as protein disulfide isomerase in ER have been shown to be responsible for proper folding of proteins in the classical secretory pathway (27). It is quite possible that chaperones facilitate a proper folding of the GIF peptide when the pro-CT–GIF chimeric molecules pass through the pathway. One might speculate that T_s cells have a similar machinery for the functional folding of GIF peptide.

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