BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 46–51 (1997)
ARTICLE NO. RC977072

Subunit of Glycosylation-Inhibiting Factor Is an Abundant Protein That Binds to Certain Glycoproteins and Sugars

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Received July 3, 1997

12 kDa subunit of glycosylation-inhibiting factor (GIF) is an abundant protein that can be isolated to homogeneity from different mammalian organs by successive application of the carboxymethylcellulose cation exchanger CM52, preparative flat-bed isoelectrofocusing and repeated application of CM52-cellulose. Several isoforms of the 12 kDa GIF subunit exist in mammalian tissues. Conformational stability of two isoforms of a 12 kDa porcine GIF subunit have been studied by CD. Conformation of the protein remains stable within the range 20° to 60°C. Over 60°C the protein undergoes irreversible denaturation. The 12 kDa GIF subunit is not stable within the pH range 2 to 3, adopts quasi-native structure within the pH range 3.5 to 5 while it remains stable between the pHs 6 to 10. The 12 kDa GIF subunit strongly binds to CM52-cellulose from which it can be eluted at concentrations of NaCl higher than 0.6 M. The GIF subunit may also be eluted from the modified cellulose using certain glycoproteins and sugars. High abundance of the 12 kDa GIF subunit in different mammalian tissues and its capacity to bind certain glycoproteins and sugars may suggest that the protein might be involved in regulatory mechanisms of glycoprotein transport (chaperone for glycoproteins) and modulation of interactions between secreted glycoproteins and the cell surface receptors.

12 kDa subunit of glycosylation-inhibiting factor (GIF) which has the same sequence as that of macrophage migration inhibitory factor (MIF) (1) was originally isolated from a T cell hybridoma (2) and various bovine tissues (3-4). It has been established that bioactive GIF is composed of two subunits, namely the 12 kDa component and a 55 kDa subunit which is a derivative of T-cell receptor α-chain (TCR-α) (5-6). GIF is a cytokine that controls selective formation of IgE-suppressive factors (2,5-7). The cDNAs encoding 12 kDa GIF(MIF) proteins were also isolated from a chicken embryonic eye lens library (8) and a mouse cDNA library (9). The cDNA encoding murine GIF hybridized to a number of human and rodent genomic DNA restriction fragments (9). Although only one copy of the gene encoding human GIF(MIF) exists in the human genome (10) other mammalian genomes may contain several copies of the gene. Secreted 12 kDa subunit of bioactive GIF by the antigen-specific T cell hybridomas has modified structure while its unmodified intracellular counterpart does not have any GIF activity (11).

In this communication we give evidence that the 12 kDa subunit of GIF binds to a number of glycoproteins and sugars. This abundant protein may regulate various cellular and immunological processes by binding with different affinities to an array of serum glycoproteins and various cellular receptors. The subunit may also function as a part of transporting vehicles for other glycoproteins, including certain cytokines. An easy purification strategy of natural GIF-like molecules and their characterization may lead to a better understanding of biological significance of these proteins.

MATERIALS AND METHODS

Materials. Acrylamide, N,N′-methylenebisacrylamide, TEMED and ammonium persulfate were purchased from Bio-Rad. Immobiline 3-10L gels, Pharmalytes 3-10, 5-7, 7-9, 8-10.5, and Ultrodex were from Pharmacia. SDS-PAGE molecular mass markers, DTT, iodoacetamid, methyl-α-D-mannopyranoside, N-acetylneuramin-lactose, 3-keto-3-deoxyoctonate, N-acetylneuramic acid, phospholipase A_2 from honey bee (*Apis mellifera*) and human transferrin were purchased from Sigma. Carboxymethylcellulose CM52 was purchased from Whatman.

Fractionation of proteins on cation exchanger and flat-bed preparative isoelectrofocusing. Two separate large scale preparations of proteins from bovine and porcine brains or kidneys were carried out.

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Abbreviations: GIF, glycosylation-inhibiting factor; MIF, migration inhibitory factor; KOD, 3-keto-3-deoxyoctonate; ANA, N-acetylneuramic acid; PIE, preparative isoelectrofocusing.

Partial sequence of 12-kDa subunit of porcine GIF has been deposited with the Protein Sequence Data Bank (EMBL), Accession No. P80928.

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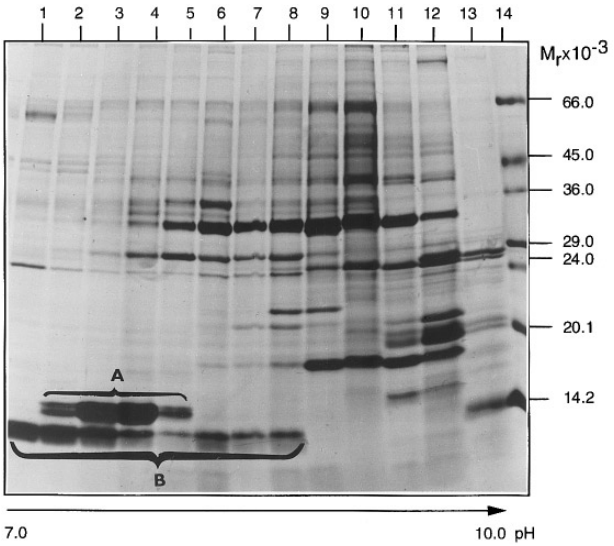

FIG. 1. SDS-PAGE of prefractionated proteins from porcine brain resolved on preparative flat-bed isoelectrofocusing. Only proteins localized between pH 7 and 10 are shown. Sequencing and amino acid composition analyses (the AAC-PI method) confirmed that the proteins designated as A are various isoforms of hemoglobins while the proteins designated as B are various isoforms of 12 kDa subunit of GIF. Molecular mass markers are shown in lane 14.

One kg of the freshly prepared organs were chopped into small pieces, frozen in liquid nitrogen and ground using a Waring blender. The powdered tissue was resuspended in 4L of buffer containing 50 mM sodium phosphate pH 7.4, 300 mM NaCl, 0.1% Triton X-100, 0.02% NaN_3, 5 mM β-mercaptoethanol, 10% ethanol and a cocktail of protease inhibitors (1mM PMSF, 5mM benzamidine, 1mg/l leupeptin, 1mg/l pepstatin, all supplied by Sigma). Protein suspension was cleared by centrifugation and the supernatant was concentrated in an AMICON 2l filtration unit (10 kDa membrane). Concentrated proteins (600 mL) were dialyzed against 50 mM sodium phosphate pH 7.4 containing 10% ethanol and 0.1% Triton X-100 and loaded onto a CM52-cellulose (Whatman) column (5cm×40cm). The proteins which were absorbed on the column were eluted with a gradient of NaCl from 100 mM to 1 M salt. The fractions of proteins eluted within 0.3-0.7 M NaCl were mixed (350 mL) and dialyzed extensively against 1 mM sodium phosphate pH 7.4 and reconcentrated. Dialyzed and concentrated mixture of proteins (50 mL) was added to 8g of ultrodex (Pharmacia) and the slurry was diluted to 100 ml with deionized water containing 5% of glycerol. The following ampholites were added to the slurry: ampholite 3-10 (3mL), ampholite 5-7 (2mL), ampholite 7-9 (1mL) and ampholite 8-10.5 (2mL). Preparative flat-bed isoelectrofocusing equipment (Pharmacia) was used and proteins were focused for 12 hours at 300V followed by 8 hours at 400V and again for 8 hours at 700V. The ultrodex bed was sliced into 15 fractions and proteins were eluted using 15 ml of water and concentrated. pH of each fraction was measured with a standard pH-meter.

Gel electrophoresis, electroblotting and amino acid composition of proteins. IEF/SDS-PAGE gels (12%, size 20×20 cm) were carried as recently described (12) and visualized by silver staining. Proteins were electroblotted on PVDF membranes (PSQ membrane, Millipore) in a semidry NovaBlot device (Pharmacia) using Tris/glycine/SDS buffer pH 8.4. PSQ membranes were stained with Coomassie Brillant Blue and destained in warm (approx. 50°C) 50% ethanol. The excised protein's blots were washed 5× in aqueous solution to remove the traces of glycine carried over from SDS-PAGE. Amino acid compositions were made under the standard conditions: pieces of PVDF blots were sealed *in vacuo* in glass tubes and hydrolyzed in 6N HCl and 0.1% phenol through 24 hours at 120°C. Amino acids were derivatized with phenylisothiocyanate (quantified as phenylisocarbonyl-amino acids, PTC-AA) and were quantified on an Applied Biosystem 420A derivatizer. Relative masses (M_r) of proteins were established using molecular mass standards supplied by Sigma. pIs of proteins on IPG 3-10 were estimated from the linear approximation of immobiline gels.

Binding of 12-kDa subunit of porcine GIF on CM52-cellulose. 0.6 ml samples of CM52-cellulose dispersed in 25 mM sodium phosphate pH 7.2 were equilibrated during 0.5 h with 10 μg of porcine 12 kDa GIF subunit. Each gel sample was washed three times with 2 ml of 50 mM sodium phosphate, 0.1% Triton X-100 pH 7.2 (SPT buffer). The gels were equilibrated at room temperature during 0.5 h with various quantities of sugars or glycoproteins dissolved in SPT buffer. The eluted GIF samples were mixed with SDS-PAGE loading buffer and the remaining CM52-gels were washed three times with SPT buffer. The CM52-gels were mixed with SDS-PAGE loading buffer and heated up during 5 min. The eluted proteins were loaded on SDS-PAGE (20cm×20cm).

Sequencing and protein data base searches. Microsequencing of proteins was made with an Applied Biosystem 477 sequencer. The sequences of proteins were extracted from the PIR and MIPSX protein data-banks (rel. 50 containing 90811 and 81755 protein sequences, respectively) using the SEQPRO program. Total hydrophobicity index (H_i) was previously defined (12).

Circular dichroism spectroscopy. Circular dichroism spectra were recorded with a Jobin-Yvon Mark VI dichrograph. The spectra were measured with quartz squared cuvettes at room temperature in 20 mM phosphate, pH 7.3. Data were averaged over 10 repetitive scans. Protein concentrations were estimated by means of molar extinction coefficient at λ 278 nm, ϵ=10,300 $M^{-1}cm^{-1}$. The circular dichroism were analyzed with the PROTEN program (13).

RESULTS

Isolation of 12-kDa Subunit of Porcine GIF and Its Properties

Soluble proteins from mammalian organs (bovine and pig brains and kidneys) were passed by CM52-

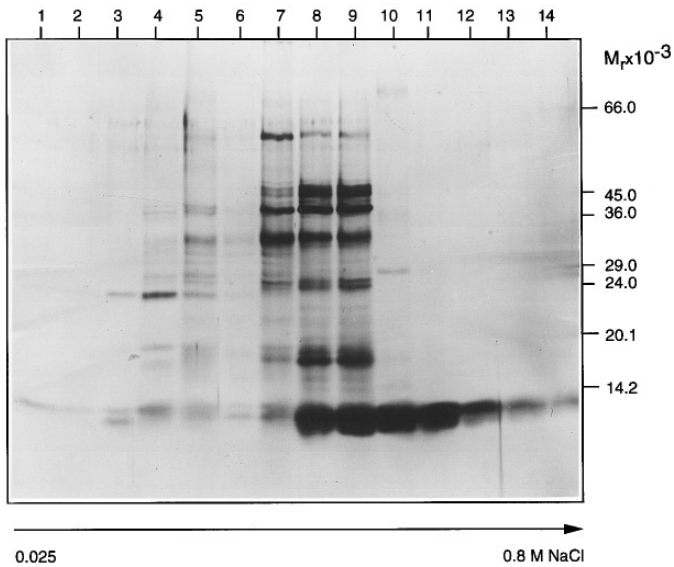

FIG. 2. SDS-PAGE of proteins eluted from CM52 column (fractions 6-8 from the preparative isoelectrofocusing, PIE). Lanes 1-3 show proteins in the flow-through of the column while 12 kDa subunit of GIF was eluted at high concentrations of salt.

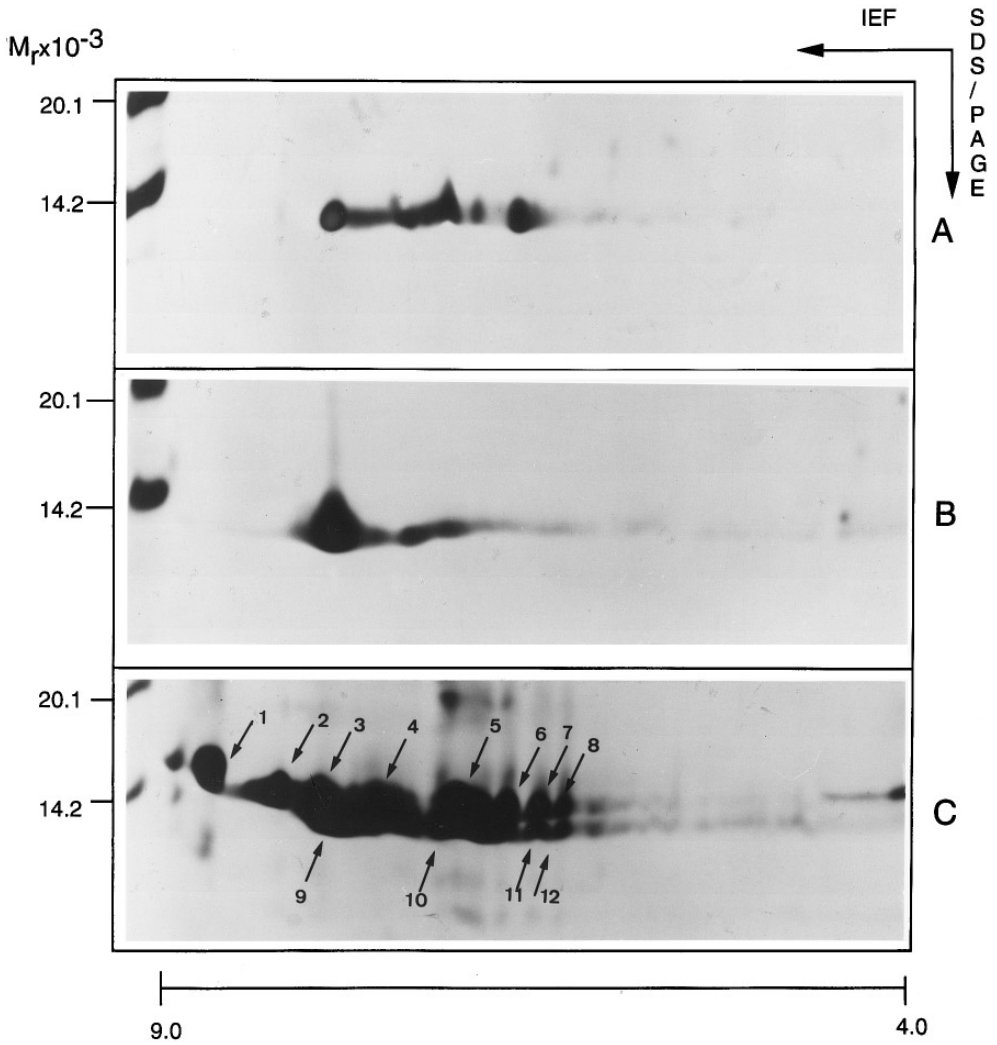

FIG. 3. 2D gels of the following proteins: (**A**) 12 kDa subunit of GIF isolated from fractions 1-2 of PIE; (**B**) 12 kDa subunit of GIF isolated from fractions 6-7 of PIE; (**C**) fractions 3 and 4 of PIE; the bands designated 1-8 are due to various isoforms of hemoglobins while those in 9-12 are due to 12 kDa subunit of GIF.

cellulose. The retained proteins were eluted from the cellulose with increasing concentration of NaCl. The fractions eluted within 0.4-0.7 M NaCl were concentrated and desalted. The desalted mixture of proteins was loaded onto preparative flat bed isoelectrofocusing (PIE) ultrodex gel and resolved into 15 fractions within pH 10.5 and 4.4 (see Fig. 1). Identities of certain proteins resolved on ultrodex were established using their amino acid compositions (the AAC-PI method) (12). Analyses of the AACs of the abundant proteins (lanes 1-8) in the range 12-14 kDa allowed to establish their identities, namely 14 kDa species are due to various isoforms of hemoglobin (14 kDa) while the 12 kDa abundant proteins are the isoforms whose sequences are homologous to that of human glycosylation inhibiting factor (GIF). Microsequencing of the N-terminals of the 12 kDa species confirmed the identities derived from AACs. The fractions containing the isoforms of GIF subunits (pI was 8.2 and 7.4) were re-applied onto CM52-cellulose and the isoforms were eluted at high concentrations of NaCl (see Fig. 2). The isoforms of 12 kDa GIF subunit have pIs between 8 and 6.5 as judged from IEF/SDS-PAGE gels of purified GIF samples from fractions 1-2 (panel A) and fractions 6-7 (panel B) (see Fig. 3) of PIE (see Fig. 1). Panel 3 on Fig. 3 well illustrates that hemoglobins and the isoforms of 12 kDa subunit of GIF share similar pI's. These proteins also have similar hydrophobicity (H_i=40-48).

Binding of Glycoproteins and Sugars to 12-kDa Subunit of Porcine GIF

Purification of 12 kDa subunit of GIF on CM52-cellulose suggests that the protein has strong binding capac-

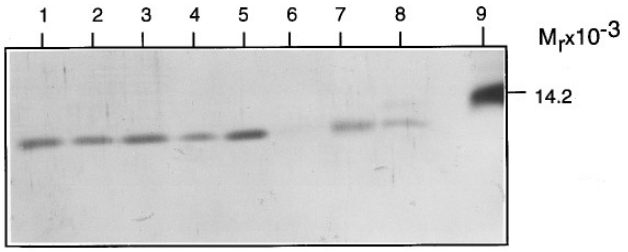

FIG. 4. SDS-PAGE of 12 kDa subunit of GIF eluted from CM52-cellulose with sugars. Odd numbers correspond to the portion of 12 kDa subunit of GIF which remains on CM52-gel while even numbers correspond to the GIF fractions eluted with the following sugars: lanes 1 and 2 (methyl-α-D-mannopyranoside, 60 mM); lanes 3 and 4 (N-acetylneuramin-lactose, 0.2 mM); lanes 5 and 6 (ANA, 5.1 mM); lanes 7 and 8 (KOD, 0.5 mM).

ity to modified sugars. Pure 12 kDa subunit of GIF binds to CM52-cellulose and the protein can be eluted from the matrix with various sugars. In Fig. 4 is shown SDS-PAGE gel of 12 kDa subunit of GIF that was prebound to CM52-cellulose and eluted with the following sugars: methyl-α-D-mannopyranoside, N-acetylneuramin-lactose, 3-keto-3-deoxyoctonate (KOD), N-acetylneuramic acid (ANA). It became apparent that methyl-α-D-mannopyranoside and acetylneuramin-lactose compete stronger with the CM52-matrix for binding to the subunit of GIF than other two sugars. In each case a part of the prebound subunit of GIF remains on the matrix which means that an equilibrium exists between the protein bound to the sugar and CM52-cellulose. The GIF subunit prebound to the cellulose can also be eluted with certain glycoproteins, e.g. phospholipase A_2 (PLA_2) from honey bee (see lanes 5 and 6) and human transferrin (lanes 7 and 8 on Fig. 5). Equilibration studies show that PLA_2 in comparison with human transferrin is more efficient to compete with CM52-gel for 12 kDa GIF subunit.

Stability of 12-kDa Subunit of GIF

12 kDa subunit of mammalian GIF is a relatively hydrophobic protein but it shows good solubility in aqueous solution (3-4). The 12 kDa GIF isoforms differ from each other by about 5% in the content of α-helix; see CD spectra published by Galat et al. (3). The secondary structure of 12 kDa bovine GIF subunit remains stable in aqueous solution within the range 20°-60°C. The secondary structure of the subunit undergoes irreversible unfolding at temperatures higher than 60°C (data not shown). The 12 kDa GIF subunit remains quasi-stable in the pH range 6 to 3.5 and it becomes unfolded at lower pH's. In contrast, change of pH within the range 6-10 does not denature the secondary structure of 12 kDa subunit of GIF.

DISCUSSION

Only one full amino acid sequence (4) and four complete cDNA sequences of GIF-related proteins have been elucidated (7-9,16) (see Fig. 6). All of these sequences have relatively high hydrophobicity indexes (H_i=39-49%) which are comparable to those of the hemoglobin family of proteins (12). Rat D-dopachrome tautomerase (17) is a hydrophobic protein (H_i=48.3) that has 27% identity to 12 kDa rat GIF. Although the pIs of 12 kDa GIF subunit and hemoglobins are similar these proteins can be well separated from each other because the GIF subunit selectively binds to CM52-cellulose while hemoglobins bind weakly to the matrix. Several different isoforms of GIF exist in mammalian tissues (12) which may reflect genetic variability of the organism but it also might be due to posttranslational modifications among which glycosylation and phosphorylation have been excluded (4,11). 12 kDa subunit of GIF has good solubility and thermal stability in aqueous solution at pH's 6-10 but exposure to the temperatures higher than 60°C caused its irreversible denaturation.

Our works show that the 12 kDa subunit of GIF is an abundant intracellular protein which can be isolated from many mammalian organs (3-4). Although the cDNAs encoding 12 kDa subunit of GIF do not consist of any classical secretion signal sequence the mature protein may be secreted in association with other glycoproteins, e.g. glycosylated cytokines. It has been reported that bioactive GIF was found in the serum of patients allergic to honey bee stings (phospholipase A_2 antigen, PLA_2) (11). PLA_2 has at least 14 different combinations of sugars linked to Asn-13 (14-15) and it binds to the 12 kDa subunit of GIF. It is thus conceivable that the presence of 12 kDa subunit of GIF in the serum of allergic patients is due to direct binding of the subunit to the glycosylated antigen fragments. 12

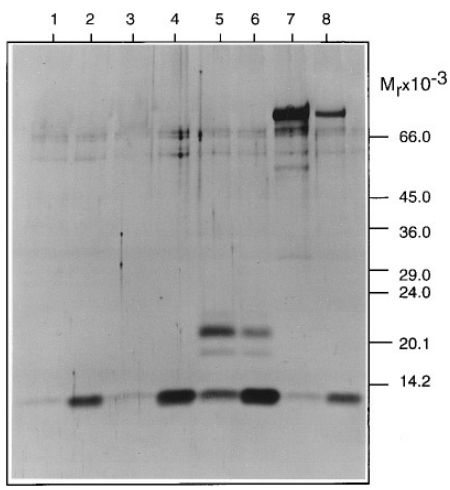

FIG. 5. SDS-PAGE of 12 kDa subunit of GIF which remains bound to CM52 gel (lanes 2, 4, 6, 8) after the gels were equilibrated with the following sugars and glycoproteins: lane 1, 0.3 mM of KOD; lane 2, 10.0 mM ANA; lane 5, phospholipase A_2 from honey bee (7.4 μM); lane 7, human transferrin (4.3 μM).

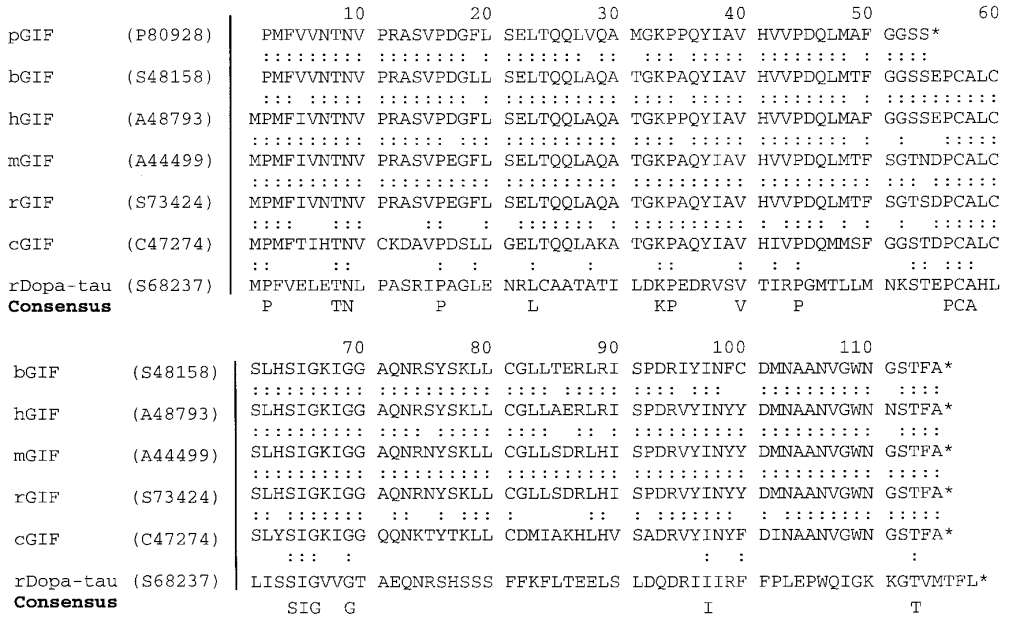

FIG. 6. Sequence alignment between the N-terminal of the 12 kDa subunit of porcine GIF with other 12 kDa GIF-related proteins: bovine GIF (bGIF), EMBL:P80177 (114aa, m=12212, pI=7.8, H_i=47.4); human GIF (hGIF), PIR:S48158 (115aa, m=12476, pI=8.0, H_i=41.7); murine GIF (mGIF), PIR:A44499 (115aa, m=12504, pI=7.1, H_i=39.1); rat GIF (rGIF), MIPS:S73424 (115aa, m=12477, pI=7.0, H_i=41.7); chicken GIF (cGIF), PIR:C47274 (115aa, m=12484, pI=7.1, H_i=49.6); rat D-dopachrome tautomerase (rDopa-tau), MIPS:S68237 (118aa, m=13133, pI=6.1, H_i=48.3).

kDa subunit of GIF also binds to the interferon-γ antagonist sarcolectin (18) which is a subfraction of serum albumin (19-20). The structure of recombinant 12 kDa subunit of GIF expressed in *E.coli* has been recently solved (21-22). The protein is composed of the β-α-β motifs whose overall spatial arrangement resembles to some degree the structures of other cytokines, e.g. interleukin-1, interleukin-8 and the peptide binding domain of human class I MHC protein (22).

It has been proposed, however, that bioactive secreted 12 kDa subunit of GIF is associated with a derivative of TCR-α chain of suppressor T-cells and the complex controls selective formation of IgE-suppressive factors (2,5-6,23). The 12 kDa subunit of GIF, however, may also bind to other extracellular glycotargets, e.g., association of glycosylated peptides with the MHC class I molecules may be strengthened by the 12 kDa subunit of GIF. Although there is limited sequence correlation between the GIF-like proteins and D-dopachrome tautomerases it remains unknown if these two classes of proteins have common natural substrate(s). Further studies are needed for elucidation the cellular signaling pathways that are regulated by these abundant proteins.

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