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Functions of the *N*-Glycans of Rat Leukemia Inhibitory Factor Expressed in Chinese Hamster Ovary Cells

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Leukemia inhibitory factor (LIF) is a pluripotent growth factor which acts in various cell systems. LIF is a glycoprotein containing six putative *N*-glycosylation sites. We established Chinese hamster ovary (CHO) cell lines to evaluate the biological roles of the *N*-glycosylation in rat LIF (rLIF). The bioactivity of rLIF was evaluated in two different bioassay systems using F9 and DA-1a cells. Employing site directed mutagenesis, six *N*-glycosylation-deficient LIF mutants were generated by replacing each asparagine residue (N) (at positions N9, N34, N63, N73, N96, and N116) by glutamine (Q). The resultant mutants showed similar activity in the bioassay using F9 cells. However, N34Q was about 3 times more potent than the wild-type rLIF in the assay using DA-1a cells. These findings suggest that the presence of *N*-glycan at N34 suppresses cell proliferation. In contrast, N63Q was about 2.5 times less potent than the wild-type rLIF indicating the pivotal role of N63 glycosylation for rLIF bioactivity. Taken together, our data suggest that the *N*-glycans of LIF play different roles depending on the cell line and that glycosylation of each specific residue contributes differently to its bioactivity.

Key words: DA-1a, F9, leukemia inhibitory factor, *N*-glycosylation, recombinant protein.

LIF is a multifunctional growth factor which acts in various cell systems including normal and leukemic hematopoietic cells, kidney epithelial cells, neural cells, adipocytes, osteoblasts, and myoblasts (*1*). LIF has different biological effects in different types of cells. For example, it promotes proliferation of leukemic DA-1a cells (*2*), while it inhibits retinoic acid (RA)-induced differentiation of embryonal carcinoma F9 cells (*3*).

LIF shares biological functions with ciliary neurotrophic factor, oncostatin M, cardiotrophin-1, and interleukin (IL)-6 and 11 (*4, 5*). LIF transduces its biochemical signal *via* the LIF receptor (LIFR) complex, which is composed of two subunits, the LIFR α-chain, which binds LIF alone, and gp130, a component of the receptor complex that binds IL-6. All the cytokines mentioned above utilize gp130, suggesting similarities in their bioactivities (*5*).

The molecular structures of human, mouse, rat, sheep, and pig LIF have been determined after cDNA cloning (*6*). The amino acid sequences of the LIF proteins for these species show 74 to 92% similarities. LIF consists of a mature polypeptide of approximately 20 kDa. Six putative asparagine-linked glycosylation (*N*-glycosylation) sites (positions 9, 34, 63, 73, 96, and 116 in rat LIF (rLIF)) are conserved in all five species. However, rLIF contains an additional site at position 150 (*6, 7*).

The LIFs produced by various eukaryotic cells are reported to be heavily glycosylated. rLIF produced by Buffalo rat liver cells has a molecular weight (MW) of 43 kDa, indicating modification by glycosyl moieties of about 23 kDa (*8*). Mapping of both the possible *N*-glycosylation sites and the receptor-binding sites in the crystal structure of mouse LIF indicated that some of the glycosyl moieties might occupy a space near the receptor-binding sites (*9*). These findings prompted us to examine the structure and function of the *N*-glycosylation of LIF protein.

We previously examined the transient expression of rLIF in COS7 cells (*10*). The proteins recognized by the anti-LIF antibody displayed size heterogeneity, and included at least seven molecular species with relative masses ranging from 43 to 21 kDa. In addition, construction and expression of a mutated form of the rLIF gene revealed that the rLIF protein contained six *N*-glycosylation sites at positions 9, 34, 63, 73, 96, and 116. Bioassays with wild-type and mutated rLIF proteins, performed in mouse leukemia DA-1a cells, showed that some mutants exhibited a small reduction in bioactivity, thus suggesting some functional role(s) for the *in vitro* activity. However, the heterogeneity of the glycosyl moieities of rLIF make it difficult to discuss the relationship between each glycosyl

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Abbreviations: LIF, leukemia inhibitory factor; LIFR, LIF receptor; IL, interleukin; *N*-glycosylation, asparagine-linked glycosylation; *O*-glycosylation, serine/threonine-linked glycosylation; RA, retinoic acid; CHO, Chinese hamster ovary.

moiety and its bioactivity (*10*).

In the present study, we established stable transformants of Chinese hamster ovary (CHO)-K1 cells in order to evaluate the biological roles of *N*-glycans using deglycosylated rLIF in two different bioassay systems, F9 and DA-1a cells.

MATERIALS AND METHODS

Plasmid DNA and Cell Lines—pABWN (*11, 12*) was a kind gift from Dr. J. Miyazaki (Tohoku University). Mouse leukemic DA-1a cells were kind gift from Dr. T. Hara (The University of Tokyo). CHO-K1 cells and mouse embryonal carcinoma F9 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo).

Construction of a Plasmid for Stable Expression of rLIF—The expression plasmid for transient expression of rLIF (prL) was constructed as described previously (*10*). Briefly, plasmid DNA, in which the rLIF gene (*13*) was expressed under the control of the SRα promoter, was constructed. Mutated rLIF genes, in which the asparagine residue at each Asn-X-Ser/Thr (X: any amino acid) site was substituted by glutamine, were also constructed and their plasmid DNAs were designated prL-N9Q, -N34Q, -N63Q, -N73Q, -N96Q, and -N116Q (*10*).

PCR was performed with primers 1 (5′-TGCGCTCGAGCCACCATGAAGGTCTTGGCC-3′) and 2 (5′-AGGTCTCGAGTCTAGAAGGCCTGGACCACCG-3′) using prL or its mutants as templates to introduce *Xho*I sites at both ends of the expression units of the rLIF genes. The absence of nucleotide misincorporation during PCR was checked by analyzing the nucleotide sequence. Each DNA fragment was then digested out by *Xho*I (Takara, Kyoto) and ligated with pABWN, resulting in plasmid pABrL (Fig. 1a). The orientation of the rLIF gene in pABWN was confirmed by restriction enzyme digestion. Plasmid DNAs for expressing rLIF mutants were constructed as described above, resulting in plasmids pABrL-N9Q, -N34Q, -N63Q, -N73Q, -N96Q, and -N116Q, respectively.

Establishment of CHO-K1 Cells Transfected by Plasmid DNA—CHO-K1 cells were routinely cultured in Ham-F12 medium (GIBCO-BRL) containing 10% Fetal Clone II (Hyclone), 50 units/ml penicillin G (Sigma), and 50 μg/ml streptomycin sulfate (Sigma) in 5% CO_2 and 95% air humidity.

Stable transfectants of CHO-K1 were produced as described previously (*14*). After incubation of selected stable cells (2×10^5) in the growth medium, the culture medium was changed to a serum-free, low-protein medium, CHO-S-SFM II (GIBCO-BRL).

Immunological Detection and Quantification of rLIF in Conditioned Medium of CHO-K1 Cells—Conditioned medium was recovered 48 h after changing from the growth medium to CHO-S-SFM. After centrifugation at 15,000× *g* for 30 min, the supernatant was desalted using a PD-10 column (Pharmacia) and concentrated. The sample was reconstituted with distilled water, subjected to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P membrane (Millipore). The membrane was incubated with anti-mouse/human LIF goat polyclonal antibody (R&D Systems, Minneapolis, USA), followed by peroxide-conjugated anti-goat IgG antibody (Jackson Immunochemicals, Baltimore, USA). The membrane was developed using enhanced-chemiluminescence reagents (Amersham). The density of each band was measured by NIH imaging. The relative amount of rLIF and its mutants was then calculated with reference to a standard curve, plotted using mouse LIF; ESGRO (AMRAD Biotech) was used as a standard. The concentration of the conditioned medium in which the band-density was the same as that of 1 μg/ml ESGRO was designated as that containing 1 μg/ml rLIF.

Glycosidase Treatment of Non-Mutated rLIF—For PNGaseF treatment, wild-type rLIF (1 μg) was denatured by boiling for 10 min in the presence of 1% SDS, and then incubated with 40 mU PNGaseF (Boehringer Mannheim) for 20 h in buffer (20 mM sodium phosphate (pH 7.2), 10 mM EDTA, 3.5% MEGA-8, and 1.0% 2-mercaptoethanol). For sialidase treatment, wild-type rLIF (1 μg) was denatured by boiling for 10 min and was then incubated with 200 mU Sialidase (Boehringer Mannheim) for 5 h in incubation buth 5 mM $CaCl_2$. rLIF proteins were detected using anti-LIF antibody as mentioned above. Wild-type rLIF which had not been subjected to enzyme treatment was used as a control.

rLIF-Dependent Inhibition of Retinoic Acid (RA)-Induced Differentiation of F9 Cells—Cell culture and the RA-induced differentiation assay were performed essentially as described by Rudnicki *et al.* (*15*). Briefly, F9 cells were maintained under 5% CO_2 and 95% air humidity in DMEM (GIBCO-BRL) with 10% fetal bovine serum and 55 mM 2-mercaptoethanol, to which were added non-essential amino acids (GIBCO-BRL) and nucleosides (Sigma) as described by Robertson (*16*). For the differentiation assay, F9 cells were plated at a density of 2,000 cells/cm^2 on a gelatin-coated plastic dish and incubated with CHO-K1 cell-derived conditioned medium containing rLIF proteins at concentrations of 10, 2.5, 0.64, 0.16, 0.04, and 0.01 ng/ml in the presence of 0.1 μM all-*trans* retinoic acid (Sigma). After 5-d incubation, 100 colonies were chosen at random and the number of undifferentiated colonies was counted essentially as described by Strickland *et al.* (*3*) and Hirayoshi *et al.* (*17*).

rLIF-Dependent Proliferation of DA-1a Cells—DA-1a cells were maintained in RPMI 1640 (GIBCO-BRL) with 10% fetal bovine serum (JRH Bioscience), 55 mM 2-mercaptoethanol, penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml) under 5% CO_2 and 95% air humidity.

The DA-1a cells proliferation assay was performed essentially as described by Moreau *et al.* (*18*). Briefly, various amounts of the conditioned medium containing rLIF proteins at concentrations of 0 to 2.0 ng/ml were prepared and applied to 96-well microtiter plates. DA-1a cells (2.0×10^5) were then added and incubated for 72 h. To evaluate the proliferation of the cells, 10 μl WST-1 solution (Dojindo, Kumamoto) was added to the wells and incubated for 2 h. The development of color was then measured at an absorbance at 450 nm.

RESULTS

Stable Expression of Rat LIF in CHO-K1 Cells—To express rLIF stably in CHO-K1 cells, non-mutated and mutated rLIF genes (*10*) were ligated to pABWN (Fig. 1a). CHO-K1 cells were transfected by pABrL and selected by drug resistance as described in "MATERIAL AND METH-

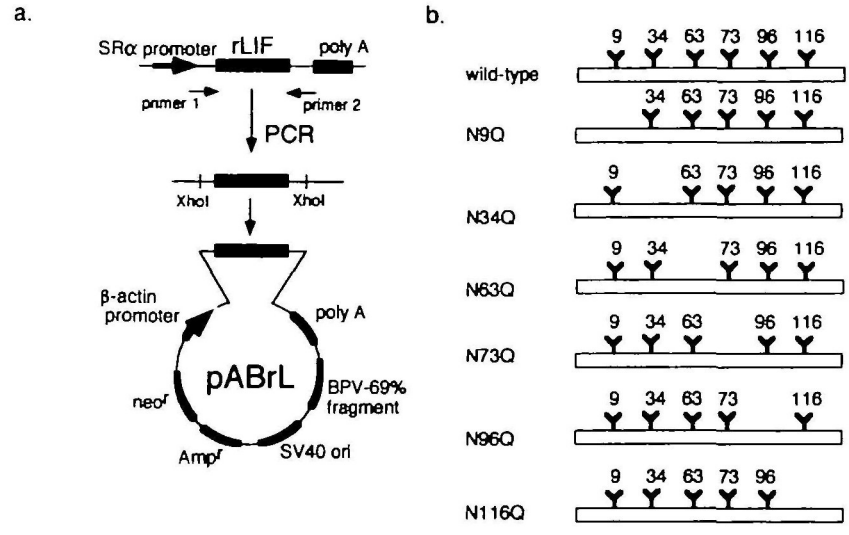

Fig. 1. **(a) Construction of an expression plasmid for rLIF.** *Xho*I sites were introduced into the rLIF gene by PCR using two primers. The DNA fragment obtained after *Xho*I digestion was then introduced into the pABWN plasmid. The final construct for wild-type rLIF was designated pABrL. **(b) Site-directed mutagenesis of rLIF.** Six deglycosylated mutants (N9Q, N34Q, N63Q, N73Q, N96Q, and N116Q), in which the asparagine residue at each Asn-X-Ser/Thr site was substituted by glutamine, were prepared by site-directed mutagenesis.

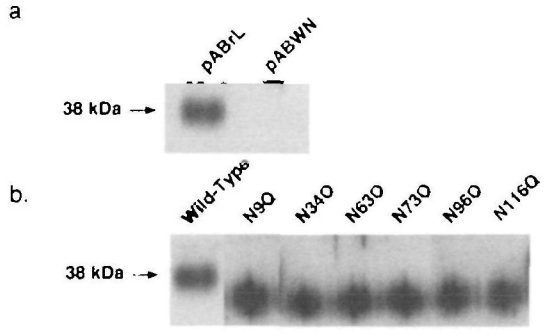

Fig. 2. **Stable expression of wild-type and deglycosylated rLIF mutants.** (a) Conditioned media from CHO-K1 cells transfected by the vector alone (pABWN) or with wild-type rLIF (pABrL) were analyzed by Western blotting using anti-LIF antibody. (b) Conditioned media from CHO-K1 cells expressing wild-type and mutated forms of rLIF are shown.

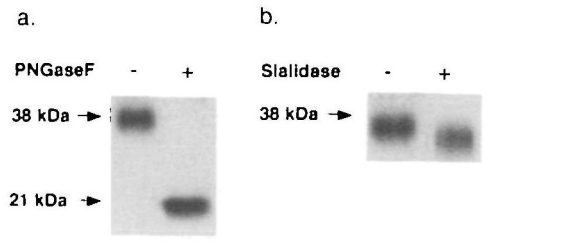

Fig. 3. **Glycosidase treatment of wild-type rLIF expressed in CHO-K1 cells.** (a) Wild-type rLIF was treated with PNGaseF and analysed by Western blotting. (b) Wild-type rLIF was treated with sialidase and analysed by Western blotting.

ODS." When the conditioned medium was examined using commercial anti-mouse/human LIF antibody, which also reacts to rLIF, recombinant rLIF was detected as molecules with a MW of 37-39 kDa (Fig. 2a). CHO-K1 cells containing mutated rLIF genes, in which the Asn of each *N*-glycosylation site was substituted by Gln (*10*), were established (Fig. 1b). These produced broad bands which were smaller than that obtained with wild-type rLIF (Fig. 2b). All deglycosylated mutants showed an approximately 2-3 kDa decrease in MW, but no site-dependent distribution of the carbohydrate chain was detected.

PNGaseF and Sialidase Treatment of rLIF—After treatment with PNGaseF, wild-type rLIF molecules were reduced to a single 21 kDa product (Fig. 3a), suggesting that the rLIF produced in CHO-K1 cells was modified by *N*-glycans of 16-18 kDa. Mutated rLIFs were also treated with PNGaseF, yielding a 21 kDa product (data not shown). When sialidase was employed to remove α2-3, α2-6, and α2-8-linked sialic acid, the MW of the wild-type rLIF molecule was reduced a little (Fig. 3b). These results suggest that the rLIF proteins expressed in CHO-K1 cells are modified by *N*-linked carbohydrate chains which are partially sialylated.

Biological Activity of Deglycosylated Mutants of rLIF in Promoting F9 Cell Differentiation—The inhibitory profile was exhibited in a dose-dependent manner (Fig. 4). To assess the biological role of each *N*-glycan, we compared the effects of the wild-type and six different mutants of rLIF. The inhibitory activity of each mutant was dependent on the amount of rLIF protein and was slightly smaller than that of the wild-type. Among the mutants, the activity of N116Q was lowest. The differences in activity were, however, not as dramatic as those observed in the assay system using DA-1a cells.

Biological Activity of Deglycosylated Mutants of rLIF in Promoting DA-1a Cells Proliferation—When the DA-1a proliferation-promoting activity of wild-type rLIF expressed in CHO-K1 cells was examined, it was found to be expressed in a dose-dependent manner (Fig. 5, inset). When conditioned medium derived from CHO-K1 cells harboring mutated rLIF genes was employed in this assay, activity was also dose-dependent (Fig. 5, inset). The 50% effective doses (ED50s) for mutated rLIFs were calculated, and could be categorized into three groups as follows: (i) the ED50 for N34Q was 3 times lower than that of the wild-type, (ii) the ED50 for N63Q was 2.5 times higher than that of the wild-type, and (iii) the ED50s for the other mutants (N9Q, N73Q, N96Q, and N116Q) were similar to that of the wild-type (Fig. 5). It should be noted that there was an 8-fold difference between the ED50 values of N34Q and N63Q. These results indicate that removal of *N*-glycans at

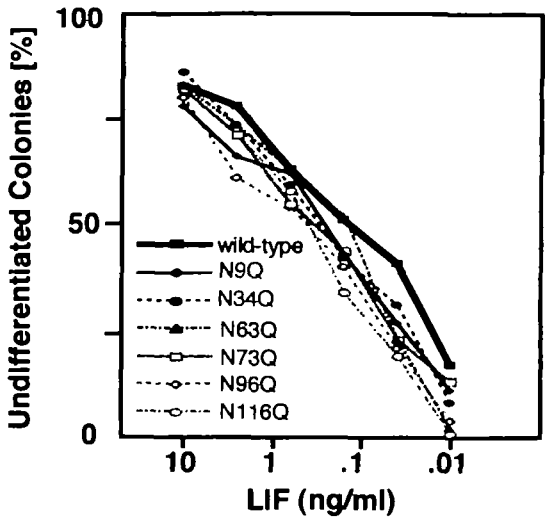

Fig. 4. **Biological activity of wild-type and deglycosylated mutants of rLIF in F9 cells differentiation assay.** RA was used to induce differentiation in F9 cells. Cells exhibiting an undifferentiated morphology were then counted as described in "MATERIALS AND METHODS." The results are expressed as the means of two individual experiments in triplicate cultures.

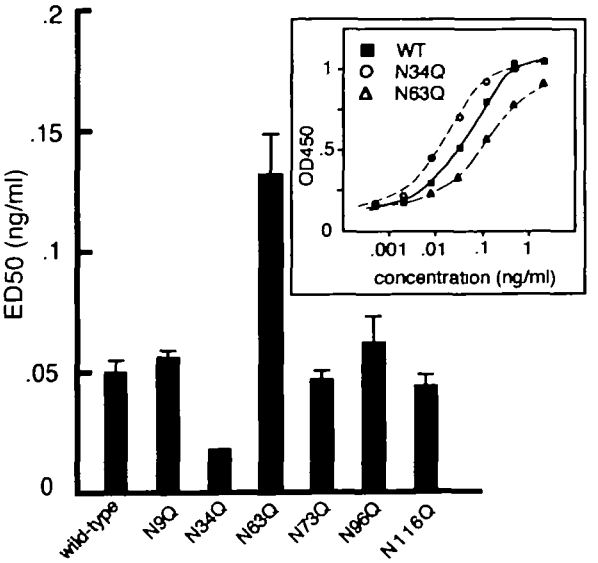

Fig. 5. **Biological activity of wild-type and deglycosylated mutants of rLIF in DA-1a cell proliferation assay.** The ED50s for wild-type and deglycosylated mutants of rLIF proteins were calculated as described in "MATERIALS AND METHODS." The results are expressed as the means with standard errors for two individual experiments in triplicate cultures. In the inset panel, the dose-dependent activities of the wild-type, N34Q, and N63Q rLIF are shown. The activity profiles of the other mutants were the same as that of the wild-type (data not shown). The results are expressed as the means of triplicate cultures in a typical experiment.

specific sites changes the bioactivity of rLIF in the DA-1a cell, and that the direction of change differs depending on the position of the mutation.

DISCUSSION

We constructed expression plasmids for both the wild-type and mutated rLIFs and expressed them into CHO-K1 cells. The conditioned medium expressed LIF-dependent activity in promoting proliferation of DA-1a cells and inhibiting RA-induced differentiation of F9 cells. In addition, this system was found to produce less heterogenous rLIF than the transient expression. Thus, the use of this expression system makes it easier to investigate the relationship between the *N*-glycosylation and the function of LIF.

We have recently identified six putative *N*-glycosylation sites (positions 9, 34, 63, 73, 96, and 116) of rLIF that were glycosylated (*10*). From our deglycosylation experiments and previous reports of recombinant proteins expressed in CHO cells (*19, 20*), the rLIF expressed in our system is thought to have several complex-type *N*-glycans with sialylated motif. After treatment with PNGaseF, the MW of rLIF was reduced to 21 kDa, which is similar to that calculated from its polypeptide sequence. Lectin blot analyses conducted in our previous study (*10*) suggest that the serine/threonine-linked glycosylation (*O*-glycosylation) sites on rLIF are either not present or not functional when rLIF is expressed by CHO cells.

To evaluate the rôle of *N*-glycans in the bioactivity of LIF, we used two mouse cell lines: DA-1a and F9. Removal of *N*-glycan from LIF had relatively little effect on its inhibitory activity toward differentiation of F9 cells. In contrast, in the proliferation of DA-1a cells, the *N*-glycans at two *N*-glycosylation sites showed unique chracteristics. The activity of the N34Q mutant was about 3 times higher than that of the wild-type rLIF, suggesting that the presence of *N*-glycan at this position played a role in suppressing the proliferation of DA-1a cells. The Asn34 of human and mouse LIF (hLIF and mLIF) is located on α-helix A, which is linked to site II of the gp130 binding site (*9, 21*). Therefore, the *N*-glycan attached to the Asn34 residue may interfere with ligand-receptor binding *in vitro*. On the other hand, the activity of the N63Q mutant was about 2.5 times lower than that of wild-type rLIF, suggesting that *N*-glycosylation at Asn63 is essential for proliferation of DA-1a cells. Asn63 is located in the AB-loop, which is not directly involved in ligand-receptor binding (*9, 21*). Hudson *et al.* (*21*) found that an N63A mutation resulted in no change of binding affinities of LIF-LIFR and LIF-gp130. This finding supports the concept that an *N*-glycan attached to the Asn63 residue is not directly involved in binding to the receptors but may be important for protein conformation or stability. On losing this *N*-glycan, the conformation of the LIF molecule might be modified, thereby affecting the interaction between LIF and the two subunits of the LIF receptor complex (LIFR α-chain or gp130). We previously prepared rat LIFR α-chain (*22*) and gp130 (data not shown) from rat placenta. Kinetic analysis of the binding of mutated LIF to the LIF receptor complex (LIFR or gp130) will provide further information on the biological significance of *N*-glycosylation.

In conclusion, we found that the *N*-glycans attached to LIF play different roles depending on the cell line, which may reflect the diverse functions of LIF. In addition, *N*-glycans at specific sites are thought to contribute differently to the bioactivity of LIF, at least in the case of the *N*-glycans at positions Asn34 and Asn63.

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