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The AATPAP sequence is a very efficient signal for *O*-glycosylation in CHO cells

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The peptide signal sequence for protein *O*-glycosylation is not fully characterized, although a recent *in vitro* study proposed that the sequence motif, XTPXP, serves as a signal for mucin-type *O*-glycosylation. Here, we show that the AATPAP sequence acts as an efficient *O*-glycosylation signal, *in vivo.* A secreted fibroblast growth factor (secFGF) was used as a model to analyze glycosylation and its effects on the biological activity of FGF. Two constructs encoding [AATPAP]secFGF in which AATPAP was introduced at the N- or C-terminus of secFGF were constructed in an eukaryotic expression vector. [AATPAP]secFGF proteins were then expressed in Chinese hamster ovary (CHO) cells and secreted into the surrounding medium, primarily as modified forms sensitive to sialidase but not to peptide *N*-glycosidase F. The modifying groups were not seen when the AATPAP sequence was converted to AAAPAP or when [AATPAP]secFGF was expressed in mutant cells incapable of UDP-GalNAc biosynthesis. The results indicate that the modifying groups were mucin-type *O*-glycans and that the AATPAP served as an efficient *O*-glycosylation signal sequence. The *O*-glycosylated forms of [AATPAP]secFGF were as mitogenic toward human vascular endothelial cells as unmodified secFGF, suggesting that introduction of the signal into biologically active polypeptides is a promising approach with which *O*-glycosylation may be achieved without affecting original activity.

***Keywords:* *O*-glycosylation signal; mucin; fibroblast growth factor; signal sequence**

***Abbreviations:* CHO cells, Chinese hamster ovary cells; FGF, fibroblast growth factor; Gal, galactose; GalNAc, *N*-acetylgalactosamine; PNGase, peptide *N*-glycosidase F; HUVEC, human umbilical vein endothelial cell**

Introduction

The biosynthetic pathway leading to *N*-glycosylation has been well-characterized: oligosaccharyltransferase recognizes the sequence, NXS/T (Asn-Xaa-Ser/Thr) and transfers a 14-sugar component from a dolichol-linked oligosaccharide [1,2] in a single step. In contrast, sequence requirement for mucin-type, *O*-linked glycosylation is not fully characterized, although recent studies by Elhammer et al. and Yoshida et al. revealed a highly efficient acceptor sequence for *O*-glycosylation [3,4]. Yoshida et al. used an *in vitro* assay system utilizing bovine UDP-*N*-acetylgalactosamine (UDP-GalNAc): polypeptide *N*-acetylgalactosaminyl transferase (*O*-GalNAcT1) to analyze a series of synthetic peptides for their potential to be *O*-acetylgalactosaminylated and found that the mucin-type *O*-glycosylation motif, XTPXP (Xaa-Thr-Pro-Xaa-Pro), was the shortest sequence motif capable of serving as an acceptor for *O*-glycosylation. The effectiveness of this motif *in vivo* is as yet unknown, however. Therefore, the aim of the present study was to determine whether a short sequence defined by this motif within a polypeptide can serve as an acceptor for *in vivo O*-glycosylation.

The FGF family is a group of biologically active polypeptides that play important roles in a variety of pathophysiological events including cellular growth and differentiation, tissue generation, wound healing and angiogenesis [5]. One of the prototype members of the family, FGF-1, lacks a signal sequence and is thus not secreted efficiently under physiological conditions. In order to use this protein as a model, we created a secreted FGF (secFGF) in which a signal peptide was attached to the N-terminus of FGF-1. Using secFGF as a model, we examined not only whether a short peptide sequence defined by the mucin-box motif can serve as an *in vivo* acceptor for *O*-glycosylation, but also the effect of *O*-glycosylation on the biological activity of secFGF.

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Materials and methods

Cell culture

Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (Rockville, MD) and initially cultured in Ham F-12 medium supplemented with 5% fetal bovine serum (FBS). ldlD-CHO mutant cells were kind gift from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA) and cultured in F-12 medium supplemented with 10% FBS. When medium conditioned by transfectants was to be recovered, both the wild-type and mutant CHO cells were transferred to ASF104 serum-free medium (Ajinomoto Co., Tokyo, Japan) because this medium produced high yields of secreted proteins.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord as previously described [6], plated onto collagen type I coated plastic dishes (Sumitomo Bakelite Co., Tokyo, Japan) and maintained in MCDB151 medium (Life Technologies, Inc., MD, USA) supplemented with 0.02% $MgSO_4-7\ H_2O$, 15% FBS, 10 ng/ml recombinant FGF-1 [7] and 5 μg/ml heparin. Mitogenic activity was analyzed in cells with less than 40 population doublings.

Construction of cDNA

secFGF was used as a model protein for introduction of the mucin-box motif. A cDNA encoding secFGF, comprised of a signal peptide plus FGF-1 (22-155), was constructed in pBluescript (KS+) plasmid. Introduction of the mucin-type *O*-glycosylation motif into secFGF was carried out using the PCR technique modified with an overlap extension protocol [8–10]. For construction of N-[AATPAP]secFGF, PCR was first performed using the secFGF cDNA plasmid as a template with primers #116 (5′-GGCCG CTCTA GAACT AGTGG AT-3′) and #144 (5′-GCAGC AACTC CGGCG CCAGC TAATT ACAAG AAGCC C-3′) for lower half and primers #115 (5′-AACAA AAGCT GGGTA CCGGG-3′) and #163 (5′-TGGCG CCGGA GTTGC TGCGT CCAGT AGCGT GCCTT G-3′) for upper half. The amplified products were mixed and primers #115 and #116 were then used for a second PCR. The resultant product was digested by EcoR I and Sal I and subcloned into pBluescript II (KS+) vector. After verifying the cDNA sequence, the inserted cDNA was cut out using EcoR I and Sal I and subcloned into the mammalian expression vector, pMEXneo. The primers used to construct C-[AATPAP]secFGF were #159 (5′-AACCC TCACTA AAGGG AA-3′) and #164 (5′/TGGCG CCGGA GTTGC TGCAT CAGAA GAGAC TGGCA G-3′) for the upper half and #157 (5′-GCAGC AACTC CGGCG CCATA AAGAA TTCCT GCAGC C-3′) and #135 (5′-ATACG ACTCA CTATA GGG-3′) for the lower half.

Site-directed mutagenesis substituting the threonine residue of AATPAP with an alanine was carried out using a procedure similar to the one described above. N- and C-[AAAPAP]secFGF were respectively constructed by PCR using N- and C-[AATPAP]secFGF as templates with primers #159 and #206 (5′-ATTAG CTGGC GCCGG AGCTG CTGC-3′) for the upper half and #210 (5′-GCAGC TCCGG CGCCA GCTAA TTAC-3′) and #116 for the lower half of N-[AAAPAP]secFGF and primers #159 and #207 (5′-TGCAG GAATT CTTTA TGGCG CCGGA GCTGC TGC-3′) for the lower half of C-[AATPAP]secFGF, respectively.

Transfection of DNA and concentration of various forms of secreted FGF-1

CHO-K1 or ldlD mutant CHO cells were transfected with the expression vector using DOSPER reagent (Roche Diagnostics, Basel, Switzerland), selected for resistance to G418 (1 mg/ml) and cloned. The growth medium was then replaced by ASF104 serum free medium while the cells were sub-confluent. Thereafter, the medium was changed daily, and the conditioned medium was pooled for later analysis [11].

The various forms of secFGF secreted into the medium were recovered by absorption with heparin-Sepharose CL-6B (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden) beads at 4 °C overnight. The beads were then washed extensively with phosphate buffer containing 0.5 M NaCl 0.001% CHAPS and 1 mM EDTA; bound proteins were eluted with phosphate buffer containing 2.5 M NaCl. Eluted fractions were desalted on a PD-10 column (Amersham Pharmacia Biotech Ltd.), after which the peak fractions, monitored by absorbance at 280 nm, were pooled and subjected to further analysis.

Analysis of secreted forms of FGF-1

The concentration of the various forms of FGF-1 secreted by transfectants into the surrounding medium was determined by enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti FGF-1 antibody (mAb-1: [12]) and HRP-conjugated goat anti mouse IgG (Zymed Laboratories Inc., CA, USA). An aliquot of each sample was denatured with 2-mercaptoethanol and subjected to 12.5% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, NH, USA) and after blocking with 5% skim milk solution, the filter was labeled with mAb-1 antibody and visualized using HRP-conjugated anti-mouse IgG and ECL (Amersham Pharmacia Biotech Ltd.). Sialidase digestion was carried out by mixing samples with immobilized sialidase (Vibrio cholerae; Sigma, MO, USA) after dilution with 0.5 M acetate buffer (pH 5) and then incubating the samples overnight at 37 °C. Peptide *N*-glycosidase F (PNGase) digestion

was carried out according the manufacturer's (Roche Diagnostics) instructions.

Mitogenic assay of expressed secFGF-1

HUVECs were seeded onto collagen type I-coated, 96-well plates at 2×10^3 cells/well in 0.1 ml of culture medium. After 24 hours, the cells were syncronized at the G_0 phase by removing recombinant FGF-1 and heparin for 24–36 hours. The starved cells were stimulated with assay sample in the presence or absence of 5 μg/ml heparin for 18 hrs and then labeled with 1 μCi of [methyl-^{3}H]-thymidine (7 Ci/mmol, Moravek Biochemicals, Inc., CA, USA) per well for 6 hours. Once the cells were metabolically labeled, they were harvested using an automatic cell harvester and the incorporated radioactivity was counted using a Microbeta system (Wallac, Inc., MD, USA).

Results

Expression of two forms of [AATPAP]secFGF in CHO-K1 cells

We used secFGF to determine whether the sequence AATPAP (Ala-Ala-Thr-Pro-Ala-Pro) serves as an acceptor for *O*-glycosylation, which enabled us to take advantage of the availability of structural information [13,14], a specific antibody [12], a sensitive biological assay system [6] and an efficient purification procedure [7]. There is no predictable *O*-glycosylation site in the structure of secFGF. The usefulness of secFGF as a model protein for glycosylation was confirmed by showing that the secFGF derivative containing a potential *N*-glycosylation signal was secreted as *N*-glycosylated forms. When expressed in mammalian cells, secFGF is translated as a single chimeric polypeptide composed of a signal peptide and a FGF-1 core peptide (Fig. 1). Upon translation, secFGF is co-translationally translocated into the endoplasmic reticulum (ER) where the signal peptide is cleaved, and the remaining core polypeptide is secreted through ER-Golgi system into the culture medium as a protein essentially having the structure of FGF-1.

The three-dimensional structure of FGF-1 indicates that the core globular structure of the protein is rigid, while the N- and C-terminal regions are flexible and project out from the globular body [13,14]. Therefore to avoid unexpected conformational changes in the protein caused by the introduction of the AATPAP sequence, the sequence was introduced at the N-terminus (N-[AATPAP]secFGF) or the C-terminus (C-[AATPAP]secFGF) of the FGF-1 region (Fig. 1).

N- and C-[AATPAP]secFGF proteins are secreted into culture medium as glycosylated forms

The N- and C-[AATPAP]secFGF proteins secreted into the conditioned medium of CHO-K1 transfectants were concentrated using heparin-Sepharose affinity beads and detected at 21 kDa on immunoblots using an anti-FGF-1 monoclonal antibody (Fig. 2, lanes 1 and 3, respectively), while secFGF was detected at 18.5 kDa (Fig. 2, lane 5). Digestion with sialidase (Fig. 3A), but not PNGase (Fig. 3B), changed the electrophoretic mobility of the N- and C-[AATPAP]secFGF proteins. In contrast, PNGase digestion of *N*-glycosylated secFGF caused its mobility to become identical to that of non-glycosylated secFGF (Fig. 3B), indicating that N- and C-[AATPAP]secFGF proteins were glycosylated with sugar chains that contained sialic acid but were not *N*-glycans.

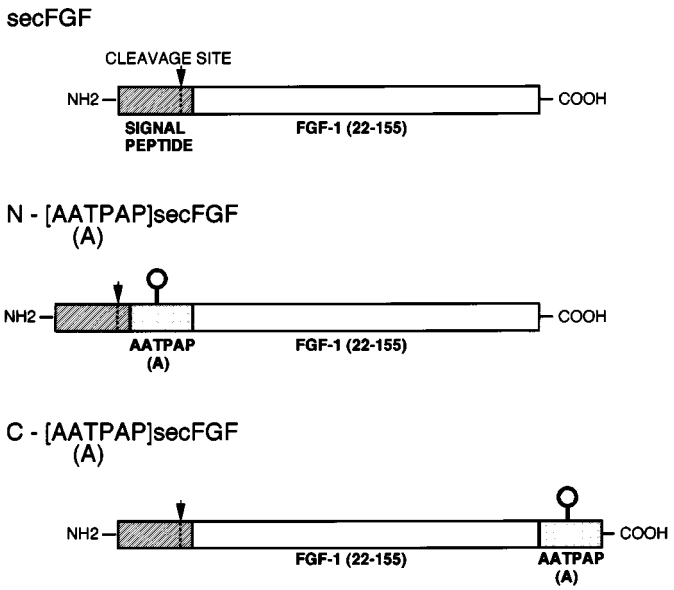

Figure 1. Introduction of the AATPAP sequence into secFGF. Schematic diagram of the structure of the proteins prepared in this study. FGF-1 (22-155) is a naturally-occurring, N-terminal truncated form with full mitogenic activity. The N- and C- [AATPAP]secFGF proteins have the AATPAP sequence attached to the FGF-1 structure at its N- or C-terminus, respectively. Complementary DNAs for the secFGF and [AAT(A)PAP]secFGF proteins were cloned in an eukaryotic expression vector, pMEXneo, and transfected into CHO cells as described in the text. The proteins were secreted into the culture medium after cleavage of the signal peptide and, in some cases, application of an *O*-glycan (indicated by ♀).

Point mutation converting AATPAP to AAAPAP abolished glycosylation

To confirm that the glycosylation induced by the introduction of the AATPAP sequence was on the threonine residue, new constructs were prepared in which the threonine residue was replaced with an alanine residue (Fig. 1). The respective expression vectors were transfected into CHO-K1 cells and the proteins secreted from the transfectants were analyzed as described. Fig. 2, lanes 2 and 4, show that both N- and C-[AAAPAP]secFGF migrate to the 19.5 kDa position, which corresponds to the calculated sizes of the

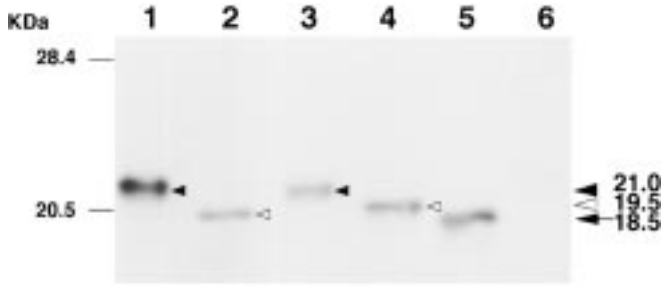

Figure 2. N- and C-[AATPAP]secFGF proteins are expressed and secreted by CHO cells as heavily modified forms in which the modifying group is on the threonine residue. Conditioned supernatants of the CHO transfectants were analyzed for the production of various forms of [AAT(A)PAP]secFGF. The N-[AATPAP]secFGF (lane 1), N-[AAAPAP]secFGF (lane 2), C-[AATPAP]secFGF (lane 3) and C-[AAAPAP]secFGF (lane 4) proteins secreted by CHO-K1 transfectants were absorbed by heparin-Sepharose beads, resolved by SDS-PAGE, transferred to a nitrocellulose membranes and immunoblotted with an anti-FGF-1 antibody. The supernatants of secFGF transfectants (lane 5) and mock transfectants (lane 6) were also analyzed. Positions of molecular weight markers are indicated by bars on the left. Closed and open arrowheads indicate the positions of the modified and unmodified forms of the [AAT(A)PAP]secFGF proteins, respectively, with their sizes. The position of secFGF is indicated by an arrow. Note that all signals were detected as modified forms for the N- and C-[AATPAP]secFGF proteins (lanes 1 and 3).

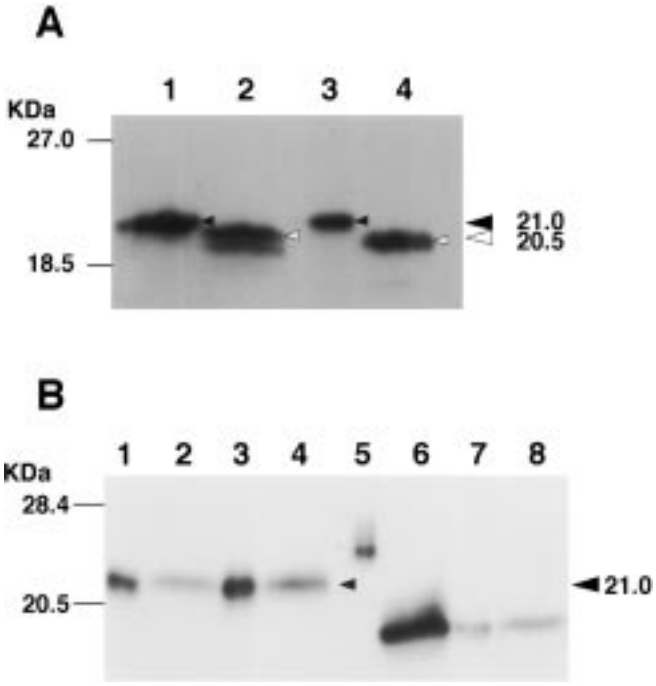

Figure 3. N- and C-[AATPAP]secFGF glycoproteins are sensitive to sialidase but not to PNGase. A, Sialidase digestion. Secreted N-[AATPAP]secFGF (lanes 1 and 2) and C-[AATPAP]secFGF (lanes 3 and 4) proteins were either not treated (lanes 1 and 3) or digested with sialidase at 37 °C for 18 hours (lanes 2 and 4). The proteins were then analyzed as described in the legend to Fig. 2. Positions of molecular weight markers are indicated by bars on the left. Closed and open arrowheads indicate the positions of the untreated and digested forms of the [AATPAP]secFGF proteins, respectively, with their sizes. **B, PNGase digestion.** Secreted N-[AATPAP]secFGF (lanes 1 and 2), C-[AATPAP]secFGF (lanes 3 and 4), secFGF mutant protein with an *N*-glycosylation sequence (lanes 5 and 6) and secFGF protein (lanes 7 and 8) were either not treated (lanes 1, 3, 5, and 7) or digested with PNGase (lanes 2, 4, 6, and 8). The proteins were then analyzed as described in the legend to Fig. 2. Positions of molecular weight markers are indicated by bars on the left. The arrowhead indicates the position of the [AATPAP]secFGF proteins.

core polypeptides of N- and C-[AAA(T)PAP]secFGF. The 21 kDa molecular masses obtained for the N- and C-[AATPAP]secFGF proteins (Fig. 2, lanes 1 and 3) likely reflect the presence of modifying groups (~1.5 kDa) on the core polypeptides at the threonine residue in the AATPAP sequence.

Expression of N- and C-[AATPAP]secFGF in CHO ldlD mutant cells confirmed mucin-type O-glycosylation

To further confirm that the secFGF modification was *O*-glycosylation, ldlD mutant CHO cells, which lack the capacity to synthesize sugar chains containing N-acetylgalactosamine (GalNAc) were used as expression hosts. These cells have a defect in UDP-Gal/UDP-GalNAc 4-epimerase [15,16]; consequently, if the salvage pathway is abolished by removing GalNAc from the culture medium, they do not synthesize *O*-glycans initiated with UDP-GalNAc. The expression vectors encoding N-[AAT(A)PAP]secFGF (Fig. 4A) and C-[AAT(A)PAP]secFGF (Fig. 4B) were respectively transfected into four sets of ldlD mutant cells. The transfectants were then cultured in the presence or absence of 200 μM galactose and/or 20 μM GalNAc in ASF104 serum free medium, and secreted proteins were concentrated and analyzed. We found that N- and C-[AATPAP]secFGF expressed in the absence of GalNAc showed the same respective electrophoretic mobilities (19.5 kDa) as N- and C-[AAAPAP]secFGF (Fig. 4A and B). When GalNAc was added to the culture medium, the molecular masses of N- and C-[AATPAP]secFGF increased to 21 kDa, thereby indicating that GalNAc is a crucial component in the glycosyl modification on N- and C-[AATPAP]secFGF. In contrast, the mobilities of the mutant N- and C-[AAAPAP]secFGF proteins did not change, regardless of the host cells or the presence of GalNAc in the culture medium (Figs. 4A and B). Thus, modification on the N- and C-[AATPAP]secFGF proteins was confirmed to be mucin-type *O*-glycosylation on the threonine residue in the AATPAP sequence.

The two forms of O-glycosylated [AATPAP]secFGF proteins are strong mitogens for endothelial cells

Because secFGF is a potent vascular endothelial cell mitogen, we investigated the mitogenicity of the two forms of

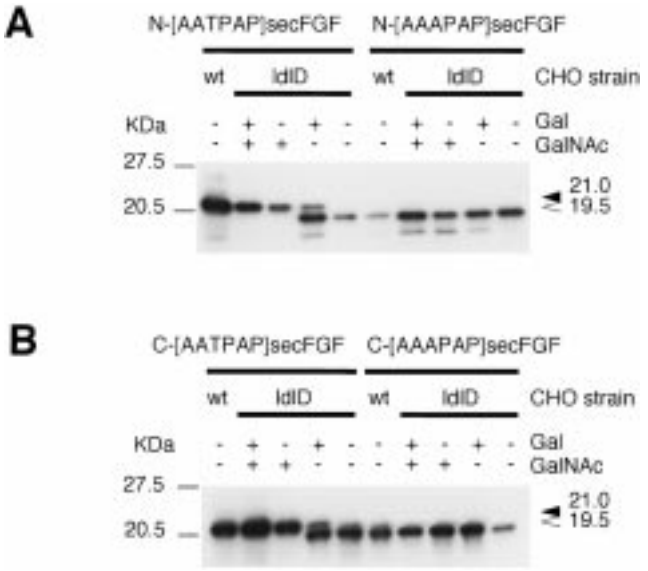

Figure 4. The sugar chains of the N-[AATPAP]secFGF and C-[AATPAP]secFGF glycoproteins require GalNAc for extension. A, N-[AATPAP]secFGF protein harbors a GalNAc-containing sugar chain. Transfectants of CHO-K1 or 1dlD mutant cells expressing N-[AATPAP]secFGF or N-[AAAPAP]secFGF were cultured in the presence or absence of 200 μM Gal and/or 20 μM GalNAc as indicated. Secreted proteins were analyzed as described in the legend to Fig. 2. **B, C-[AATPAP]secFGF protein harbors a GalNAc-containing sugar chain.** Transfectants of CHO-K1 or 1dlD mutant cells expressing C-[AATPAP]secFGF or C-[AAAPAP]secFGF were cultured in the presence or absence of 200 μM Gal and/or 20 μM GalNAc as indicated. Secreted proteins were analyzed as described in the legend to Fig. 2. Positions of molecular weight markers are indicated by bars on the left. Closed and open arrowheads indicate the positions of the modified and unmodified forms of the [AAT(A)PAP]secFGF proteins, respectively, with their sizes.

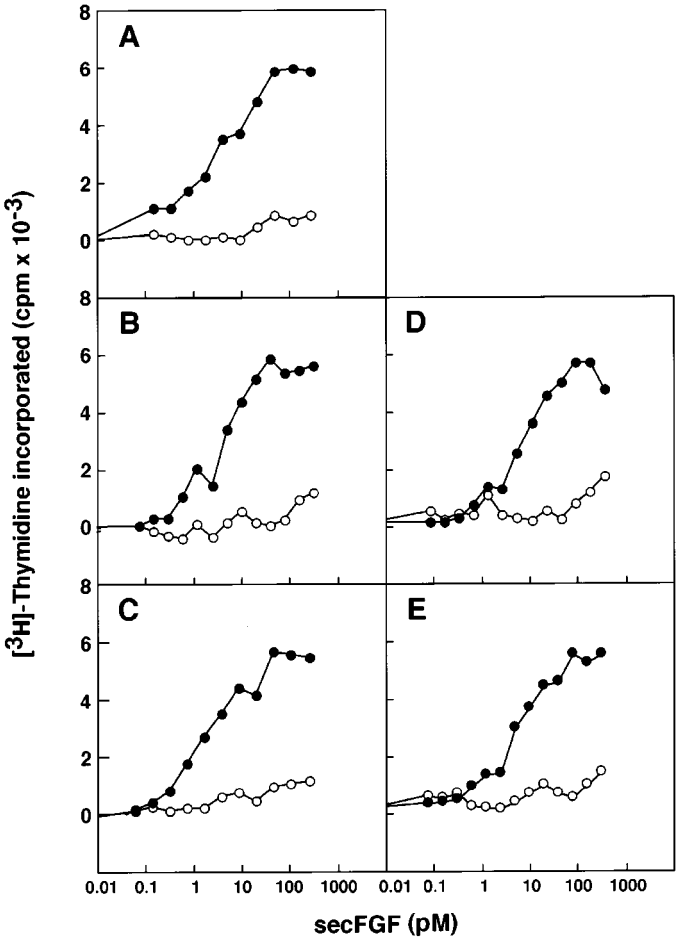

Figure 5. secFGF and [AATPAP]secFGF proteins are equally mitogenic toward endothelial cells. The secFGF (A), N-[AATPAP]secFGF (B), C-[AATPAP]secFGF (C), N-[AAAPAP]secFGF (D) and C-[AAAPAP]secFGF (E) proteins secreted from CHO-K1 transfectants were purified as described in the text. Concentrations were determined, and mitogenic activity toward HUVECs was analyzed by [^{3}H]-thymidine incorporation in the presence (closed circles) or absence (open circles) of heparin (5 μg/ml). Values represent means of triplicate samples; standard deviations were within 3% of the means. Multiple experiments using different preparations of the sample proteins yielded essentially the same results.

O-glycosylated secFGF. As shown in Fig. 5, both N- and C-[AATPAP]secFGF were potently mitogenic toward HUVECs (Fig. 5). The mitogenicity was equivalent to that of FGF-1 simple protein (Fig. 5A) and was potentiated in the presence of heparin in a manner similar to that of FGF-1. In addition, N- and C-[AAAPAP]secFGF possessed activity equal to that of N- and C-[AATPAP]secFGF, respectively. Culture medium of the mock CHO transfectants did not contain mitogenic activity (data not shown). Our findings indicate that neither insertion of the AATPAP sequence nor *O*-glycosylation on the sequence affected the mitogenic activity of FGF-1 significantly.

Discussion

Although the XTPXP peptide motif has been identified as an effective acceptor of GalNAc-transferase 1 in small synthetic oligopeptides *in vitro* [4,17], its effectiveness *in vivo* has remained uncharacterized, and the effect of neo-*O*-glycosylation on the activity of biologically active polypeptides is as yet unknown. We demonstrate in this study that the AATPAP sequence serves as an efficient signal for mucin-type *O*-glycosylation in a biologically active polypeptide (secFGF) expressed in mammalian cells. Furthermore, we succeeded in obtaining neo-*O*-glycosylated derivatives of secFGF that retain full biological activity.

The identity of the sugar modifications on the [AATPAP]secFGF proteins are confirmed to be mucin-type O-glycans by the following findings: (1) replacement of the threonine residue of AATPAP with an alanine residue abolished modification; (2) the sugar chain contained a GalNAc residue that was crucial for extension of the chain; and (3) the sugar chain is sensitive to sialidase but not to

PNGase. Sialidase-treated *O*-glycans were not further digested by *O*-glycanase, which is specific for *O*-glycans that have Gal β1-3 GalNAc (data not shown). Thus, although we still do not know the precise structures of the *O*-glycans on [AATPAP]secFGF proteins, the aforementioned results and the sizes of the sugar chains (1.5 kDa) suggest that the dominant form of the *O*-glycans is composed of a few saccharides with one or more sialic acid(s) at the nonreducing end.

The efficacy of the AATPAP sequence is remarkable; almost all the [AATPAP]secFGF proteins migrated as *O*-glycosylated forms in electrophoresis (Figs. 2–4). Our selection of the sites for insertion of the AATPAP sequence may have also favored efficient *O*-glycosylation; when applied to *O*-glycosylation prediction software (www.cbs.dtu.dk/services/NetOGlyc/[18–20]), our neoprotein designs yielded very high values for both sites (data not shown). We also made use of available information on the three dimensional structure of FGF-1 to avoid any unexpected loss of the biological activity caused by introduction of the AATPAP sequence.

The efficiency of the *O*-glycosylation machinery in CHO cells may be another factor determining the overall efficiency. By using polymerase chain reaction primers for mouse GalNAc-T1, we have detected high-level expression of this enzyme in the CHO-K1 host cells (data not shown). This findings agrees with the efficient *O*-glycosylation of the AATPAP-containing peptides by bovine GalNAcT-1 *in vitro* [4]; however, the enzyme responsible for the efficient *O*-glycosylation in CHO cells remains to be elucidated because Chinese hamster GalNAcT-1 may not have a similar acceptor specificity to that of bovine or human. The availability of mutant cells with specific defects in particular parts of the glycosylation machinery also favored the use of this cell line for study. Other advantages of using CHO cells as expression hosts include the facts that they are well known to express exogenous proteins and that serum free medium for CHO cells is well characterized and widely available. Because CHO cells are adaptable and can be used to enhance expression levels in gene amplification systems, future expansion of the present study for the purpose of large-scale production of *O*-glycosylated proteins is possible.

The successful introduction of *O*-glycans into a biologically active polypeptide while maintaining the original activity shows a promising new approach for future creation of neo-*O*-glycosylated protein drugs. However, because *O*-glycans are believed to affect the pharmacodynamics and/or physical stability of the core polypeptides, whether the nature of secFGF is modified by neo-*O*-glycosylation as compared to the original polypeptide is a subject for future study.

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