Endocrine, *vol. 24, no. 1, 25–31, June 2004* 0969–711X/04/24:25–31/$25.00

Human α-Subunit Analogs Act as Partial Agonists to the Thyroid-Stimulating Hormone Receptor

Differential Effects of Free and Yoked Subunits

Krassimira Angelova,[1] Valerie Fremont,[2] Renita Jain,[1] Meng Zhang,[2] David Puett,[1] Prema Narayan,[1,*] and Mariusz W. Szkudlinski[2,*]

[1]*Department of Biochemistry and Molecular Biology, University of Georgia, Life Sciences Building, Athens, GA 30602, USA;* [2]*Section of Protein Engineering, Laboratory of Molecular Endocrinology, IHV-UMBI, University of Maryland, Baltimore, MD 21201, USA and Research and Development Group, Trophogen, Inc., Rockville, MD 20850, USA*

The α-subunit is common to the heterodimeric glycoprotein hormones and has been highly conserved throughout vertebrate evolution. In an effort to determine if wild-type and engineered human α analogs can serve as agonists or antagonists to the human thyroid-stimulating hormone (TSH) receptor (TSHR), a potent α mutant, obtained by replacing four amino acid residues with lysine (α4K), was assayed and compared with the wild-type α-subunit. When added to CHO cells expressing TSHR, α4K, and to a very limited extent the fused homodimer, α4K-α4K, but not α, exhibited agonist activity as judged by cAMP production. When yoked to TSHR to yield fusion proteins, neither α, α4K, α-α, nor α4K-α4K activated TSHR, although yoked α4K and α4K-α4K were weak inhibitors of TSH binding to TSHR. The yoked subunit–receptor complexes were, however, functional as evidenced by increased cAMP production in cells co-expressing human TSHβ and α-TSHR, α4K-TSHR, α-α-TSHR, and α4K-α4K-TSHR. These results demonstrate that agonists to TSHR can be obtained with α-subunit analogs and suggest that rational protein engineering may lead to more potent α-based derivatives. The differences found between the experimental paradigms of adding free α analogs to TSHR and covalent attachment are attributed to conformational constraints imposed by fusion of the α-subunit analog and receptor, and may suggest an important role for a free (C-terminal) α-carboxyl in the absence of the β-subunit.

Key Words: TSH; TSHR; fusion protein; glycoprotein hormone α-subunit analogs.

Introduction

The classical glycoprotein hormone family is highly conserved in vertebrates *(1)* and consists of four members, TSH and three gonadotropins, LH, FSH, and CG *(2)*. The individual hormones are heterodimers containing a common α-subunit and a hormone-specific β-subunit, both of which are glycosylated. Interestingly, another member of this family, present in vertebrates and invertebrates, was identified by comparative genomic analysis *(3)* and shown to be capable of activating the TSH receptor (TSHR) *(4)*. The distinct gene products of the glycoprotein hormone family are members of a larger family of cystine knot-containing growth factors that act as homodimers and heterodimers *(2,5)*. The glycoprotein hormones bind to and activate three distinct G protein-coupled receptors—TSHR, FSHR and LHR—to which CG also binds *(2)*.

Crystallographic structures of human (h) CG *(6–8)* and hFSH *(9)* demonstrated that the conformations of the α and β subunits were similar, suggesting a common evolutionary origin. As the α-subunit has remained common and highly conserved throughout evolution, there is considerable interest in elucidating possible biological functions of α that could be mediated via the three glycoprotein hormone receptors, or possibly other receptors. Peng et al. *(10)* showed that the α-subunit, derived from bovine (b) LH, was capable of forming homodimers, and physiological activity has been attributed to the α-subunit *(11)*. In addition, a number of early studies suggested direct, albeit weak, effects of α on LHR binding, but these studies were compromised by the contamination of subunit preparations with intact heterodimer *(12)*. Because it is possible to produce constitutive activation of LHR by yoking or fusing a single-chain gonadotropin, e.g., hCGβ-α or α-hCGβ, to the LHR N-terminus *(13–15)*, Narayan et al. *(16)*, studying potential actions of α and hCGβ, characterized complexes of

Received January 10, 2004; Revised March 18, 2004; Accepted April 7, 2004.

*Authors to whom all correspondence and reprint requests should be addressed: Prema Narayan, Department of Biochemistry and Molecular Biology, University of Georgia, Life Sciences Building, Athens, GA 30602, USA, and Mariusz W. Szkudlinski, Section of Protein Engineering, Laboratory of Molecular Endocrinology, IHV-UMBI, University of Maryland, Baltimore, MD 21201, USA and Research and Development Group, Trophogen, Inc., Rockville, MD 20850, USA. E-mail: narayan@bmb.uga.edu or mszkudlinski@trophogen.com

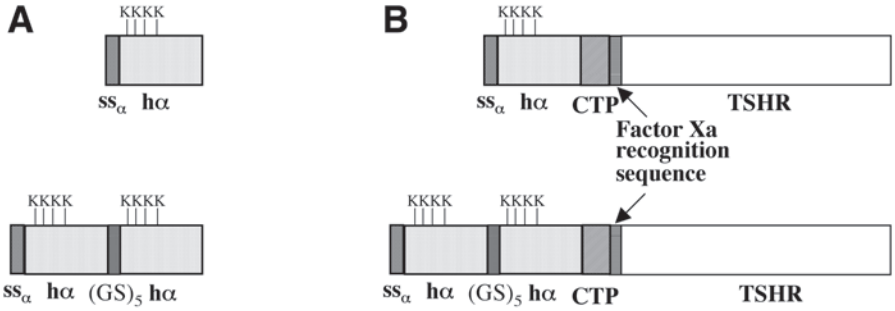

Fig. 1. Schematic of free α-subunit analogs and yoked TSHR constructs. (**A**) The free human (h) α-subunit monomer and single-chain homodimer in which the α-subunits are linked by alternating Gly-Ser residues [$(GS)_5$]. The substitution of Q13, E14, P16, and Q20 with K in the 4K analog is shown. (**B**) The monomeric or homodimeric wild-type hα-subunit or the α-4K analog was linked to hTSHR via the CTP (30 amino C-terminal peptide sequence of hCG) and a Factor Xa recognition sequence. The signal sequence of the hα-subunit is denoted as ssα.

yoked α-LHR and hCGβ-LHR and found no intrinsic activity in the individual subunits using the subunit–receptor fusion system; moreover, co-transfection of LHR with α or hCGβ failed to give receptor activation.

In order to investigate the possible effects of human α-subunit monomers and homodimers on human TSHR, we determined receptor activation in response to free and yoked recombinant α and single-chain α homodimers (wild type and analogs). The use of recombinant subunits alleviates the aforementioned problem of contamination with intact heterodimer. Mutants of the α-subunit were chosen that have been shown to confer exceptionally high potency to TSH *(17)*. In particular, the α-subunit mutant, with Lys replacements of Gln-13, Glu-14, Pro-16, and Gln-20 (α4K), was analyzed both in monomeric and single-chain homodimer forms. Our hypothesis is that an effect of α, albeit expected to be of low potency, may be detected in TSHR since this receptor appears to be less constrained in the inactive state than LHR *(18–20)*.

Results

Expression and Characterization of Monomeric and Homodimeric (Fused) Wild-Type and Mutant α-Subunits

The wild-type and engineered human α-subunits (Fig. 1A) were expressed in CHO-K1 cells, and concentrations were estimated by immunoreactivities to a panel of different monoclonal and polyclonal antibodies *(17)*. The average expression levels in the media from several transient transfections were as follows: wild-type α, 2.8 μg/mL; α4K, 0.2 μg/mL; and α4K-α4K, 0.04 μg/mL.

Wild-type human TSH and human α-subunit, as well as various analogs, were added to the media of CHO cells expressing TSHR, followed by determination of cAMP in the media after 2 h and representative dose response plots are shown in Fig. 2. The results indicate that, at the highest concentrations assayed, wild-type α was unable to activate TSHR; however, α4K and α4K-α4K elicited modest and weak responses, respectively, compared to TSH. Preliminary results with a mutant containing six Lys replacements, α4K + N66K + G73K *(21)*, indicated that it too was effective in activating TSHR, e.g., at approx 20 ng/mL the cAMP response was about 19 pmol/mL, well above basal levels (data not shown). The limited quantities of recombinant materials unfortunately prevented full dose–response measurements with determinations of EC_{50} values for the α analogs.

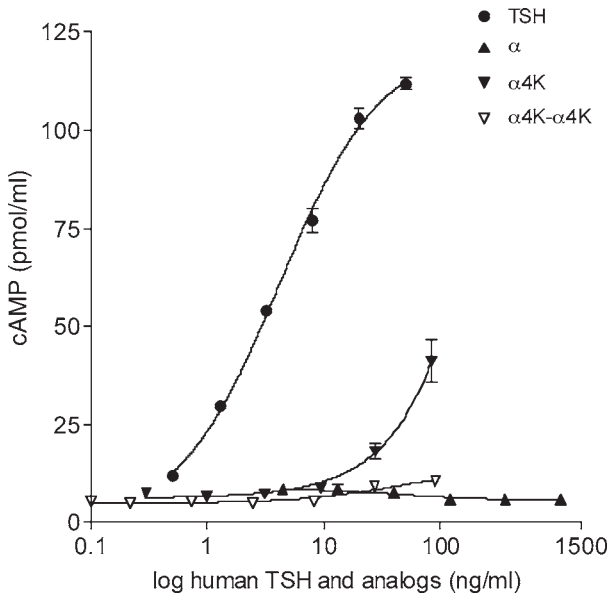

Fig. 2. Activation of TSHR by hTSH and α-subunit analogs. Using hTSH as a positive control, the media from CHO-K1 cells expressing wild-type α, α4K, and α4K-α4K were added to TSHR-expressing CHO cells (JPO9), followed by measurement of cAMP. Representative plots are shown from several independent transfections. Similar studies were performed with wild-type α, α4K, and hTSH using CHO cells devoid of TSHR (JPO2 cells) and with concentrated media from CHO-K1 cells that had been transfected with empty vector added to TSHR-expressing cells (JPO9 cells); in neither case was there an elevation of cAMP (data not shown).

These effects, however, appear to be specific for TSHR, because (a) addition of comparable amounts of α (wild type and analogs) to control CHO cells (JPO2), i.e., with no TSHR, failed to elicit a response, and (b) concentrated media from CHO-K1 cells transfected with empty vector did not stimulate cAMP production in cells expressing TSHR (JPO9) (data not shown).

Expression and Characterization of Monomeric and Homodimeric (Fused) Wild-Type and Mutant α-Subunits Yoked to TSHR

As an alternative to expressing high levels of the α-subunits and analogs, we generated fusion proteins of the subunits with TSHR. This strategy permits the study of receptor activation by subunits that may bind with low affinity *(16, 32)*. The yoked constructs (Fig. 1B) were transiently transfected into COS-7 cells and cell surface expression of several yoked constructs was monitored by two methods, binding of an anti-TSHR antibody and binding of ^{125}I-bTSH (Fig. 3A). Overall, the relative expression was similar as measured by the two techniques. Basal and maximal TSH-mediated cAMP levels in cells expressing TSHR and the four yoked complexes are shown in Fig. 3B. There is no evidence of constitutive activation of the receptor by fused α, α4K, α-α, and α4K-α4K, and all are responsive to added TSH. In Fig. 3C the maximal cAMP obtained after addition of TSH is presented after correction for the expression level based on immunoreactivity with the TSHR antibody and on ^{125}I-bTSH binding. To facilitate comparisons, wild-type TSHR was normalized to 100% in each case. These results indicate that the variations in TSH-mediated maximal cAMP production result from the different TSHR expression levels. A similar conclusion is obtained for basal cAMP production when corrected for ^{125}I-bTSH binding (data not shown).

In order to ascertain if yoked α and α analogs altered TSHR responsiveness to exogenous TSH, competitive binding (with ^{125}I-bTSH) and cAMP measurements were made at various concentrations of TSH (see Fig. 4A,B for representative plots of competitive binding and cAMP production, respectively). The IC_{50}s, EC_{50}s, and coupling ratios, i.e., IC_{50}/EC_{50}s, are summarized in Table 1 for all experiments. The IC_{50} values for TSHR, α-TSHR, and α-α-TSHR ranged from about 65 to 85 p*M*, and the EC_{50}s were 85–95 p*M*, leading to coupling ratios of 0.7–1.0. The IC_{50}s of α4K-TSHR and α4K-α4K-TSHR were, on the other hand, somewhat higher,

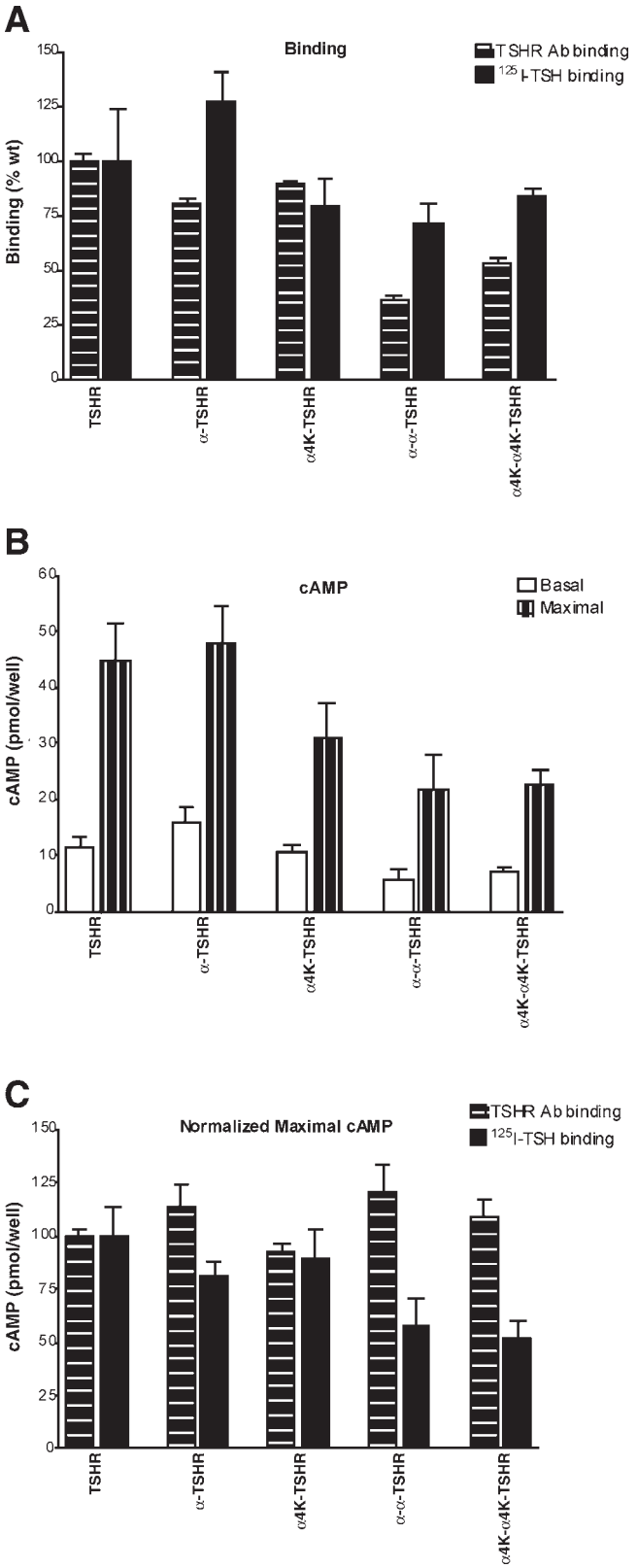

Fig. 3. Cell surface expression and signaling properties of yoked wild-type α-TSHR and analogs. (**A**) Expression based on immunoreactivity of α-TSHR (wild type and analogs) with a polyclonal antibody directed against the extracellular domain of TSHR and determined by binding of ^{125}I-bTSH to TSHR in the yoked products, i.e., B_0. In both cases, the expression of wild-type TSHR is normalized to 100% to facilitate comparisons. (**B**) Basal levels of cAMP and maximal TSH-mediated cAMP production in transfected COS-7 cells expressing wild-type and analog α-TSHRs. Maximal cAMP production was the response to a saturating concentration of TSH (100 ng/mL). (**C**) Maximal TSH-mediated cAMP levels in transfected COS-7 cells corrected for surface expression via TSHR immunoreactivity and ^{125}I-bTSH binding. To facilitate comparisons, the maximal amount of cAMP per unit expression was normalized to 100% in each system.

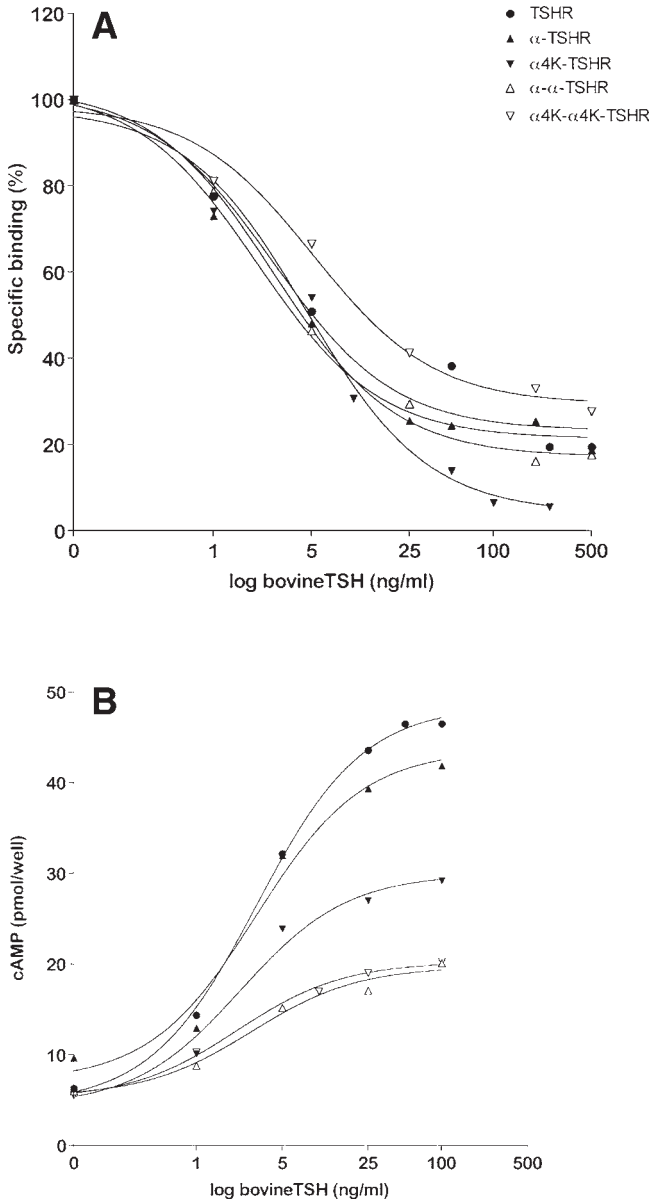

Fig. 4. Representative plots of competitive TSH binding and cAMP response of yoked wild-type α-TSHR and analogs. (**A**) Competitive binding with 100–200 p*M* ^{125}I-bTSH and various concentrations of bTSH. (**B**) cAMP production by various concentrations of bTSH. The IC_{50} and EC_{50} values from all experiments are given in Table 1.

Table 1
IC_{50} and EC_{50} Values
of Wild-Type and Yoked Human TSHR[a]

TSHR	IC_{50} (*n*), p*M*	EC_{50} (*n*), p*M*	IC_{50}/EC_{50}
TSHR (wild type)	65.8 ± 10.1 (4)	95.7 ± 3.5 (3)	0.7
α-TSHR	64.0 ± 8.6 (4)	88.3 ± 7.9 (3)	0.7
α-α-TSHR	83.8 ± 8.9 (4)	85.3 ± 4.6 (3)	1.0
α4K-TSHR	145.0 ± 17.0 (2)	94.0 ± 7.0 (3)	1.5
α4K-α4K-TSHR	149.7 ± 27.7[b] (3)	91.7 ± 10.3 (3)	1.6

[a]Wild-type human TSHR and yoked forms were expressed in COS-7 cells, followed by competitive binding (with bTSH and ^{125}I-bTSH) and cAMP measurements at various concentrations of bTSH. The IC_{50}s and EC_{50}s are given as mean ± SEM with the exception of the IC_{50} of α4K-TSHR, which is shown as mean ± range.

[b]Significantly different from wild-type TSHR (the IC_{50} of α4K-TSHR is also much higher than that of wild-type TSHR, but the limited *n* prohibits a statistical analysis).

145–150 p*M*, while the EC_{50}s were comparable, 90–95 p*M*, giving coupling ratios of 1.5–1.6. These results suggest that, while not activating TSHR as evidenced by the lack of an increase in basal cAMP production, the fusion of α4K and α4K-α4K to TSHR is slightly inhibitory to TSH binding, but not TSH-mediated cAMP production.

Responsiveness of α-TSHR Analogs to TSHβ

In order to examine the functionality to TSHβ of yoked complexes of α, α4K, α-α, and α4K-α4K to TSHR, cells were co-transfected with cDNAs to each of the subunit–TSHR constructs and TSHβ. Figure 5 shows a summary of the results on specific ^{125}I-bTSH binding (A), basal cAMP production (B), and maximal cAMP concentrations (C). Notably, the presence of TSHβ does not alter basal cAMP levels of control wild-type TSHR, but the yoked α-TSHR analogs exhibit increased levels of cAMP in the presence of TSHβ (Fig. 5B). These cAMP levels are similar to the maximal level of cAMP obtained with a saturating dose of exogenous TSH (Fig. 5C). Co-transfection with TSHβ did not produce an additional increase in cAMP levels. These results suggest that the fusion of α, α4K, α-α, and α4K-α4K to TSHR still permits a functional interaction with TSHβ, and the functional complex formed by interaction of the co-transfected TSHβ subunit with the yoked subunit–receptor is similar to that formed by the exogenously added heterodimer.

Discussion

These results are the first to demonstrate that monomeric and fused homodimeric human α analogs are weak agonists to human TSHR. Such findings are important in delineating structure–function relationships between the hormone and receptor and suggest that the design of hormone analogs may be feasible.

A comparison of the effects of free and TSHR-yoked wild-type α and various mutant forms yielded some surprising differences. α4K and, to a lesser extent, α4K-α4K, acted as partial agonists when added to TSHR. In contrast, when yoked to TSHR, neither elevated basal cAMP levels nor altered TSH-mediated cAMP production. It is of course conceivable that weak activation of yoked subunit–recep-

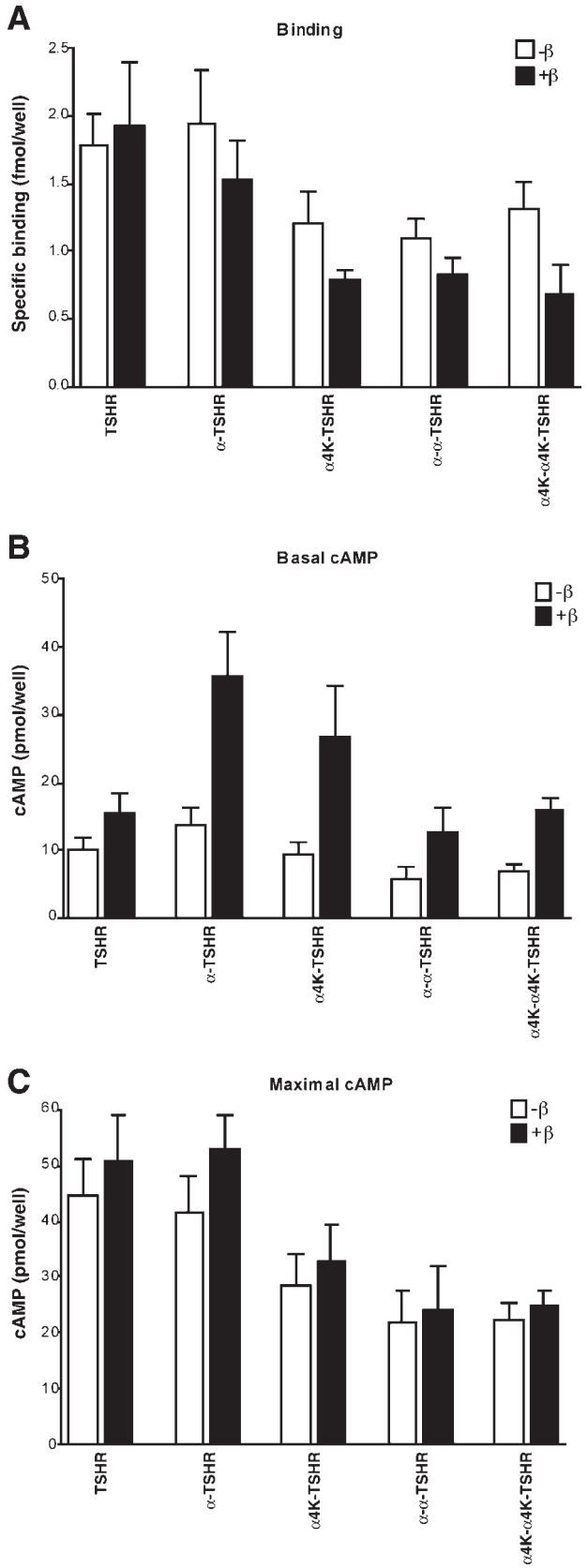

Fig. 5. Responsiveness of α-TSHR analogs to TSHβ. Cells expressing TSHR, α-TSHR, α4K-TSHR, α-α-TSHR, and α4K-α4K-TSHR, along with TSHβ (via co-transfection), were used to measure specific ^{125}I-bTSH binding (**A**), basal cAMP (**B**), and maximal hTSH-mediated production, cAMP (**C**).

tor complexes could be masked by the different expression levels compared to wild-type TSHR. This caveat aside, there appears, nonetheless, to be differences in the actions of α4K when free and yoked to TSHR. A weak inhibition of TSH binding to the α4K-TSHR and α4K-α4K-TSHR complexes was evidenced by slightly higher IC_{50}s than that of wild-type TSHR. It is noteworthy, however, that α and the α analogs yoked to TSHR were functional as evidenced by activation of the receptor when TSHβ was present. In addition, a previous study indicated that both linked wild-type and analog α-subunit (α6K) decreased constitutive activity of a TSHR deletion mutant devoid of about 98% of the extracellular domain *(19)*.

These various results using free and yoked α derivatives suggest that ligands may act somewhat differently with TSHR when in a free or fused state, presumably due to the conformational constraints imposed by the covalent attachment and the co-expressed β-subunit may release some of these constraints. Another possible contributory factor is that of a free C-terminal α-carboxyl group on the untethered α-subunit that has been eliminated in the fusion proteins. However, this seems less likely considering that receptor activation of the yoked constructs is restored by co-expression with TSHβ.

Of the various α analogs examined, only α4K and, to a lesser extent, α4K-α4K exhibited some type of apparent functionality. The α4K derivative was initially designed and tested to provide potent agonists of hTSH and hCG *(17)*. The heterodimer composed of human α4K and TSHβ was vastly more potent than wild-type TSH, and the potency of the heterodimer of α4K-hCGβ was several fold greater than that of wild-type hCG. Thus, it is not entirely surprising that, of the various analogs tested, the α4K derivative was the one to exhibit effects on TSHR. The results also support many other studies suggesting a direct role of α in receptor binding *(22–30)*, rather than serving simply to alter the conformation of the β-subunits in the heterodimer.

The yoked products obtained by fusing a single-chain hCG to LHR have been studied extensively in intact transfected cells *(13–15)* and more recently in transgenic mice *(31)*. Consistent with the present finding that α-plus-TSHR and yoked α-TSHR failed to elicit receptor activation, Narayan et al. *(16)* showed that α-plus-LHR (via co-transfection) and yoked α-LHR (as well as yoked hCGβ-LHR) were also devoid of constitutive activity. In other structure–function studies using a ligand–LHR fusion system, Schubert and Puett *(32)* found that a single-chain hCG analog consisting of the hCGβ determinant loop and C-terminal peptide (residues 93–145) fused to intact α, and in turn yoked to LHR, resulted in, at best, marginal constitutive activity. Thus, with two receptors, TSHR and LHR (cf. ref. *16*), there is fairly compelling evidence that the wild-type human α-subunit, either free or fused, is unable to activate the receptor. Potent receptor agonists and antagonists, based on the cognate ligand structures, will undoubtedly require analogs,

as demonstrated herein, or fragments from both the α and β subunits as indicated elsewhere *(32)*. The latter suggestion is strengthened by the finding that hCG can weakly activate the two non-cognate receptors, TSHR and FSHR, whether added free or yoked *(33)*.

In summary, this study has shown that α-subunit analogs exhibit agonist activity only in free, non-tethered forms. In contrast, yoked α-subunit analogs were weak inhibitors of TSH binding. Differences found between the experimental paradigms can be attributed to conformational constraints imposed by fusion of the subunit and intact receptor, and suggest an important role of the α-subunit (C-terminal) α-carboxyl group in binding to and activation of TSHR in the absence of TSHβ. This study has not addressed the intriguing possibility of α-subunit monomers or homodimers serving as ligand(s) to other receptors.

Materials and Methods

Cell Culture, Mutagenesis, α-Subunit–Receptor Fusion Constructs, and Transfection

COS-7 cells were grown and maintained as described earlier *(34)*. Two stable lines of CHO cells expressing hTSHR, clone, JP26 (2,000 receptors/cell), and JPO9 (90,000 receptors/cell), were kindly provided by Dr. Gilbert Vassart, Université Libre de Bruxelles, Belgium, as was a TSHR-negative control line (JPO2).

Because replacement of amino acid residues Gln-13, Glu-14, Pro-16, and Gln-20 of human α with Lys results in TSH and hCG heterodimers of increased potency *(17)*, this α analog (α4K) was prepared as a monomer and a single-chain homodimer (α4K-α4K) (Fig. 1A). The human α-subunit mutants were prepared using the megaprimer method *(17)*, and the dimeric α-subunit constructs were made via the PCR-ligation-PCR method *(35)*. The $(Gly\text{-}Ser)_5$ flexible 10-amino acid residue linker was used to maintain close association and relatively unconstrained folding of dimeric α-subunits *(36,37)*. After subcloning into the expression vectors, the entire PCR products were sequenced to verify the mutations and ensure no undesired polymerase errors.

Fusion constructs of the α-subunit analogs (monomer and dimer) and TSHR were generated by standard PCR amplification and cloning methods. The hCGβ carboxy terminal peptide (CTP) sequence consisting of amino acid residues 116–145 and the Factor Xa protease recognition sequence (IEGR) were used to link the subunits to hTSHR (Fig. 1B). The DNA was subcloned into the expression vector pcDNA3 (Invitrogen) and sequenced to ensure accuracy of the cloned sequences. COS-7 cells were transiently transfected (Lipofectamine, Invitrogen Life Technologies) with wild-type α-subunit and mutant fusions with TSHR in pcDNA3 *(17,34)*.

Expression and Quantification of Subunit Analogs

CHO-K1 cells were transiently transfected (Lipofectamine) in 60 mm culture dishes with wild-type or mutant α-subunit cDNAs. After recovery for 12 h in regular growth medium, transfected cells were cultured in CHO-serum-free medium (CHO-SFM, Invitrogen Life Technologies) for 48 h. Subsequently, the conditioned media, including control medium from cells mock transfected with the expression plasmids without cDNA inserts, were harvested and concentrated with Centriprep 10 concentrators (Amicon, Beverly, MA). The concentrations of the analogs were determined with a panel of different monoclonal and polyclonal antibodies recognizing different TSH epitopes as described in detail previously *(17)*.

Cell Surface Expression, TSH Binding, and Signaling

Cell surface receptor expression of wild-type TSHR and fusion proteins was measured by ELISA in transiently transfected COS-7 cells that were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Cells were then incubated for 2 h at 37?C in Dulbecco's modified Eagle's medium containing a polyclonal antibody directed against the TSHR extracellular domain followed by incubation for 1 h with a peroxidase-conjugated goat anti-rabbit antibody as previously described *(19)*. After washing, the peroxidase substrate (TMB, BioFX Labs) was added and incubated for 5 min; the reaction was then stopped by acidification and the absorbance determined at 450 nm.

Competitive binding assays were performed using COS-7 cells expressing wild-type TSHR and fusion products of α (wild type and analogs) with TSHR as described earlier *(17,34)*. ^{125}I-Bovine (b)TSH binding was measured in the presence of various concentrations of bTSH, with corrections being made for non-specific binding. cAMP production in CHO cells and COS-7 cells was determined by radioimmunoassay *(17,34)*. The binding and signaling data were analyzed by Prism software (Graph Pad Software, San Diego, CA) using nonlinear regression analysis. The results represent an average of two to six independent experiments, each performed in duplicate or quadruplicate.

Acknowledgments

We thank Dr. Bruce Weintraub for continuous support and guidance. This work was supported in part by NIH Research Grant DK33973 and by an American Cancer Society Institutional Grant (UMB-MS).

References

1 Li, M. D. and Ford, J. J. (1998). *J. Endocrinol.* **156,** 529–542.
2 Hearn, M. T. W. and Gomme, P. T. (2000). *J. Mol. Recog.* **13,** 223–278.
3 Hsu, S. Y., Nakabayashi, K., and Bhalla, A. (2002). *Mol. Endocrinol.* **16,** 1538–1551.
4 Nakabayashi, K., Matsumi, H., Bhalla, A., et al. (2002). *J. Clin. Invest.* **109,** 1445–1452.
5 Isaacs, N. W. (1995). *Curr. Opin. Struct. Biol.* **5,** 391–395.

6 Lapthorn, A. J., Harris, D. C., Littlejohn, A., et al. (1994). *Nature* **369,** 455–461.
7 Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. (1994). *Structure* **2,** 545–558.
8 Tegoni, M., Spinelli, S., Verhoeyen, M., Davis, P., and Cambillau, C. (1999). *J. Mol. Biol.* **289,** 1375–1385.
9 Fox, K. M., Dias, J. A., and Van Roey, P. (2001). *Mol. Endocrinol.* **15,** 378–389.
10 Peng, K.-C., Puett, D., and Brewer, J. M. (1997). *J. Mol. Endocrinol.* **18,** 259–265.
11 Nemansky, M., Moy, E., Lyons, C. D., Yu, I., and Blithe, D. L. (1998). *J. Clin. Endocrinol. Metab.* **83,** 575–581.
12 Williams, J. F., Davies, T. F., Catt, K. J., and Pierce, J. G. (1980). *Endocrinology* **106,** 1353–1359.
13 Wu, C., Narayan, P., and Puett, D. (1996). *J. Biol. Chem.* **271,** 31638–31642.
14 Narayan, P., Gray, J., and Puett, D. (2000). *Endocrinology* **140,** 67–71.
15 Narayan, P., Wu, C., and Puett, D. (2000). *Methods* **21,** 59–66.
16 Narayan, P., Gray, J., and Puett, D. (2002). *Mol. Endocrinol.* **16,** 2733–2745.
17. Szkudlinski, M. W., Teh, N. G., Grossman, M., Tropea, J. E., and Weintraub, B. D. (1996). *Nature Biotech.* **14,** 1257–1263.
18 Cetani, F., Tonacchera, M., and Vassart, G. (1996). *FEBS Lett.* **378,** 27–31.
19 Zhang, M., Tong, P., Fremont, V., et al. (2000). *Endocrinology* **141,** 3514–3517.
20 Szkudlinski, M. W., Fremont, V., Ronin, C., and Weintraub, B. D. (2001). *Physiol. Rev.* **82,** 473–502.
21 Leitolf, H., Tong, K. P., Grossmann, M., Weintraub, B. D., and Szkudlinski, M. W. (2000). *J. Biol. Chem.* **275,** 27457–27465.
22 Yoo, J., Ji, I., and Ji, T. H. (1991). *J. Biol. Chem.* **266,** 17741–17743.
23 Yoo, J., Zeng, H., Ji, I., Murdoch, W. J., and Ji, T. H. (1993). *J. Biol. Chem.* **268,** 13034–13042.
24 Chen, F., Wang, Y., and Puett, D. (1992). *Mol. Endocrinol.* **6,** 914–919.
25 Liu, C., Roth, K. E., Shepard, B. A. L., Shaffer, J. B., and Dias, J. A. (1993). *J. Biol. Chem.* **268,** 21613–21617.
26 Furuhashi, M., Ando, H., Bielinska, M., et al. (1994). *J. Biol. Chem.* **269,** 25543–25548.
27 Zeng, H., Ji, I., and Ji, T. H. (1995). *Endocrinology* **136,** 2948–2953.
28 Liu, C. and Dias, J. A. (1996). *Arch. Biochem. Biophys.* **329,** 127–135.
29 Xia, H., Chen, F., and Puett, D. (1994). *Endocrinology* **134,** 1768–1770.
30 Grossmann, M., Szkudlinski, M. W., Dias, J. A., et al. (1996). *Mol. Endocrinol.* **10,** 769–779.
31. Narayan, P., Meehan, T., and Puett, D. (2003). *Biol. Reprod.* **68(Suppl. 1),** 212–213.
32 Schubert, R. L. and Puett, D. (2003). *J. Mol. Endocrinol.* **31,** 157–168.
33 Schubert, R. L., Narayan, P., and Puett, D. (2003). *Endocrinology* **144,** 129–137.
34 Angelova, K. and Puett, D. (2002). *Endocrine* **19,** 147–154.
35 Ali, S. A. and Steinkasserer, A. (1995). *BioTechniques* **18,** 746–750.
36. Agros, P. (1990). *J. Mol. Biol.* **211,** 943–958.
37. Heikoop, J. C., Van Beuningen-De Vann, M. M. J. A. C. M., Van Den Boogaart, P., and Grootenhuis, P. D. J. (1997). *Eur. J. Biochem.* **245,** 656–662.