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Crosslinked bifunctional gonadotropin analogs with reduced efficacy

Michael P. Bernard, Win Lin, Rebecca Myers, Donghui Cao, Yongna Xing, William R. Moyle *

Department of OBGYN, Robert Wood Johnson (Rutgers) Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA

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Abstract

The N-linked oligosaccharides on human choriogonadotropin (hCG) and follitropin (hFSH) α-subunit loop 2 (α2) have a dominant influence on hormone efficacy. hCG analogs lacking this oligosaccharide retain approximately 40% the efficacy of the fully glycosylated hormone in cyclic AMP accumulation assays. Previous efforts to reduce efficacy further have involved removing the other N-linked oligosaccharides. We found that some intersubunit disulfide crosslinks reduced the efficacies of hCG analogs lacking only the α2 oligosaccharide. The least active analog was an hCG/hFSH chimera containing hFSH residues 95–108 in place of hCG residues 101–114 and a disulfide bond between α-subunit residue 37 and β-subunit residue 33. While it bound lutropin receptors 2- to 3-fold better than hCG and follitropin receptors 10–30% as well as hFSH, it had less than 10% and 5% the efficacies of either hormone. This suggests that complete deglycosylation will not be required to produce glycoprotein hormone analogs that have low efficacies.

Keywords: Gonadotropin analogs; hCG; hFSH; Bifunctional partial agonist; Disulfide crosslinking

1. Introduction

The decline in the efficacy of hCG following its sequential digestion with neuraminidase, β-galactosidase, N-actylglucosidase, and mannosidase (Moyle et al., 1975) suggested the oligosaccharides of hCG and possibly all glycoprotein hormones might have essential roles in signal transduction. This notion was subsequently confirmed in many laboratories (Fares et al., 1996; Flack et al., 1994; Matzuk et al., 1989; Min et al., 1996; Sairam and Bhargavi, 1985; Valove et al., 1994), to name a few.

The oligosaccharides on both subunits contribute to the full efficacy of hCG, but that on α2 has a greater role in signaling than either of those on β1 (Matzuk et al., 1989). Elimination of the α2 oligosaccharide reduced the efficacy of hCG by approximately 60% (Matzuk et al., 1989). When tested in cell lines that have relatively few LH receptors, analogs of hCG lacking the α2 oligosaccharide and both β1 oligosaccharides had efficacies of 10% or less (Matzuk et al., 1989). Removal of the β-subunit oligosaccharides alone had much less influence on efficacy than removal of the α2 oligosaccharide, however (Matzuk et al., 1989).

We have been attempting to devise bifunctional gonadotropin antagonists, i.e., hormone analogs that interact with LH and FSH receptors to block the actions of LH and FSH (Trout et al., 1999). In theory, bifunctional gonadotropin antagonists may be useful for removing the cystic follicles in polycystic ovary syndrome (PCOS) patients, either by blocking the activities of endogenous LH and FSH directly or, more likely, by delivering apoptosis inducing agents to these unwanted gonadotropin receptor bearing cells. This type of "chemical wedge resection" has the potential to provide the same therapeutic benefit as surgery (Shanti and Murphy, 1997; Stein and Leventhal, 1935) without the risk of adhesion formation. It might also permit the procedure to be repeated whenever the PCOS condition returned in patients seeking to become pregnant or in women having PCOS who wished to reduce ovarian androgen production.

Abbreviations: PCOS, polycystic ovary syndrome; hCG, human choriogonadotropin; LH, lutropin; FSH, follitropin; α1, α2, α3, α-subunit loops 1, 2, and 3; β1, β2, β3, β-subunit loops 1, 2, and 3

* Corresponding author. Tel.: +1 732 235 4224; fax: +1 732 235 4225.
E-mail address: moyle@umdnj.edu (W.R. Moyle).

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Our efforts to develop bifunctional antagonists have been based on the observation that the hCG seatbelt controls its ability to distinguish LH, FSH, and TSH receptors (Campbell et al., 1991; Grossmann et al., 1997; Moyle et al., 1994). Analogs of hCG in which β-subunit residues between cysteines 11–12 are replaced with their FSH β-subunit counterparts bind LH and FSH receptors with high affinities (Moyle et al., 1994). These chimeric analogs can be expressed in a single chain format wherein the α-subunit is fused to the C-terminal end of the chimeric β-subunit (Trout et al., 1999). Elimination of the α2 glycosylation signal of these analogs reduced their efficacies in assays employing cell lines engineered to over express either LH or FSH receptors (Trout et al., 1999). The efficacies of the single chain bifunctional analogs appear to be too high for them to be useful therapeutics, however, and we have been attempting to prepare bifunctional antagonists having lower efficacy by other approaches.

Due to the contributions of the oligosaccharides on both subunits to efficacy, efforts to prepare hCG antagonists usually involve removing them from α2 and β1 (Matzuk et al., 1989). Studies described here were initiated to learn if the efficacy of hCG and bifunctional analogs could be reduced to low levels by removing only the α2 oligosaccharide and were based partly on our notions of hormone receptor induced signaling (Moyle et al., 2004). We report here that some intersubunit disulfides are capable of reducing the efficacies of hCG analogs lacking only the α2 oligosaccharide. This observation conflicts with conclusions of Heikoop et al. (1998), who suggested that the loss in efficacy caused by the absence of the α2 oligosaccharide was due to heterodimer dissociation. We also found that introduction of FSH residues between cysteines 11–12 further reduced the efficacy of the crosslinked analog. Although the efficacies of the resulting bifunctional analogs are lower than those of other partial agonists, we have not yet been able to prepare bifunctional antagonists devoid of signal transduction activity. The observations described here are a significant step in reaching this goal, however, and have important implications for the mechanism of glycoprotein hormone induced signal transduction.

2. Materials and methods

hCG was purified in this laboratory as described (Bahl, 1969) or obtained from Dr. Robert Campbell (Serono Research Institute, Rockland, MA). hFSH used for radioiodination was also obtained from Dr. Campbell. Deglycosylated hCG lacking an intersubunit disulfide was produced by dissociating the hCG heterodimer at low pH, neutralizing the pH with a buffer containing N-glycanase to remove the α2 oligosaccharide, and permitting the deglycosylated α-subunit to recombine with the hCG β-subunit (Xing and Moyle, 2003). The sources of all monoclonal antibodies have been described (Campbell et al., 1991; Cosowsky et al., 1995; Moyle et al., 1995, 1990). Radio-iodinated hormones and monoclonal antibodies were produced using an Iodo-Gen procedure (Bernard et al., 2004).

Constructs that encoded the analogs described here (Table 1) were prepared by standard mutagenesis methods similar to those used earlier (Campbell et al., 1991; Cosowsky et al., 1995; Moyle et al., 1995, 1990) and expressed transiently in COS-7 cells (Campbell et al., 1991). Analogs secreted into the medium were assayed by sandwich immunoassays as described (Moyle et al., 1982), except that α-subunit antibody A113 was used for capture and β-subunit antibody B110 was used for detection. The presence of a disulfide crosslink was determined by monitoring the resistance of the heterodimer to low-pH induced dissociation (Xing et al., 2004a). Receptor-binding and cyclic AMP signal transduction assays used to monitor the activities of these analogs were described earlier (Cosowsky et al., 1995; Moyle et al., 1994, 1995). All dose response curves were analyzed using Prism (GraphPad Software, San Diego, CA). Each study was repeated three or more times and the results described are typical.

Table 1
Nomenclature and structures of the analogs used in these studies

Abbreviation	Analog composition	α2-Oligosaccharide	β-Subunit residues 101–115	β-Subunit amino acids
α5–β8	αQ5C/hCGβR8C	Yes	GGPKDHPLTCDDPRF	145
dgα5–β8	dgαQ5C/hCGβR8C	No	GGPKDHPLTCDDPRF	145
α37–β33	αY37C/hCGβI33C	Yes	GGPKDHPLTCDDPRF	145
dgα37–β33	dgαY37C/hCGβI33C	No	GGPKDHPLTCDDPRF	145
α37–β33CF	αY37C/CF101-109βI33C	Yes	TVRGLGPSYCDDPR	114
dgα37–β33CF	dgαY37C/CF101-109βI33C	No	TVRGLGPSYCDDPR	114
α37–β33CFC	αY37C/CFC101-114βI33C	Yes	TVRGLGPSYCSFGEF	145
dgα37–β33CFC	dgαY37C/CFC101-114βI33C	No	TVRGLGPSYCSFGEF	145
α27–β44	αQ27C/hCGβV44C	Yes	GGPKDHPLTCDDPRF	145
dgα27–β44	dgαQ27C/hCGβV44C	No	GGPKDHPLTCDDPRF	145
α76–β44	αV76C/hCGβV44C	Yes	GGPKDHPLTCDDPRF	145
dgα76–β44	dgαV76C/hCGβV44C	No	GGPKDHPLTCDDPRF	145

Notes: CF101–109 refers to a truncated hCG/hFSH β-subunit chimera in which hCG residues 101–109 are replaced with hFSH residues 95–103. CFC101–114 refers to a full-length hCG/hFSH β-subunit chimera in which hCG residues 101–114 are replaced with hFSH residues 95–108. All other residues correspond to those of hCG β-subunit.

3. Results

The contribution of the α2 oligosaccharide to efficacy may depend on its proximity to the subunit interface (Fox et al., 2001; Lapthorn et al., 1994; Wu et al., 1994). This oligosaccharide is also near the "seatbelt," a 20-residue sequence in the β-subunit that contributes to the abilities of lutropins and follitropins to distinguish their receptors (Campbell et al., 1991; Dias et al., 1994; Grossmann et al., 1997; Moyle et al., 1994). The abilities of antibodies and additional oligosaccharides to enhance the efficacy of hCG analogs lacking the α2 oligosaccharide (Moyle et al., 2004) led us to propose that the efficacy of hCG depends on its ability to increase the distance between portions of the leucine-rich repeat and signaling-specificity domains of the LH receptor extracellular domain. We reasoned that the efficacy of some hCG analogs lacking the α2 oligosaccharide would be reduced further by disulfide bonds that stabilized the heterodimer in a conformation that rendered it less capable of altering this distance.

To test this possibility, we introduced disulfide bonds between intersubunit residues α5–β8, α37–β33, and α76–β44. These did not appear to influence the efficacy of hCG in LH signaling assays (Fig. 1a). Addition of a disulfide between residues α27–β44 appeared to reduce the efficacy of hCG slightly in most, but not all experiments (Fig. 1a; Table 2). These observations extend those reported for fully glycosylated hCG by Heikoop et al. (1997) and show that disulfide crosslinks can be introduced between a residue in β-subunit loop 2 with one in α1 or α3 without preventing hCG binding to LH receptors.

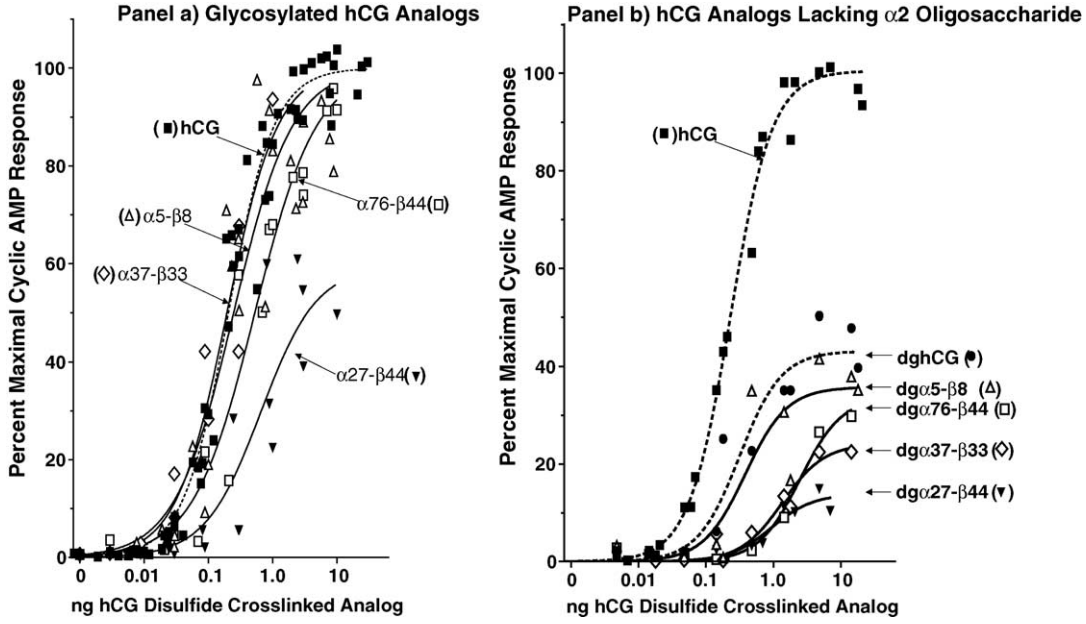

Fig. 1. Influence of intersubunit disulfide bonds on the cyclic AMP accumulation signal transduction activities of hCG analogs containing all four N-linked glycosylation signals (panel a) and those lacking the α2 glycosylation signal (panel b). *Note*: the error bars are omitted to make the panels more visible. The average error was less that 10% of the mean.

Table 2
Activities of crosslinked hCG and dghCG in cyclic AMP accumulation assays

	Glycosylated analogs of hCG				
	hCG	5α–8β	27α–44β	37α–33β	76α–44β
EC50	0.21	0.25	3.49	0.19	0.53
95% CL	0.18–0.23	0.19–0.32	2.31–5.28	0.15–0.25	0.41–0.67
	hCG analogs missing the loop α2 oligosaccharide				
	dghCG	dg5α–8β	dg27α–44β	dg37α–33β	dg76α–44β
EC50	0.29	0.38	0.93	1.35	2.48
95% CL	0.20–0.42	0.20–0.72	0.56–1.55	0.91–2.01	1.81–3.40
Efficacy (%)	42	36	14	24	34
95% CL	38–45	29–42	11–17	20–28	30–38

Notes: These values were determined by pooling the results from multiple assays. EC refers to the half-maximum of the dose response curve. Efficacy reflects the maximum response relative to that of hCG which for each experiment was defined as 100%. Values shown as "95% CL" refer to the 95% confidence level. All values were determined with Prism.

Removal of the α2 oligosaccharide from hCG by N-glycanase treatment reduced its efficacy to approximately 40% that of hCG (Fig. 1b). This is consistent with the observations of Matzuk et al. (1989), who removed this oligosaccharide genetically by eliminating the glycosylation signal at α-subunit residue Asn52. In contrast to Heikoop et al. (1998), we found that introduction of intersubunit disulfides into this analog did not increase the efficacy of analogs lacking the α2 oligosaccharide (Fig. 1b). In fact, some of the disulfide crosslinks reduced the efficacy of hCG analogs lacking the α2 oligosaccharide below that observed by removing the α2 oligosaccharide alone. This phenomenon is consistent with our current notions of signal transduction in which the α2 oligosaccharide functions by increasing the distance between the leucine-rich repeat and the signaling specificity domain of the LH receptor (Moyle et al., 2004). Thus, dgα37–β33, an analog having a disulfide between α2 and β1, had a lower efficacy than dghCG (Fig. 1b; Table 2). The finding that the α5–β8 disulfide bond did not reduce the efficacy of the deglycosylated heterodimer suggested that efficacy depended in part on the location of the disulfide bond, a phenomenon that is also consistent with our notion of signal transduction. We found that dgα27–β44 appeared to have a lower efficacy than dgα5–β8 (Fig. 1b; Table 2), a phenomenon that may have been due to the observation that this disulfide tended to reduce the efficacy of hCG (Fig. 1a). The α27–β44 disulfide appeared to reduce the binding of hCG to LH receptors, however, and we did not consider it further.

Deglycosylation of α2 appears to have a greater influence on the activity of hFSH than that of hCG (Trout et al., 1999; Valove et al., 1994). This may be related to differences in residues located in the carboxyterminal halves in their seatbelts, a portion of the β-subunit in contact with α2 that can influence FSH activity (Lindau-Shepard et al., 1994; Moyle et al., 1994). It may also be due to the manner in which hFSH appears to interact with its receptor (Moyle et al., 2004). To test the possibility that bifunctional analogs of hCG would have lower efficacies than hCG and hFSH, we monitored the LH and FSH activities of dgα37–β33 analogs in which the C-terminal half of the seatbelt was derived from the hFSH β-subunit (Fig. 2). As expected on the basis of previous studies (Moyle et al., 1994), substitution of hFSH residues into this region of the seatbelt led to analogs that interacted with FSH receptors (Fig. 2c) in addition to LH receptors (Fig. 2a). The abilities of dgα37–β33CF and dgα37–β33CFC to block binding of ^{125}I-hFSH to FSH receptors were similar to those we had found for other bifunctional analogs, which have approximately 10–30% the affinity of hFSH for FSH receptors (Moyle et al., 1994). The efficacies of dgα37–β33CF and dgα37–β33CFC were less than 10% that of hCG and 5% that of hFSH in cyclic AMP assays, however (Fig. 2b and d). Both analogs inhibited hCG and hFSH induced signal transduction (Fig. 2b and d; broken lines). As expected from their lower affinities for FSH receptors than LH receptors, greater amounts of the chimeras were required to block FSH induced signal transduction than were capable of inhibiting hCG induced signaling.

To determine the relative influence of the disulfide crosslink, the α2 oligosaccharide, and the seatbelt on the efficacy of these analogs in LH assays, we compared the signal transduction activities of α37–β33, α37–β33CF, dgα37–β33, and dgα37–β33CF in LH receptor assays (Fig. 3). As can be seen from the activities of α37–β33, dgα37–β33, and α37–β33CF, deglycosylation of loop α2 had a much greater influence on the efficacy of hCG than changes to the seatbelt. For example, in LH receptor assays the efficacy of α37–β33 was comparable to that of α37–β33CF, an analog that can also interact with FSH receptors. Removing the α2 oligosaccharide from α37–β33 to create dgα37–β33 reduced its efficacy substantially, but not nearly as much as removing the α2 oligosaccharide from α37–β33CF to create dgα37–β33CF (Fig. 3).

4. Discussion

These observations showed that removal of the α2 oligosaccharide reduced the signal transduction activity of hCG, even when the heterodimer was crosslinked by a disulfide. In fact, none of the added disulfide crosslinks restored the efficacy of heterodimers lacking the α2 oligosaccharide to that of hCG. This finding is in marked contrast to the report of Heikoop et al. (1998) who concluded that intersubunit disulfides offset the loss in efficacy caused by removing the α2 oligosaccharide. The differences in their findings and ours may be due to the assays employed. Deglycosylation has long been known to have a much more dramatic influence on efficacy when occupancy of a large numbers of receptors is required to elicit a response than when the receptors are present in excess (Moyle et al., 1977, 1980). In our assays, there are relatively few steps between ligand binding and the measured response, i.e., cyclic AMP accumulation. Heikoop et al. (1998) used an assay that depends on the ability of hCG to induce the expression of a luciferase reporter gene in LH receptor transfected CHO cells, a procedure that requires many more steps between ligand binding and response. Due to the relatively small difference between the basal and maximal responses and the inherent amplification in the signal transduction pathway, this assay is more likely to reach a maximal response when relatively few LH receptors are occupied. This would result in an overestimation of efficacy relative to hCG. Furthermore, removal of the α2 oligosaccharide appeared to increase the affinity of the crosslinked analogs for the LH receptor (Fig. 2a). Thus, the crosslinked deglycosylated analogs tested by Heikoop et al. (1998) may have bound lutropin receptors better than their glycosylated counterparts. In combination, the apparent overestimation of efficacy and increased affinity of the crosslinked analogs for the receptor would account readily for the similarities in the dose response curves of hCG and the crosslinked analogs

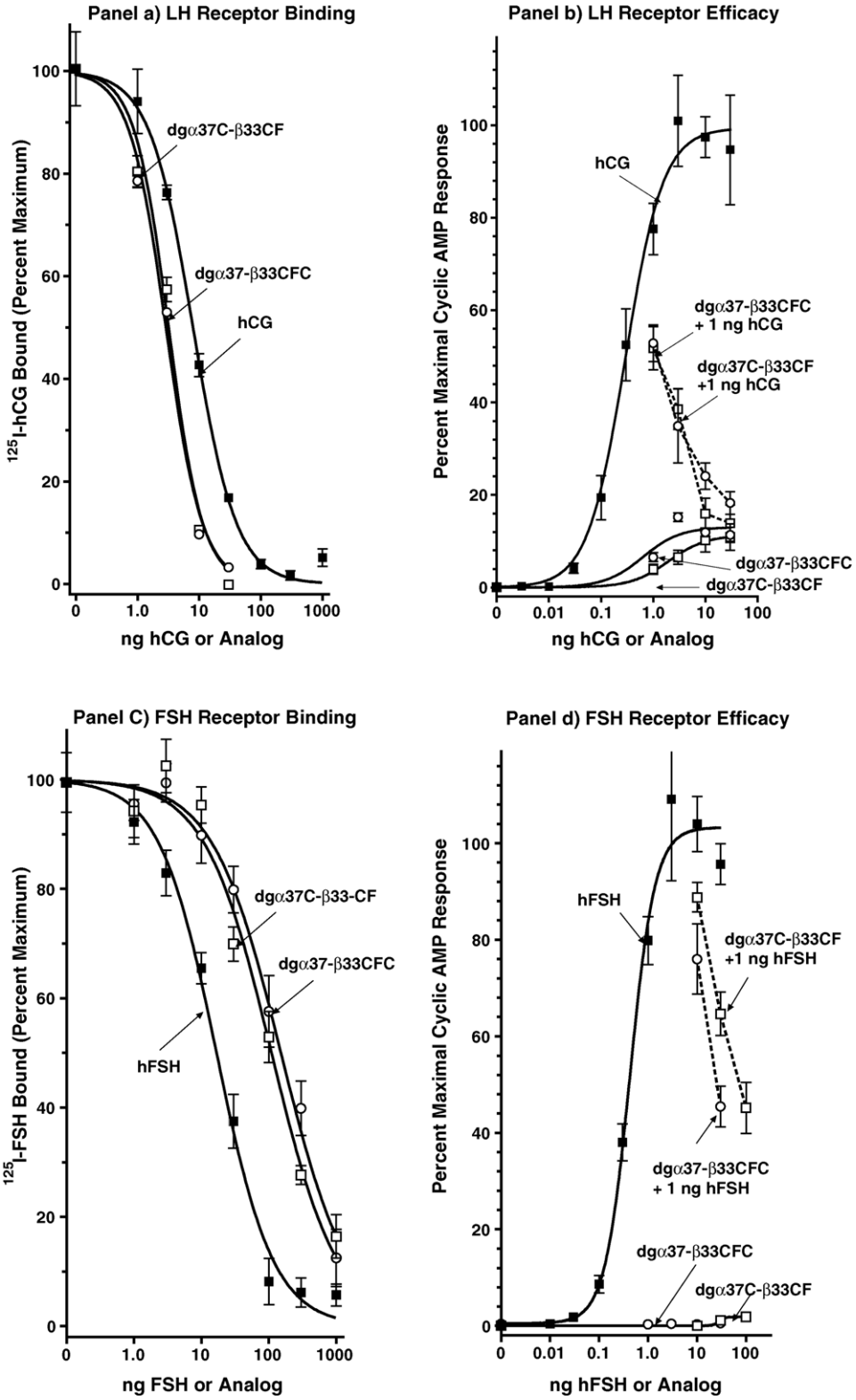

Fig. 2. Activities of bifunctional α37–β33 disulfide crosslinked analogs lacking the loop α2 oligosaccharide in LH and FSH receptor binding assays (panels a and c) and cyclic AMP accumulation signal transduction assays (panels b and d). The dgα37C–β33CF and dgα37–β33CFC bound LH receptors 2.75 (2.33–3.25) and 2.49 (2.22–2.99)-fold better than hCG (mean and 95% confidence limits). These analogs bound FSH receptors 0.15 (0.12–0.20) and 0.11 (0.08–0.15)-fold less well than hFSH. Both analogs blocked cyclic AMP accumulation in response to 1 ng hCG and 1 ng hFSH as illustrated by the broken lines in panels b and d, respectively. As would be expected from the differences in their abilities to interact with LH and FSH receptors relative to hCG and hFSH, the analogs were much more potent inhibitors of hCG induced signaling.

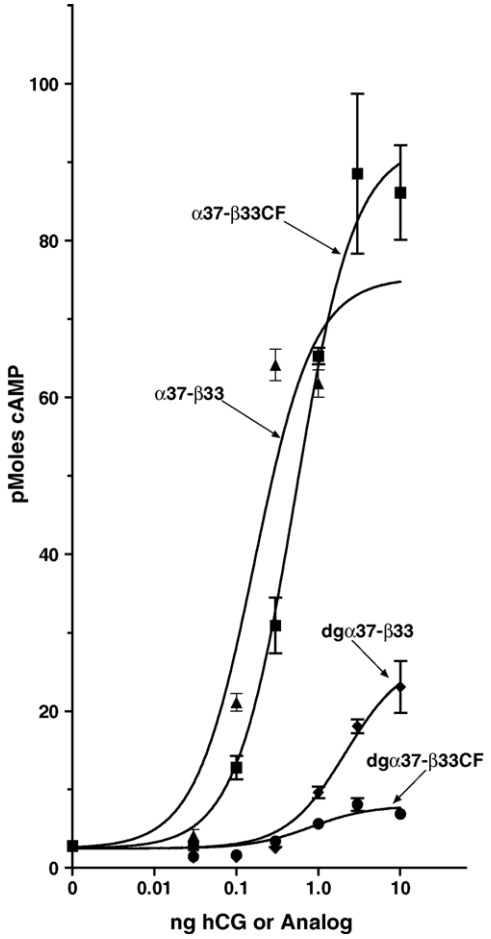

Fig. 3. Relative influence of the seatbelt and the loop α2 oligosaccharide on hormone efficacy in LH assays. Analogs were tested for their abilities to elicit cyclic AMP accumulation using CHO cells that over express rat LH receptors as described in the text.

observed by (Heikoop et al., 1998). We anticipate that use of the luciferase assay made the crosslinked deglycosylated analogs appear to be more active than they actually were.

Heikoop et al. (1998) also suggested that the lowered efficacy of dghCG observed by Matzuk et al. (1989) may have been due to the instability of the dghCG heterodimer. Dissociation of the heterodimer appears to require passage of the loop α2 oligosaccharide though the hole in the β-subunit created by the seatbelt (Xing et al., 2004a). Consequently, removal of the α2 oligosaccharide might be expected to reduce the stability of the heterodimer. The stability of hCG at neutral pH does not depend on the α2 oligosaccharide, however. We found that removal of this oligosaccharide enhanced hCG subunit recombination at neutral pH (Xing and Moyle, 2003), most likely because the heterodimer appears to be stabilized by hydrogen bonds that are formed by interactions between backbone atoms in α2 with residues in the β-subunit (Xing et al., 2004b). Therefore, the deglycosylated heterodimer would not be expected to dissociate rapidly in culture media at the physiological pH employed for receptor-binding and signal-transduction assays. In addition, since the free subunits are essentially inactive in hCG bioassays, hCG analogs lacking the α2 oligosaccharide would not have been capable of competing with hCG in assays described by Matzuk et al. (1989) if they were markedly unstable as had been implied by Heikoop et al. (1998).

The residual efficacy of dgα37–β33CFC is 10% or less in assays employing cells that over express the LH receptor and 5% or less in assays employing cells that over express the FSH receptor. Although low, this may remain too high for this analog to be useful clinically as a gonadotropin antagonist. Stimulation of testosterone formation in isolated Leydig cells requires only 1% receptor occupancy in vitro (Dufau and Catt, 1978; Moyle et al., 1977). Therefore, we anticipate that the efficacies of dgα37–β33CFC and related analogs may need to be reduced below 1% for them to be useful antagonists, even in combination with GnRH agonists and GnRH antagonists, agents capable of suppressing endogenous LH and FSH secretion (Coccia et al., 2004; Shalev and Leung, 2003). This is because bifunctional gonadotropin antagonists would need to occupy a high fraction of the receptors to prevent binding of circulating LH and FSH. In contrast, the efficacies of dgα37–β33CFC and dgα37–β33CFC may already be low enough to permit their use as vehicles for targeting apoptosis inducing agents to the ovary, e.g., toxin filled liposomes, or to enable them be used as fusion protein partners with other agents that are known to promote apoptosis of ovarian tissues, e.g., interferon-γ (Quirk et al., 2000). This is because their use as targeting agents is likely to require occupancy of many fewer LH and FSH receptors. Consequently, there would be less reason to anticipate that the low residual activities of these analogs would trigger cellular survival.

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