Random mutagenesis and screening of complex glycoproteins

Linskens, Maarten; Grootenhuis, Peter D.J.; Blaauw, Mieke; Huisman-de Winkel, Bianca; Ravestein, Arno van; van Haastert, Petrus; Heikoop, Judith C.

Published in:
European Journal of Biochemistry

Publication date:
1999

Random mutagenesis and screening of complex glycoproteins: expression of human gonadotropins in *Dictyostelium discoideum*

MAARTEN H. K. LINSKENS,*,[1] PETER D. J. GROOTENHUIS,† MIEKE BLAAUW,* BIANCA HUISMAN-DE WINKEL,† ARNO VAN RAVESTEIN,† PETER J. M. VAN HAASTERT,* AND JUDITH C. HEIKOOP†,[2]

*Cell Engineering Facility GBB, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands; and †N.V. Organon, P.O. Box 20, 5340 BH Oss, The Netherlands

ABSTRACT **The soil amoeba *Dictyostelium discoideum* is a host cell that provides simple genetics in combination with complex protein synthesis. We show that the complex human heterodimeric gonadotropins can be produced and secreted by this organism. Furthermore, both follicle stimulation hormone and choriogonadotropin produced by *D. dictyostelium* bind to their human receptors and elicit a biological response comparable to the wild-type hormones. We also show that structure-function analysis using random mutagenesis and screening of recombinant glycoprotein hormones is feasible. Thus, expression of gonadotropins in *D. dictyostelium* opens the way to the engineering of potential new therapeutic analogues. Linskens, M. H. K., Grootenhuis, P. D. J., Blaauw, M., Huisman-de Winkel, B., van Ravestein, A., van Haastert, P. J. M., and Heikoop, J. C. Random mutagenesis and screening of complex glycoproteins: expression of human gonadotropins in *Dictyostelium discoideum*. *FASEB J.* 13, 639–645 (1999)**

Key Words: heterologous protein expression · follicle-stimulating hormone · infertility

DRUG DISCOVERY PROCESSES are undergoing revolutionary changes due to rapid scientific and technological developments in genomics, bioinformatics, biotechnology, combinatorial chemistry, and high throughput screening (1). Rather than synthesizing and testing molecules one by one, mass generation and screening of compounds have become feasible. In the area of low molecular weight compounds, several lead compounds have already been identified using this new approach (2). However, in the case of high molecular weight protein drugs, a number of bottlenecks render the high throughput approach more difficult. Research on glycoprotein hormones (gonadotropins), which are used clinically in the treatment of infertility (3), illustrates this problem.

The gonadotropins human choriogonadotropin (hCG),[3] luteinizing hormone (LH), and follicle-stimulating hormone (FSH) regulate the cellular and endocrine function of the reproductive organs (4). These glycoproteins consist of two subunits (α and β) that are associated by hydrogen bonds and hydrophobic contacts. The α-subunit of the gonadotropins is identical for a given species, whereas the β-subunits are different and are responsible for the receptor specificity. The glycosylation (*N*-linked; for hCG, also *O*-linked) is important for the folding, secretion, and stability (5) of these proteins and also influences their *in vivo* behavior. Since the biosynthesis of the gonadotropins is a very complex process that also involves so-called cystine knot formation (6) and attachment/modification of oligosaccharide side chains (7), only eukaryotic cells can be used to express these glycoproteins. Thus, the recombinant gonadotropins used clinically (rec FSH, rec LH) have been expressed in Chinese hamster ovary (CHO) cells (3).

Despite the efforts of many research groups over two decades, we estimate that fewer than 500 gonadotropin mutants have been reported in the scientific literature. Although these site-directed mutagenesis studies have led to the identification of structural determinants for receptor binding and bioactivity, there are still many issues to be addressed. Perhaps even more important, there is an ongoing clinical need for novel gonadotropin drugs like LH antagonists (to treat ovarian hyperstimulation syndrome) for which there are no candidates (8). It may be anticipated that random mutagenesis approaches in

[1] Correspondence: Cell Engineering Facility, GBB, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. E-mail: m.linskens@chem.rug.nl

[2] Present address: McKinsey & Company, Amstel 344, 1017 AS Amsterdam, The Netherlands.

[3] Abbreviations: CHO, Chinese hamster ovary; FSH, follicle-stimulating hormone; hCG, human choriogonadotropin; LH, luteinizing hormone; PCR, polymerase chain reaction; rec, recombinant.

combination with high throughput screening may lead to the generation of such compounds.

However, due to the limited number of mutants that can be generated, mammalian cells such as CHO cells are not suitable for random mutagenesis of protein domains. In addition, the use of CHO cells is expensive and labor intensive. The soil amoeba *Dictyostelium discoideum* is an organism that provides an attractive alternative for heterologous expression of the human glycoprotein hormones (9, 10). Though it can be grown and transformed with the same ease as the yeast *Saccharomyces*, it has some complex features that resemble mammalian cells, such as glycosylation and chemotaxis. Furthermore, it has been shown that *Dictyostelium* provides a useful system for random mutagenesis approaches (11). Several heterologous proteins have been successfully expressed in *Dictyostelium*, such as the human muscarinic receptor (12), the rat GLUT 1 glucose transporter (13), and human antithrombin III, which could be recovered from growth medium (14). Very recently, we have demonstrated that *Dictyostelium* is also able to express a single-chain variant of hCG (15), which shows bioactivity albeit somewhat less than CHO-produced material (16).

In this paper we explain that heterodimeric hCG and FSH can be expressed by *Dictyostelium* cells and that these secreted hormones display bioactivities of the same order as the corresponding CHO-generated gonadotropins, suggesting that the α- and β-subunits are folded and assembled correctly. We selected two amino acid positions of hCG (β94, β95) that are part of an established hot spot (the so-called 'determinant loop') (17, 18) of hCG and generated a sample of 87 random mutants. We studied the immuno- and bioactivity of these mutants and found mutants with widely varying properties that provide new insights into the structure-function relationships. Since a complete generation testing cycle of several thousands of mutants can be completed within 2 months, this approach may allow the identification of variant forms of hCG that are of therapeutic interest.

MATERIALS AND METHODS

Plasmids and DNA cloning

The structure and overall organization of the vectors used for expression of the α- and β-subunits of the gonadotropins is essentially identical to that of MB12n, which has been described previously (16). In short, each subunit was amplified from cDNA containing plasmids by using the appropriate 5′ and 3′ primers to generate a DNA fragment that is cloned in a *Dictyostelium* extra-chromosomal expression vector (16). To facilitate expression of two independent plasmids in *Dictyostelium,* we required two plasmids with different selection markers. For this purpose, we replaced the blasticidin resistance cassette in MB12n with the 2.4 kb *Kpn*I-*Xba*I fragment from p155d1 (19) containing a neomycin cassette, creating MB12neo. For construction of the expression vector for the α-subunit, its natural cDNA sequence, including the mammalian secretion signal sequence, was produced by polymerase chain reaction (PCR) by using primers that introduce a *Bgl*II restriction site both at the 5′ and 3′ end of the fragment, so that it could be cloned in MB12neo. For construction of the expression vector for the β-subunit of hCG, MB12n was modified to contain another unique restriction site (*Sph*I) 3′ of the *Bgl*II cloning site (16). About 40% of the codons in the β-subunit of CG are infrequently used in *Dictyostelium* (20). To limit possible problems in mRNA translation at the start of the open reading frame, the first 30 bases of the leader sequence of the β-subunit were altered conform to *Dictyostelium* preferred codon usage (16). The hCG β-subunit cDNA was amplified using a 5′ primer (16), resulting in alteration of the first 30 bases of the coding sequence conform the *Dictyostelium* preferred codon usage. The primers also introduced appropriate restriction sites at both the 5′ (*Bgl*II) and 3′ (*Sph*I) end of the fragment to facilitate directional cloning.

To construct the expression vector for the β-subunit of FSH, the cDNA was amplified using a 5′ primer resulting in the alteration of the first 27 bases of the coding sequence conform the *Dictyostelium* preferred codon usage [CGACTC ACTATAGGGC AGATCTCACC *ATG* AAG ACT CTC CAA TTT TTC TTC CTC TTC TGT TGC TGG], and cloning was performed as described for the β-subunit of hCG. All DNA sequences were confirmed by using an automated sequencer.

PCR mutagenesis and mutant library construction

Specific base substitutions were introduced by site-specific mutagenesis and combining PCR fragments that overlap in sequences, as described, by using standard PCR conditions (21). The primers were designed to alter amino acids 94 and 95 of the β-subunit of hCG. The first two nucleotides of both codons were altered fully randomly (A,C,T or G), whereas the third base was restricted to G or T to minimize the introduction of stop codons. After PCR and subcloning in the *Bgl*II and *Sph*I sites of pCR 2.1 (Invitrogen, Leek, The Netherlands), the pool of constructs was transformed to *Escherichia coli.* Subsequently, DNA was isolated from a pool of 400 transformants and, after restriction digestion, the *Bgl*II/*Sph*I mutated fragments were subcloned in MB12n containing the BgIII and *Sph*I site (see above).

Expression of recombinant hormones

After plating *E. coli* transformants of MB12 plasmids containing the random mutant fragments, 400 colonies were pooled, DNA was prepared, and *Dictyostelium* was transformed by electroporation as described (16). Selection with blasticidin (10 μg/ml) was introduced 5 h after electroporation. The next day, cells were clonally diluted in 96-well plates using fourfold dilutions and neomycin selection (10 μg/ml) was added. In total, sixteen 96-well plates were used. Medium was replaced every 3–4 days, maintaining selective conditions. Positive wells were identified 11–14 days after electroporation, and the transformation efficiency was estimated from the dilution series. Typically, ~500 transformants were obtained by electroporation of 10^7 cells with 1 μg of both the hCG α and hCG β vectors.

Transformants from single wells were then selected for further experiments. A single well contains 200 μl of medium. Larger amounts of media for dose-response *in vitro* analysis were harvested from 10 cm culture dishes. Concentrations of wild-type and mutant hCG were measured using a DELFIA

hLH assay (Wallac Oy, Turku, Finland), which has a 100% cross-reactivity with hCG, as described by the manufacturer. FSH was quantified by a sandwich immunoassay as described previously (22). Both assays are based on the enzyme-linked immunoassay principle, with a solid-phase anti-β chain antibody and a soluble anti-α chain antibody. In the DELFIA assay, 15,000 units is equivalent to ~1 mg hCG protein; in the FSH assay, 10,000 units is equivalent to ~1 mg FSH protein.

In vitro *bioassay*

In vitro bioactivity was determined on either the human FSH receptor or the human LH/CG receptor, as described previously (5). In short, CHO cells expressing human HL/CG or FSH receptors also contain a reporter construct with luciferase driven by a cAMP responsive element. On exposure of the cells to the hormone, the reporter gene is activated through the receptor-mediated signal transduction pathway and luciferase activity is measured (5). Highly purified, recombinant gonadotropins produced by CHO cells were used as standards.

Sequence analysis of the hCG mutants

Total DNA of several selected *Dictyostelium* clones was isolated and used to transform *E. coli*. For each *Dictyostelium* clone, a number of transformants was analyzed by colony PCR for the presence of the α-subunit plasmid or the β-subunit plasmid. Subsequently, DNA was isolated from several transformants containing the β-subunit plasmid and sequence analysis was performed on the mutated region by using an automated sequencer (Pharmacia, Piscataway, N.J.).

RESULTS AND DISCUSSION

Design of the random mutagenesis and screenings strategy

Figure 1 shows an overview of the different steps involved in selection of gonadotropin analogues by random mutagenesis in conjunction with a screen for immuno- and biological activity. The first step in a random mutagenesis approach is the selection of a protein domain for analysis. Based on the X-ray structure of hCG (23, 24) combined with site-directed mutagenesis studies, we selected part of the so-called 'determinant loop' for construction of the mutant library. Since this region of hCG has been demonstrated to be involved in receptor binding and signal transduction (18), we expected to generate a set of gonadotropin analogues with altered biological profiles. The X-ray structure of hCG is shown at the top of Fig. 1 (Protein Data bank code 1hcn) in which the selected amino acids β94 and β95 are shown in red.

Gonadotropin expression in Dictyostelium

A major obstacle to overcome was the functional expression of gonadotropins in *Dictyostelium*. For the expression of the α- and β-subunit of hCG, two expression plasmids were generated. The first construct contained the α-subunit of hCG together with the neomycin gene as a selectable marker, whereas the second construct contained the hCG β-subunit with the blasticidin gene as a selectable marker. The two wild-type expression plasmids were transformed simultaneously to *Dictyostelium*. After transformation, cells were plated and clonal transformants that express both selection markers were identified and further grown for analysis. The amount of hCG secreted by *Dictyostelium* was determined by a sandwich immunoassay that indicated that heterodimeric, immunologically active hCG was produced by *Dictyostelium* (200-400 mU/ml), which is equivalent to ~13-26 ng/ml. This amount is produced in 4-5 days in a petri dish with $\sim 10^6$ cells per ml of medium. For comparison, the expression level of wt hCG in CHO cells is 800mU/10^6 cells per 24 h (22).

We also studied the production of FSH by *Dictyostelium*. In line with the strategy for hCG (see Materials and Methods), two expression plasmids were generated and transformed simultaneously. An FSH specific sandwich immunoassay demonstrated that immunologically active FSH was produced by *Dictyostelium* (200–400 mU/ml, ~20-40 ng/ml). We expressed each subunit by using their own signal peptide, confirming our earlier observation that mammalian signal sequences are able to facilitate secretion of these proteins in *Dictyostelium* (16).

Biological activities of gonadotropins produced by Dictyostelium

Although the presence of heterodimeric hCG was demonstrated in the medium by means of epitope detection, additional experiments were necessary to establish whether hCG produced by *Dictyostelium* is biologically active. The bioactivity of heterodimeric hCG from *Dictyostelium* was analyzed by examination of its ability to activate the human LH/CG receptor in a luciferase reporter assay. The results demonstrate that the heterodimeric hCG produced by *Dictyostelium* is able to activate the human LH/CG receptor (**Fig. 2***A*). Moreover, its bioactivity is comparable (IC_{50} value approximately twofold higher) to the bioactivity of wild-type hCG produced by CHO cells. This suggests that the overall conformation of heterodimeric hCG produced by *Dictyostelium* is comparable to the material produced in CHO cells. Heterodimeric FSH produced by *Dictyostelium* is also able to activate the human FSH receptor (Fig. 2*B*).

The gonadotropins probably belong to the most complex glycoproteins produced by mammalian cells. It is axiomatic that gonadotropin subunit assembly is vital for the biological activity of the hormones. Furthermore, correct pairing of the intrachain disulfide bonds is essential for the conformation of the subunits and assembly. Finally, both

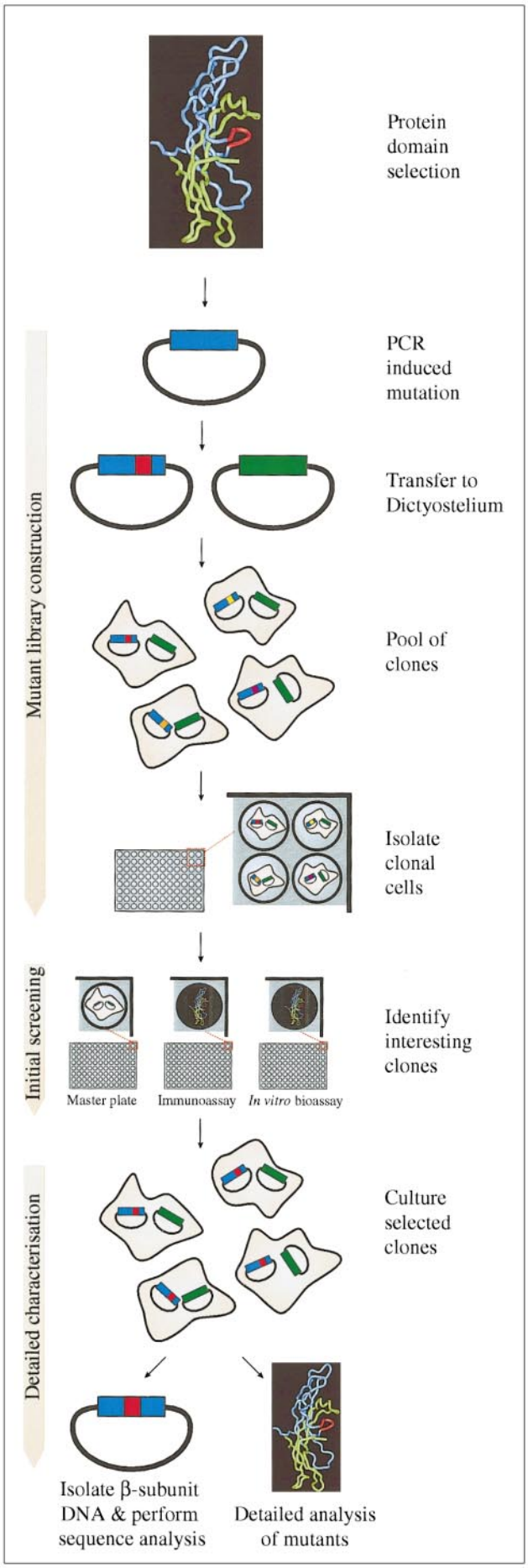

Figure 1. Schematic view of various steps in the random mutagenesis and screening for hCG analogies. Throughout the figure, the α- and β-subunits are colored green and blue, respectively. In this example, two amino acids of the determinant loop of hCG, namely, β94 and β95 (red), were selected for PCR induced mutagenesis. The pool of mutated β-subunits plasmids was transferred simultaneously with the α-subunit plasmid to *Dictyostelium* cells. A mutant library was obtained by serial dilution (96-well format) and isolation of clonal cells. Interesting clones were identified by initial screening of the supernatants for immuno- and *in vitro* bioactivity. Selected clones were further grown and cell culture supernatants were analyzed in detail. In addition, the β-subunit plasmid from individual clones was isolated for sequence analysis.

hCG and FSH contain four *N*-linked sugars that are essential for intracellular folding and transport, as well as for their biological activity. Thus, we conclude that *Dictyostelium* contains an extensive set of proteins involved in assisted folding and glycosylation. The fact that *Dictyostelium* is able to perform all posttranslational modifications necessary for the proper folding and bioactivity of gonadotropins suggests that this organism is probably also capable of folding a variety of other complex mammalian glycoproteins.

Random mutagenesis of a selected region of hCG

Since *Dictyostelium* was found to be capable of producing biologically active gonadotropins, the construction of the mutant library became feasible. Using site-directed mutagenesis, six random base substitutions were introduced in the codons for amino acids 94 and 95 in the expression vector for the hCG β-subunit. Simultaneously with this pool of altered expression vectors for the β-subunit of hCG, the expression vector for the α-subunit was transformed to *Dictyostelium* (see Fig. 1 for schematic overview). After transformation and serial dilution in 96-well plates, clonal transformants were selected and analyzed.

Initial screen for bioactivity

To identify interesting clones, we screened supernatants of the *Dictyostelium* clones for the presence of immuno- and bioactivity (see lower part of Fig. 1). As controls, wild-type hCG producing *Dictyostelium* clones and cells transformed with control plasmids were present on the 96-well plates (9 wells in total). The amount of hCG produced by different *Dictyostelium* clones varied considerably. The relatively high variation in production levels is most likely due to differences in growth rate and/or production levels between the individual clones. However, by calculating the biological activity per amount of immunological active protein, it is possible to eliminate the influence of this variation in the assay. Nevertheless, since each activity in this random mutagenesis screen

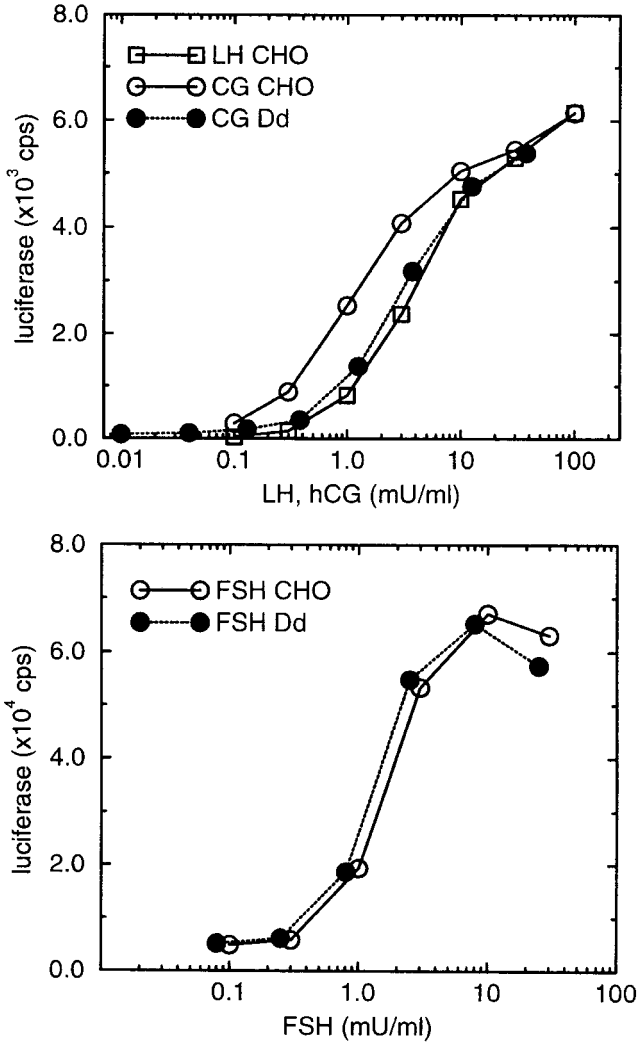

Figure 2. *In vitro* biological activity of wild-type (*A*) hCG and (*B*) FSH produced in CHO cells and *Dictyostelium discoideum* (Dd). Luciferase production was measured after 4 h incubation at 37°C and stimulation of CHO cells stably expressing the human (*A*) LH/CG or (*B*) FSH receptor.

is determined by single-point measurement, some variation and scatter in the data can be expected, as demonstrated by the ratio of biological and immunological activity (B/I) of three individual wild-type hCG-producing clones (**Fig. 3**, open bars). As expected, all *Dictyostelium* clones that were transfected with a control plasmid showed no immuno- or biological activity (not shown).

The results of a typical mutant screen are displayed in Fig. 3. The *in vitro* bioactivity of individual clones is depicted as a function of their immunological activity. Of the 87 mutants analyzed, 12 clones do not show any hCG production (these clones are not presented in Fig. 3). This is probably due to interference of the altered amino acids with appropriate folding of the mutated β-polypeptide and/or association with the α-subunit. The value of the three wild-type hCG-producing clones is in the same range (0.42-0.79). About half of the remaining 75 mutants show B/I ratios that are not significantly different from wild-type hCG produced by *Dictyostelium*. One of the clones (#78, Fig. 3) shows an increased B/I ratio of ~1.9. Remarkably, ~40% of the mutants show significantly decreased B/I ratio's. This indicates that a significant number of mutant proteins show decreased biological activity compared with wild-type hCG, suggesting that amino acids 94 and/or 95 of the β-subunit are indeed involved in receptor binding and/or signal transduction. From the 75 hCG-producing clones, we selected 18 individual clones with varying B/I ratios for detailed analysis.

Bioactivity and sequence analysis of selected mutants

The 18 selected clones were further grown on 10 cm plates until confluence and cell culture supernatants were collected after 4 additional days for detailed analysis of the mutants (see bottom part of Fig. 1). Of the 18 clones, 4 did not produce any detectable hCG, both in an immuno- and an *in vitro* bioassay. This is in agreement with the results obtained in the initial screen. Among the other clones, the amount of hCG produced varied considerably (116-2118 mU/ml). Since all clones were grown until confluence, the difference in their production levels can only be explained by differences in assembly and/or secretion of the mutants. This is not unlikely, as the determinant loop is also known to be involved in assembly of the α- and β-subunit of hCG (25).

To identify the mutations that are responsible for biological activities of the selected mutants, we isolated total DNA from selected *Dictyostelium* clones and transformed it to *E. coli*. First, *E. coli* transformants were analyzed by PCR for the presence of an α- or a β-subunit containing expression vector. Subsequently, sequence analysis of β-subunit containing plasmids was performed on multiple *E. coli* colonies (on average, 5.2 *E. coli* clones per *Dictyostelium* DNA isolation). In 15 of 18 *Dictyostelium* DNAs tested, a single sequence was detected in all *E. coli* colonies. These findings indicate that in the majority of cases the gonadotropin expressed by the *Dictyostelium* clones is encoded by a unique mutant sequence in the hCGβ expression vector. In addition, the sequence data show no obvious sequence bias indicating that the mutagenesis has been random.

The cell culture supernatants were analyzed for the presence of *in vitro* biological activity of hCG at a range of concentrations based on their immunological activities. The dose/response curves for nine of the mutants are shown in **Fig. 4**. Most mutants display a decreased biological activity compared with wild-type hCG. This result could be anticipated, since amino acid β94 and β95 are involved in receptor binding and/or signal transduction. However, to our

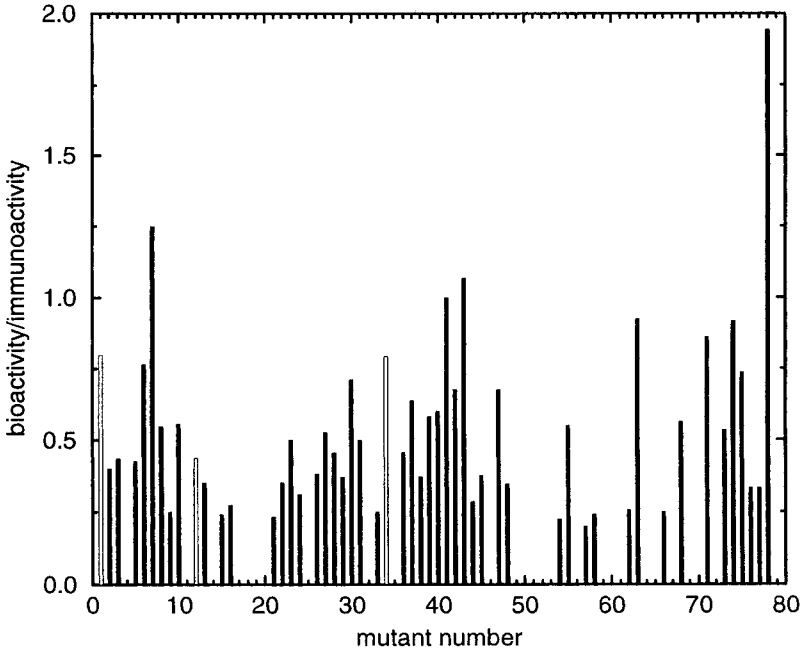

Figure 3. Distribution of mutant phenotypes in an initial screen. The ratio between the *in vitro* bioactivity and immunoactivity of individual clones is depicted as function of the mutant number. Twelve mutants had an immunoactivity of 0 and are not displayed. Three of the 78 clones (#1, 12, 34) produce wild-type hCG and are depicted with open bars.

surprise, the mutant with a Pro-Ser sequence showed the same bioactivity as wild-type hCG, illustrating that the effect of different mutations on the biological activity of the hCG analogies cannot be predicted beforehand on the basis of structure/function relationships. Another interesting observation is that one of the mutants with a large charge alteration (Arg-Arg to Gly-Asp) showed an unexpected high bioactivity as compared with wild-type (Fig. 4) (26). It also illustrates that popular techniques like Ala scanning of regions in order to determine the 'hot' amino acids may lead to too simple views on which amino acids are essential for receptor binding and/or bioactivity.

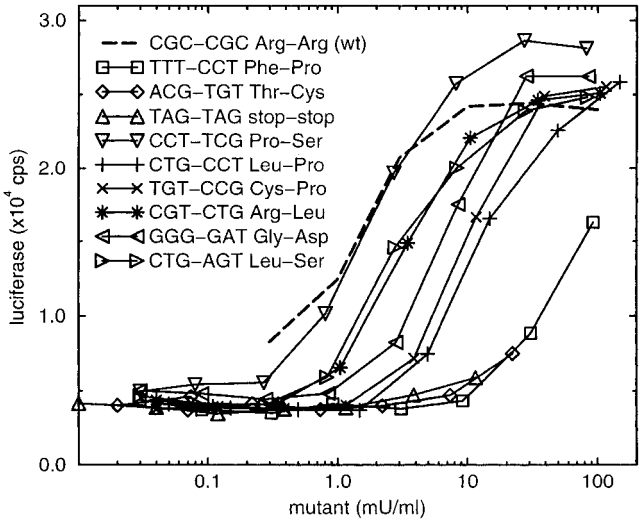

Figure 4. *In vitro* biological activity of wild-type hCG (dashed line) and selected hCG mutants (closed lines) produced by *Dictyostelium discoideum.* Luciferase production was measured after 4 h incubation at 37°C and stimulation of CHO cells stably expressing the human LH/CG receptor.

In a random mutagenesis screen, it is important to check that the first high throughput assay is robust and representative for most samples. The Arg-Leu mutant (#78, Fig. 3), which showed an increased B/I ratio in the initial screen, now displayed wild-type activity. Probably one or both activities of this mutant were not measured accurately in the initial screen. On the other hand, all mutants that showed B/I ratios in the range of wild-type hCG in the initial screen also displayed a wild-type *in vitro* biological activity when analyzed in more detail. Furthermore, all mutants with decreased B/I ratios in the initial screen indeed show a clear increase in IC_{50} in the more extensive biological activity assay. Their B/I ratios varied from a 2- to >100-fold lower than wild-type hCG produced by *Dictyostelium.* Thus, the initial screen is sufficient to identify the clones that display decreased B/I ratios, as the majority of clones give rise to comparable results when analyzed in more detail. As discussed earlier, some variation and scatter cannot be excluded, since in the initial screen both immunological activity and *in vitro* biological activity are determined by single-point analyses. A successful screening approach should therefore aim to identify activities that sufficiently deviate from wild-type activity, such as the significant decreased B/I ratios in this study, which can then be studied in more detail.

We conclude that screening of thousands of mutants for a specific activity or property is now feasible, because only a small portion of the gonadotropin analogs will display the desired biological properties and need further analyses. The current random mutagenesis and screening approach is applicable in different ways. First, although several structural determinants for receptor binding and bioactivity of

gonadotropins have been elucidated (4, 23), the function of the majority of the domains in these large glycoproteins is more or less unknown. The large number of mutants that can be screened for facilitates exhaustive screening of different domains of these molecules without any bias whatsoever. A much more complete structure/function analysis may be the starting point for the design of low molecular weight lead compounds. Several recent studies have shown how, by a combination of random mutagenesis approaches with structural analyses and screening, protein-protein interactions could be effected by relatively small oligopeptides (27, 28). Second, regions of interest can be analyzed in detail, facilitating the optimization of desired biological activities without the limitations of site-directed mutagenesis.

From the results described in this paper, we conclude that *Dictyostelium discoideum* is an attractive alternative host cell for the expression of complex mammalian glycoproteins. The use of *Dictyostelium discoideum* for random mutagenesis of gonadotropins in combination with screening now opens the way to the generation of new fertility drugs. We anticipate that this method also may be generally useful for engineering potential new therapeutic analogies of other clinically important glycoproteins. FJ

We thank Nathascha Hanzen, Maurice van Loosbroek, and Linda Snels-Stabel for expert technical assistance.

REFERENCES

1. Glaxo-Wellcome (1996) Redesigning drug discovery. *Nature (London)* **384** (Suppl.), 1–5
2. Plunkett, M. J., and Ellman, J. A. (1997) Combinatorial chemistry and new drugs. *Sci. Am.* 55–59
3. Shoham, Z., and Insler, V. (1996) Recombinant technique and gonadotropins production: new era in reproductive medicine. *Fertil. Steril.* **66,** 187–201
4. Combarnous, Y. (1992) Molecular basis of the specificity of binding of glycoprotein hormones to their receptors. *Endocr. Rev.* **13,** 670–691
5. Heikoop, J. C., van den Boogaart, P., de Leeuw, R., Rose, U. M., Mulders, J. W. M., and Grootenhuis, P. D. J. (1998) Partially deglycosylated human choriogonadotropin, stabilized by intersubunit disulfide bonds, shows full bioactivity. *Eur. J. Biochem.* **253,** 354–356
6. Isaacs, N. W. (1995) Cystine knots. *Curr. Opin. Struct. Biol.* **5,** 391-395
7. Thotakura, N. R., and Blithe, D. L. (1995) Glycoprotein hormones: glycobiology of gonadotrophins, thyrotrophin and free α subunit. *Glycobiology* **5,** 3–10
8. Jennings, J. C., Moreland, K., and Peterson, C. M. (1996) In vitro fertilisation. A review of drug therapy and clinical management. *Drugs* **52,** 313–343
9. Slade, M., Emslie, K., and Williams, K. L. (1997) Expression of recombinant glycoproteins in the simple eukaryote Dictyostelium discoideum. *Biotech. Genet. Engin. Rev.* **14,** 1–35
10. Jung, E., and Williams, K. L. (1997) The production of recombinant glycoproteins with special reference to simple eukaryotes including Dictyostelium discoideum. *Biotechnol. Appl. Biochem.* **25,** 3–8
11. Kim, J-Y., Caterina, M. J., Milne, J. L. S., Lin, K. C., Borleis, J. A., and Devreotes, P. N. (1997) Random mutagenesis of the cAMP chemoattractant receptor, cAR1, of Dictyostelium. *J. Biol. Chem.* **272,** 2060–2068
12. Voith, G., and Dingermann, T. (1995) Expression of the human muscarinic receptor gene m2 in Dictyostelium discoideum. *Bio/Technology* **13,** 1225–1229
13. Cohen, N. R., Knecht, D. A., and Lodish, H. F. (1996) Functional expression of rat GLUT 1 glucose transporter in Dictyostelium discoideum. *Biochem. J.* **315,** 971–975
14. Dingermann, T., Troidl, E.-M., Broker, M., and Nerke, K. (1991) Expression of the human antithrombin III in the cellular slime mould Dictyostelium discoideum. *Appl. Microbiol. Biotechnol.* **35,** 496–503
15. Heikoop, J. C., van Beuningen-de Vaan, M. J. A.C. M., van den Boogaart, P., and Grootenhuis, P. D. J. (1997) Evaluation of subunit truncation and the nature of the spacer for single chain human gonadotropins. *Eur. J. Biochem.* **245,** 656–662
16. Heikoop, J. C., Grootenhuis, P. D. J., Blaauw, M., Veldema, J. S., van Haastert, P. J. M., and Linskens, M. H. K. Expression of a bioactive, single chain choriogonadotropin in Dictyostelium discoideum. *Eur. J. Biochem.,* **256,** 359–363
17. Moore, W. T., Burleigh, B. D., and Ward, D. N. (1980) Chorionic gonadotropins: comparative studies and comments on relationships to other glycoprotein hormones. In *Chorionic Gonadotropin* (Segal, S. J., ed) pp. 89–126, Plenum Press, New York
18. Huang, J., Ujihara, M., Xia, H., Chen, F., Yoshida,, H., and Puett, D. (1993) *Mol. Cell. Endocr.* **90,** 211–218
19. Hughes, J. E., Kiyosawa, H., and Welker, D. L. (1994) Plasmid maintenance functions encoded on Dictyostelium discoideum nuclear plasmid Dpdl. *Mol. Cell. Biol.* **14,** 6117–6124
20. Sharp, P. M., and Devine, M. (1989) Codon usage and gene expression level in Dictyostelium discoideum: highly expressed genes do 'prefer' optimal codons. *Nucleic Acids Res.* **17,** 5029–5039
21. Higuchi, R. (1989) Using PCR to engineer DNA. In *PCR Technology; Principles and Applications for DNA Amplification* (Erlich, H. A., ed) pp. 61–70, Stockton Press, New-York
22. Heikoop, J. C., van den Boogaart, P., Mulders, J. W. M., and Grootenhuis, P. D. J. (1997) Structure-based design and protein engineering of intersubunit disulfide bonds in gonadotropins. *Nature Biotechnol.* **15,** 658–662
23. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) Crystal structure of human chorionic gonadotropin. *Nature (London)* **369,** 455–461
24. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure of the human chorionic gonadotropin at 2.6 Å resolution from MAD analysis of the selenomethionyl protein. *Structure* **2,** 545–558
25. Chen, F., and Puett, D. J. (1991) Delineation via site-directed mutagenesis of the carboxyl-terminal region of human choriogonadotropin beta required for subunit assembly and biological activity. *J. Biol. Chem.* **266,** 6904–6908
26. Han, Y., Bernard, M. P., and Moyle, W. R. (1996) HCG-β residues 94-96 alter LH activity without appearing to make key receptor contacts. *Mol. Cell. Endocr.* **124,** 151–161
27. Yanofsky, S. D., Baldwin, D. N., Butler, J. H., Holden, F R., Jacobs, J. W., Balasubramanian, P., Chinn, J. P., Cwirla, S. E., Peters-Bhatt, E., Whitehorn, E. A., Tate, E. H., Akeson, A., Bowlin, T. L., Dower, W. J., and Barrett, R. W. (1996) High affinity type I interleukin 1 receptor antagonists discovered by screening recombinant peptide libraries. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7381–7386
28. Wrighton, N. C., Farrell, F. X., Chang, R., Kashyap, A. K., Barbone, F. P., Mulcahy, L. S., Johnson, D. L., Barrett, R. W., Jolliffe, L. K., and Dower, W. J. (1996) Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* **273,** 458–463

Received for publication September 9, 1998.
Revised for publication November 23, 1998.