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On the Biological Functions of Equine Chorionic Gonadotropin

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말의 융모성 성선자극 호르몬의 생화학적 기능

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ABSTRACT

In horse, a single gene encodes both eCG and eLH β subunits. The difference between eCG and eLH lies in the structure of their glycoresidues, which are both sialylated and sulfated in LH and sialylated in CG. eCG consists of highly glycosylated α- and β-subunits and is an unique member of the gonadotropin family because it elicits response characteristics of both FSH and LH in other species than the horse. This dual activity of eCG in heterologous species is of fundamental interest to the study of gonadotropin structure-function relationships and the understanding of the molecular bases of the specific interactions of these hormones with their receptors. Thus, eCG is a dintinct molecule from the view points of its biological function and glycoresidue structures.

The oligosaccharide at Asn 56 of the α-subunit plays an indispensable role, whereas the carboxyl -terminal extension of the eCG β-subunit with its associated O-linked oligosaccharides is not improtant for, the *in vitro* LH-like activity of eCG. In contrast, both N- and O-linked oligosaccharides play important roles for FSH-like activity and increase FSH-like activity by removal of N- and O-linked oligosaccharides. Therefore, the dual LH- and FSH-like activities of eCG can be clearly separated by removal of either the N-linked oligosaccharide on the α-subunit or CTP-associated O-linked oligosaccharides from its β-subunit. The glycoresidues seem to play crucial roles for biological activities.

The tethered-eCG was efficiently secreted and showed similar LH-like activity to the dimeric eCG α/β and native eCG. FSH-like activity of the tethered-eCG was also shown similarly in comparison with the native and wild type eCG α/β. Our data for the first time suggest that the tethered-eCG can be expressed efficiently and the produced product by the CHO-K1 cells is fully LH- and FSH-like activities in rat *in vitro* bioassay system. Our results also suggest that this molecular can imply particular models of FSH-like activity not LH-like activity in the eCG. Taken together, these data indicate that the constructs of tethered molecule will be useful in the study of mutants that affect subunit association and/or secretion.

(Key words : Equine chorionic gonadotropin, Biological activity, rec-eCG)

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Ⅰ. INTRODUCTION

eCG is a unique member of the gonadotropin family since it appears to be a single molecule that possesses both LH- and FSH-like activities in other species than the horse (Moore & Ward, 1980; Apparailly & Combarnous, 1994; Min et al., 1996, 1997). This dual activity of eCG in heterologous species is of fundamental interest to the study of the structure-function relationships of gonadotropins and their receptors.

The glycosylation sites of eCG are illustrated in Fig. 1. There have two N-glycosylation sites on the α-subunits of all these glycoproteins, whereas the numbers of such sites on their β-subunits differ depending on the hormone: LH β- and FSH β-subunits bear one and two sites, respectively, there are two on hCG, whereas eCG has only one. The α-subunit of eCG has two N-glycosylation sites (Asn 56 and Asn 82) and its β-subunit has one (Asn 13). In addition to these N-glycosylation sites, the β-subunits of human and baboon CGs are heavily glycosylated C-terminal extensions, which increase the average lengths of these β-subnuits from 120 to 145 amino acids (Pierce & Parsons, 1981; Crawford et al., 1986; Min, 2000c,d). Analysis of a purified preparation of eCG revealed that its β-subunit consists of 149 amino acids (Sugino et al., 1987), which was confirmed by the molecular cloning of its cDNA described previously (Min et al., 1994, 1996). There seem to be at least four to six, or even as many as 11, O-glycosylation sites on the extended C-terminal region of the eCG β-subunit (Bousfield et al., 1992; Min et al., 1996, 1997). In that point, the role of oligosaccharides in the function of eCG is distinct from that in the case of hLH, hFSH and hCG. These oligosaccharides are required for efficient signal transduction (Sairam & Bhargavi, 1985; Calvo et al., 1986; Min et al., 1996). We have studied the signal transduction and function/roles of LH/CG receptor to analyse the gonadotropin biologcial activity (Min et al., 1998; Min, 1999, 2000a,c).

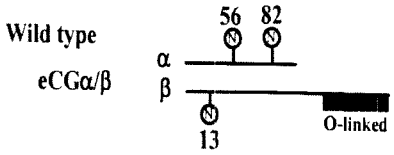

Fig. 1. Glycosylation sites of eCG the α- and β-subunits. The α-subunit has two Asn glycosylation sites located at homologous positions(Asn^{56} and Asn^{82}). eCG β-subunit has a 25~29 amino acid residue extension peptide at its carboxyl terminus, and it also possesses several O-glycosylation sites in its C-terminal extension, at least 11 of which are glycosylated.

We published a review in this journal in which we summarized on the secretion and functions of equine chorionic gonadotropin (Min, 2000b). In this review, we will only brief touch on the biological roles of oligosaccharides on LH-and FSH-like activities in the culture system of rat Leydig cells and granulosa cells by using recombinant eCG mutants, of which the codon for Asn 56 was substituted with Gln on the α- subunit or the unique eCG β-subunit CTP was deleted by site-directed mutagenesis. And we also constructed the single chain eCG molecule. These results indicate that the tethered-eCG is secreted efficiently, and that is similar to wild type eCG in the LH-and FSH-like activities.

Ⅱ. BIOLOGICAL ACTIVITIES

1. Biological Activities of rec-eCG

We investigated the roles of oligosaccharides on LH- and FSH-like activities in the culture systems of rat Leydig cells and granulosa cells by using recombinant eCG wild type (WT) (Min et al., 1996,

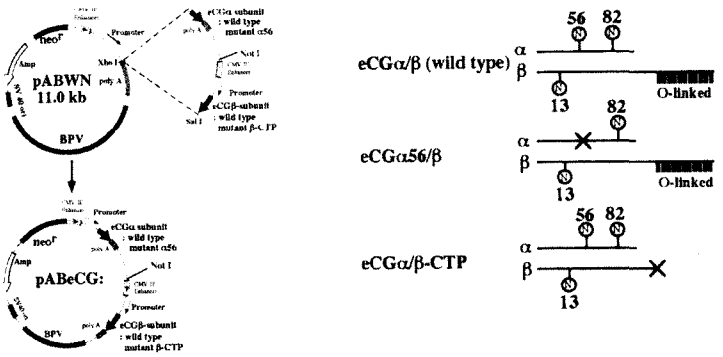

Fig. 2. Expression vector construction. The expression transfer vector pABWN (11Kb) comprises a promoter based on the chicken β-actin promoter, containing cytomegalovirus-immediate early (CMV-IE) enhancer and a subregion of the bovine papilloma virus (BPV) genome. Mutagenesis for changing the N- and O-linked oligosaccharide sites on eCG.

1997; Min, 2001). The expression transfer vector pABWN (11 kb) comprises a ubiquitously strong promoter based on the chicken β-actin, a 69% subregion of the bovine papilloma virus (BPV) genome and a mutant neomycin phosphotransferase II-encoding gene driven by a weak promoter, which confers only marginal resistance to G418 (neor) (Miyazaki et al., 1989; Niwa et al., 1991; Min et al., 1996, 1997; Min, 2000c) (Fig. 2).

The expression vectors were transfected into chinese hamster ovary (CHO-K1) cells by the liposome formulation (Lipofectamine) transfection method according to the supplier's instruction. Stable cell transfectants were selected for G418 (800 μg/ml) resistance. The clonal cell lines isolated with G418 were subjected to reverse transcription polymerase chain reaction (PCR) and Northern blotting analyses to select and establish those which stably expressed α- and β-subunit mRNAs (Fig. 3).

Recombinant eCGs secreted into the medium were collected and quantified by radioimmunoassay. These competitive curves were directly proportional to each other, showing that there were no quantitative differences in the recognition of native, wild type and mutant eCGs by the antibody (Fig. 4). The

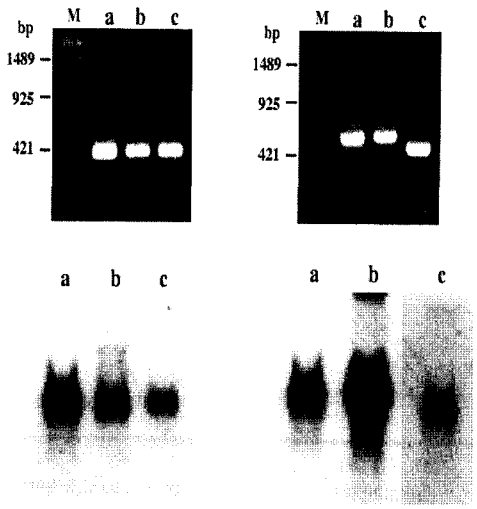

Fig. 3. RT-PCR and Northern blotting analyses of wild type and mutant eCGs expressed by stable CHO-K1 cell clones. a: wild type; b: 56 α/β; c: α/β-CTP.

effects of the recombinant eCG on the testosterone secretion in primary cultures of rat Leydig cells were determined to evaluate thier LH-like activity. The testosteorne concentration increased in direct proportion to the concentration of native eCG (Fig. 5). Over the range of 20 ~ 200 ng/ml, wild type

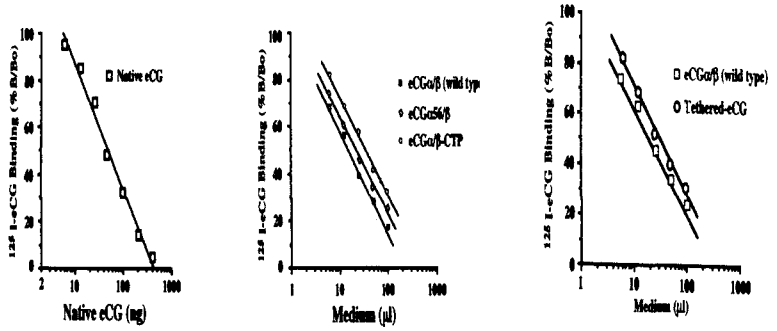

Fig. 4. Quantification of recombinant eCGs by RIA. The recombinant eCGs [eCG α/β (wild type), mutants and tethered-eCG] secreted by the stably expressed cells into serum free media were collected and subjected to RIA. Based on the result, the quantity of the recombinant proteins is expressed as an equivalent of the native eCG standard (1,000 IU/mg) as described in the text.

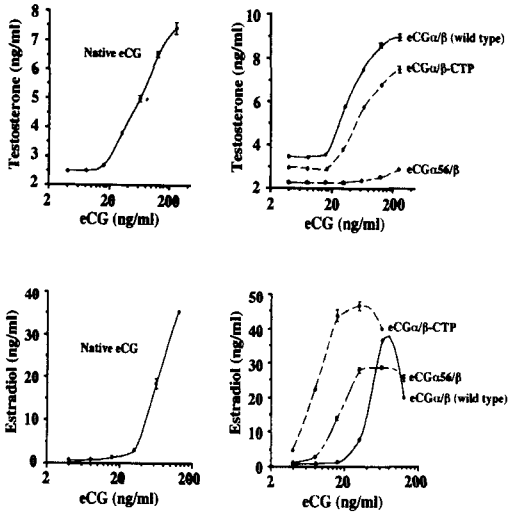

Fig. 5. LH-and FSH-like activities of wild type and mutant eCGs. Leydig cells (LH-like activity: upper) were incubated with various concentrations of native eCG (left) and recombinant eCGs (right). And granulosa cells (FSH-like activity: lower) from immature rats were incubated for 3 days with native (left) and rec-eCGs (right). A representative experiment, repeated three times, is shown.

eCG showed a similar concentration-response curve. The effects of the recombinant eCGs on aromatase activity were evaluated by measuring estradiol production by primary cultures of rat granulosa cells. Native and wild type eCGs showed very similar concentration-response curves (Fig. 5). The recombinant eCG produced by the CHO-K1 cells showed LH- and FSH-like activities in rat *in vitro* bioassay system, and these activities were very similar to those of native eCG.

The hCG and hFSH were expressed in a heterologous cell system. Pulse-chase experiments revealed that, when transfected alone FSH β was very slowly secreted similar to lutropin β and thyrotropin β but unlike choriogonadotropin β which is efficiently secreted (Bielinsk et al., 1989; Matzuk et al., 1989). The result indicated that the glycoprotein hormone of pituitary origin have determinants for secretion that differ from those on the placental hormone, choriogonadotropin. Recombinant FSH stimulated steroidogenesis comparable to purified human FSH isolated from pituitary in an *in vitro* rat granulosa cell assay. Recombinant hCG also stimulated MA-10 cell steroidogenesis. Human FSH and hCG produced by this cell line like eCG provide a source of recombinant bioactive glycoprotein hormones for experimental and clinical use.

2. Site-directed Mutagenesis

We also determined the roles of oligosaccharide for mutant eCGs, of which the codon for Asn 56 was substituted with Gln on the α-subunit or the unique eCG β-subunit carboxy-terminal peptide (CTP) was deleted by site-directed mutagenesis. Expression vectors for the mutant at α56 (pABeCG $\alpha 56/\beta$, 14.4 kb) and β-CTP (pABeCG α/β-CTP, 14.3 kb) were prepared as shown in Fig. 2. The concentration-response curve of eCG $\alpha 56/\beta$ was essentially flat, and this mutant showed no LH-like activity, indicating that the carbohydrate residue at position 56 on the α-subnit of eCG is indispensable for such activity (Fig. 5). The eCG α/β-CTP was similar to that of the wild type eCG, although its activity was slightly lower (77.2%, Table 1). This shows that the presence of the CTP with O-linked carbohydrate is not essential for the LH-like activity of eCG. Interestingly, eCG $\alpha 56/\beta$ stimulated estradiol production markedly, and it was calculated to be 2.3 times more potent than the wild type eCG. The biopotency of eCG α/β-CTP was also 6.7 times higher than that of the wild type of eCG (Fig. 5) (Table 1), and thus the maximal estradiol production was enhanced by this mutation (Min et al., 1996, 1997).

Site-directed mutagenesis of hCG has revealed that the N-linked oligosaccharide at position Asn 52 on its α-subunit is essential for its biological function (Bielinska et al., 1989; Matzuk et al., 1989). Similarly, an N-linked oligosaccharide at the same position on hFSH appears to be necessary to express its biological activities (Flack et al., 1994; Bishop et al., 1994; Valove et al., 1994). It is therefore expected that N-linked glycoresidues at position Asn 52 are very important to all glycoprotein hormones. The biological role fo the CTP of the hCG β-subunit is unknown, although the carbohydrate residues attached to it appear to contribute to a prolonged biological half-life *in vivo*, but are not critical for *in vitro* biological activity (Matzuk et al., 1990; Chen & Bahl, 1991; Chen & Puett, 1991; Huang et al., 1993). We cloned eFSH α-subunit and expressed wild-type re-eFSH and a partially deglycosylated mutant FSH(eFSH $\alpha 56/\beta$) to investigate the biological role of the oligosaccharide at Asn56 in FSH activity (Saneyoshi et al., 2001). eFSH $\alpha 56/\beta$ did not show any FSH activity, indicating the oligosaccharide at Asn 56 was necessary for eFSH (Fig. 6) . Thus, FSH-like activities of two gonadotropins, eCG and eFSH, are evoked through the distinct molecular mechanisms regarding the biological role of oligosaccharide at Asn 56 of α -subunit.

An important finding is that the FSH-like activity of the mutant eCG $\alpha 56/\beta$ was much higher than those of the wild type eCG and native eCG. Removing the Asn 56 oligosaccharide from eCG α -subunit didn't reduce the FSH-like activity, despite resulting in complete loss of the LH-like activity, so that the FSH-like activity of eCG was very

Table 1. Summary of the hormone expression, maximal responses and bioactivities of wild type and mutant rec-eCGs

rec-eCG	Hormone expression	Maximum response (%)		*In vitro* bioactivity (%)	
	(ng/100 μl)[a]	Testosterone	Estradiol	LH	FSH
Wild type	208±29	100.0	100.0	100.0	100.0
$\alpha 56/\beta$	198±35	0	86.5	1.0>	233.3
α/β-CTP	155±15	84.1	126.5	77.2	677.4

[a] Determined by RIA. Values are the means ±SEM of triplicate experiments.

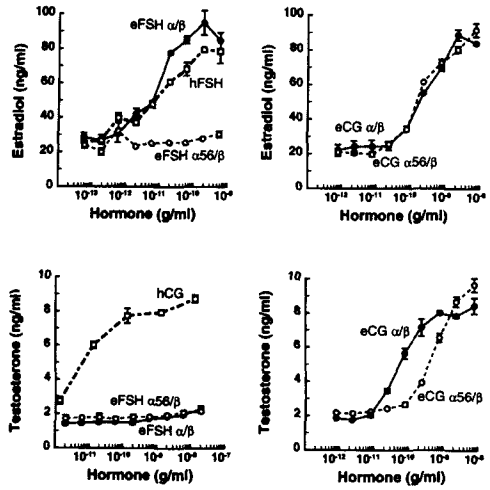

Fig. 6. Biological activity of recombinant eFSH (left) and eCG (right). The activities of recombinant eFSHs and eCGs were evaluated. Values are expressed as the mean ±SEM for at least three separate experiments.

resistant to N-linked oligosaccharide removal, and its dual FSH- and LH-like activities could by separated by removing the N-linked oligosaccharides from their α-subunit. This contrasts with the general concept that the N-linked oligosaccharide on Asn 52 of the α-subunit is essential for the expression of the activity of all gonadotropins. The concept that the N-linked glycoresidue on the Asn52 of hCG or hFSH is indispensable for their biological, not the FSH-like activity of eCG.

It is generally accepted that O-linked oligosaccharides on the CTP of the hCG β-subunit are not important for *in vitro* LH-like activity (Matzuk et al., 1990; Chen & Bahl, 1991; Chen & Puett, 1991; Huang et al., 1993; Narayan et al., 1995). Unexpectly, because the FSH-like activity of eCG α/β -CTP was also considerably higher than that of the the wild type eCG, the O-linked oligosaccharides appeared to play some role, albeit not necessarily a major one, in the *in vitro* LH-like activity. Matzuk et al. (1990) suggested that the O-linked oligosaccharides on the hCG β-subunit participated in prolonging circulating half-life of LH *in vivo*, and resulted in increased biological activity. The N-linked oligosaccharide on Asn 52 of hFSH, which does not have a CTP, is essential for its biological activity (Flack et al., 1994; Bishop et al., 1994). The O-linked oligosaccharides on the CTP of the β-subunit may be responsible for maintaining and potentiating the FSH-like activity of eCG lacking an N-linked glycorresidue at Asn 56. In order to explore this possibility, studies of eCG from which the oligosacchairdes on both subunits have been reomved are required. The role of oligosaccharides in the function of eCG is distinct from that in the case of hLH, hFSH and hCG.

3. Biological Activity of Single-chain rec-eCG

To determine whether α and β subunits can be synthesised as a single polypetide chain(tethered-eCG) and also display biological activity, the tethered-eCG molecule by fusing the carboxyl terminus of the eCG β-subunit to the amino terminus of the α-subunit was constructed (Fig. 7). The concentration-response curves of the recombinant tethered -eCG were shown in Fig. 4. The result indicates that eCG heterodimer can be expressed as a single chain encoding both subunits: α and β. Thus, the noncovalent heterodimeric structure is not critical

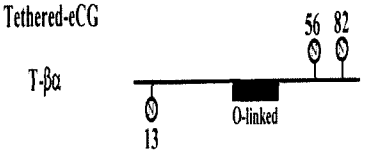

Fig. 7. Schematic diagrams of recombinant tethered-eCG. The model of tethered-eCG was shwon. A circled "N" denotes an N-linked oligosaccharide and "O-linked oligosaccharide denotes O-linked."

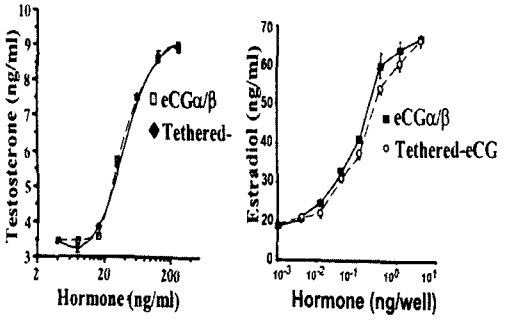

Fig. 8. Biological activity of eCG α/β (wild type) and tethered-eCG. LH (left)- and FSH (ritht)-like activities of recombinant eCGs were evaluated. Values are expressed as the mean ± SEM for at least three separate experiments.

Table 2. Hormone expression in CHOK1-cells and bioactivities of recombinant tethered -eCG relative to wild type eCG

rec-eCGs	Hormone expression (ng/100 μl)[a]	*In vitro* bioactivity(%)	
		LH	FSH
Wild type	208±29	100.0	100.0
Tethered-eCG	201±22	100.0	96.0

* [a] Determined by RIA. Values are the means ±SEM of triplicate experiments.

for the function of the glycoprotein hormone family. This data for the first time indicates that the recombinant tethered-eCG can be expressed efficiently and the produced product by the CHO-K1 cells is fully LH- and FSH-like activities in rat *in vitro* bioassay system (Fig. 8) (Table 2) (Min, 2001). Recently, the tethered molecule has been proven by several experiments on the hCG and hFSH. Sugahara et al. (1995, 1996) suggested that the tethered hCG and FSH not only was efficiently secreted but also displayed an increased biological activity *in vitro* and *in vivo* and retain a biologically active conformation similar to that seen in the heterodimer of CHO-K1 cells. Narayan et al. (1995) and Furuhashi et al. (1995) reported a similar result that the receptor binding and signal transduction of the two single chain hCGs (single chain hCG or single chain hCG devoid of CTP) were as active as wild type heterodimeric hCG that was expressed in baculovirus-infected insect cells.

Finally, these new findings are the result of the producing combination of the information gained from both genetic and biochemical approaches. Tethered-eCG can be permit development of potent new analogues that stimulate ovarian development in rodents. The constructs of tethered molecule will also be useful in the study of mutants that affect subunit association and/or secretion. A single-chain analog can also be constructed to include additional hormone-specific bioactive generating potentially efficacious compounds that have only FSH-like activity. Recombinant eCGs including the mutants, which lack oligosaccharides, will be useful tools for analyzing the structure-function relationships of gonadotropins in the horse as well as other species.

Ⅲ. 요 약

eCG(말 융모성성선자극 호르몬)β와 eLH (말 황체형성호르몬)β는 하나의 유전자로 코드 되어 있으며, eCG와 eLH는 당쇄의 구조에 있어서 차이가 있는데, LH는 sulfate가 CG는 sialylate가 수식되어 있다. eCG는 다른 동물에 있어서 강력한 FSH (난포자극)와 LH의 이중활성을 나타내어 아주 특이하고, 많은 탄수화물로 수식되어 있는 당단백질 호르몬이다. eCG의 이러한 이중활성은 성선자극호르몬의 구조, 기능 및 수용체와 이들 호르몬과의 특이결합에 대하여 분자 생물학적인 관점에서 연구하는데 아주 흥미롭다. 따라서, eCG는 당쇄의 구조와 생화학적인 기능에서 아주 특이한 분자이다.

이러한 중요점을 당쇄첨가부위의 돌연변이를 통하여 분석한 결과, LH의 활성에서는 eCGα의

56번 당쇄가 필수불가결한 역할을 하지만, eCGβ의 카르복실기 말단의 O-linked 당쇄는 중요하지 않은 것으로 관찰되었다. 한편, N- 및 O-linked 당쇄 모두는 FSH활성에는 중요한 기능을 가지고 있는데, 양쪽 당쇄의 제거는 오히려 FSH 활성을 증가시켰다. 따라서, eCG의 LH와 FSH의 이중활성은 α의 N-linked 당쇄의 제거와, β의 O-linked 당쇄를 제거함으로써 완전히 분리할 수 있으며, eCG에 있어서 당쇄는 생화학적 활성에 대하여 아주 중요하게 작용한다는 새로운 사실이 밝혀졌다.

단일체인 eCG(β의 C-terminal에 α를 연결한)도 eCGα/β 및 천연형 eCG와 비교한 결과 효율적으로 분비되어지고 완전한 LH와 FSH 활성을 나타내었다. 이러한 결과들은 eCG 분자에 있어서 지금까지 문제시되어왔던 LH활성을 나타내지 않고, 높은 FSH 활성만을 나타내는 특이한 모델을 만들 수가 있으며, 현재 단일체인 분자에 있어서 당쇄의 기능에 대한구축은 각 단체의 결합, 분비에 영향을 미치는 당쇄 돌연변이 연구에 아주 유용할 것으로 사료된다.

Ⅳ. ACKNOWLEDGMENTS

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