RESEARCH COMMUNICATION

Analysis of glycosylation sites on gp91*phox*, the flavocytochrome of the NADPH oxidase, by site-directed mutagenesis and translation *in vitro*

Timothy M. WALLACH and Anthony W. SEGAL*

Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, U.K.

Flavocytochrome b_{558} of the NADPH oxidase which generates superoxide in phagocytic cells, is a $\alpha_1\beta_1$ heterodimer of gp91*phox* and p22*phox*, which together form a membrane-spanning electron-transport chain that transfers electrons from NADPH in the cytosol to oxygen. The C-terminal portion of gp91*phox* is a member of the ferredoxin-NADP⁺ reductase family of reductases. Little is known of the organization of the N-terminal section of

this molecule, which is associated with the two haem structures. It is N-glycosylated, and site-directed mutagenesis has been used to eliminate the five potential N-linked glycosylation consensus sites. Mutated cDNAs were expressed *in vitro*. This approach provided evidence for glycosylation of residues Asn¹³¹, Asn¹⁴⁸ and Asn²³⁹, but not of Asn⁹⁶ and Asn⁴²⁹.

INTRODUCTION

Over the last few years there has been accumulating evidence on the topology of flavocytochrome b_{558} , and a great deal has come from the study of the functional consequences in patients with chronic granulomatous disease (CGD) in which this molecule is defective (reviewed in [1,2]). The molecule consists of two subunits, p22*phox* and gp91*phox*, which are present in a stoichiometry of 1:1 [3]. Secondary structure analysis indicates two to three potential transmembrane regions in p22*phox* and four to six in gp91*phox* [4,5]. The C-terminus of gp91*phox* is a member of the FNR family of reductases and contains both FAD and NADPH binding sites [6]. It has been modelled on the coordinates of FNR [7]. Regions of this part of the protein must be on the cytosolic face of the plasma membrane and thus exposed to the substrate, NADPH, and a small helix has been defined that might regulate access of NADPH to its binding cleft.

The topology of the N-terminal half of gp91*phox* is of interest because it is the probable location of the histidine residues which bind the two haems in this molecule. Some evidence for the location of these histidine residues comes from sequence homology between gp91*phox* and plasma membrane proteins, including RbohA (rice), FRE2 (*Saccharomyces cerevisiae*), FrpI⁺ (*Schizosaccharomyces pombe*), and organellar cytochromes, cytochrome *b* (*S. cerevisiae*) in the inner mitochondrial membrane and cytochrome b_6 (spinach) of the b_6f complex in the chloroplast membrane [8]. These conserved histidine residues in gp91*phox* are in positions 101 and 115 in one predicted transmembrane helix, and 209 and 222 in another [8].

gp91*phox* is heavily glycosylated through N-linkages [9], which probably helps to protect the flavocytochrome from the degradative contents of the phagocytic vacuole [10], and has five consensus sites for N-linked glycosylation at amino acid positions 96, 131, 148, 239 and 429, fairly close to the conserved histidine residues. To assist the development of the model of this region of gp91*phox*, an analysis of the glycosylation state of these sites has

been undertaken in order to identify those regions of the protein located on the outside of the membrane.

EXPERIMENTAL

cDNA and vector

cDNA of gp91*phox* (a gift from Dr. C. Porter, Chester Beatty Laboratories, London), bases –1 to 1776, was inserted into the *Bam*HI site of a pcDNA1/Amp vector (Invitrogen) under the control of a T7 promoter.

Design of primers

Each of the asparagine residues in the five Asn-Xaa-(Ser/Thr) consensus sites was changed into a threonine residue by a single base change. The five mismatch primers (mismatch sites shown in bold), purchased from Genosys, were:

- (1) Asn⁹⁶ → Thr:
CTGGACAGGACTCTCACCTTTCATAAAATGG
- (2) Asn¹³¹ → Thr:
GAATGCCCGAGTCACTAATTCTGATCC
- (3) Asn¹⁴⁸ → Thr:
GGAGACAGGCAAACCTGAAAGTTATCTC
- (4) Asn²³⁹ → Thr:
CGCAGAGAGTTTGGCTGTGCATACTATAACAGTTTGTG
- (5) Asn⁴²⁹ → Thr:
CAAATATTGCAATACCGCCACCAATCTGAAGC

The primers used to toggle between the unique non-essential restriction sites (*Xho*I, CTCGAG and *Sma*I, CCCGGG) were: *Xho*I–*Sma*I unique site elimination (USE), CCATCAGCTG–

GCGGCCGCCCGGGCATGCATCTAGAGGG and *Sma*I-XhoIUSE, CCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGG. Mutagenesis primers were phosphorylated with T4 polynucleotide kinase before use.

The following sequencing primers were used: forward 1, TGCCTGAATTTCAACTGCATG; forward 2, GTCTTTTG-GTACACACATCA; forward 3, ACCAAGGTGGTCACTC-ACC; and reverse 1, ACTATCCATTTCCAAGTCATA.

USE mutagenesis

The USE mutagenesis kit (Pharmacia) was used as recommended by the suppliers. Briefly, a mixture of circular construct, primers 1–5 and *Sma*I–XhoI USE primer were used at a molar ratio of 1:50:50. The construct was denatured and the primers were annealed. The reaction mixture (containing T4 DNA ligase, T4 DNA polymerase and T4 gene 32 protein) and nucleotide mixture (containing dNTPs and ATP) were added and the primers were extended and ligated. At this stage, digestion with *Sma*I was performed (non-mutated copies of the construct have the *Sma*I site and are linearized). The mixture was then transfected into competent *Escherichia coli* mutS, a recombination-deficient host. Since linearized (original) plasmid transfects have a much lower efficiency than circular (mutant) plasmids, this selects for mutated forms. A plasmid preparation, made from the transformed bacteria, was subjected to a second round of restriction selection with *Sma*I and re-transformed into competent normal *E. coli* with ampicillin selection. The introduction of the desired mutation into plasmids was verified by sequencing. A toggle system was devised using the XhoI–*Sma*I USE and *Sma*I–XhoI USE primer pair to allow repeated rounds of mutagenesis. Automated sequencing was carried out using 400 ng of poly(ethylene glycol)-purified plasmid and 3.2 pmol of sequencing primer.

Protein synthesis *in vitro*

The TNT[®]-coupled reticulocyte lysate system (Promega) was used for protein synthesis from the mutated gp91*phox* cDNA under the control of a T7 promoter. This allows simultaneous transcription and translation of DNA *in vitro* in the presence of [³⁵S]methionine. The newly synthesized ³⁵S-labelled proteins can then be visualized by autoradiography after SDS/PAGE. Canine pancreatic microsomal membranes were added for cotranslational glycosylation, and a synthetic N-linked glycosylation inhibitor, benzyl-Asn-Leu-Thr-N-methylamide (30 mM), was used in some experiments. SDS sample buffer was added and the samples were incubated at 37 °C for 30 min. SDS/10% (w/v) PAGE was performed according to Cleveland et al. [11] using the Hoefer Mighty Small II system. The gels were dried and exposed to autoradiography film for 48 h. Rainbow molecular-mass standards were from Pharmacia.

RESULTS AND DISCUSSION

Figure 1(A) shows the banding patterns from the various mutations chosen to elucidate the glycosylation sites of gp91*phox*. The background was minimal (lane 1). Wild-type gp91*phox*, synthesized in the absence of microsomal membrane, had a molecular mass of 50 kDa (lane 2). When microsomal membrane was added (lanes 3 and 11), the glycosylated gp91*phox* was resolved into a series of bands of 50, 53, 56 and 60 kDa. When heat-denatured membrane was added (results not shown) no glycosylation was observed. Increasing the concentration of

Figure 1 gp91*phox* glycosylation-site analysis by TNT[®] lysate

1(A) shows a representative autoradiograph of the various gp91*phox* glycoforms synthesized *in vitro* from the mutated cDNA samples shown in 1(B). Membrane was added where indicated and the positions of the unglycosylated proteins and that of the three glycosylated species are indicated on the right. 1(B) is a schematic representation of the mutated forms of gp91*phox* cDNA used in the corresponding lanes of 1(A). The filled boxes represent the wild-type glycosylation sites and the empty boxes indicate where the asparagine residue has been substituted. 1(C) is a topological scheme for gp91*phox*. The N-terminal section is based on hydrophathy profiles and the six potential transmembrane helices (I–VI) are included. The four potential haem-binding histidine residues are marked (◆). The asparagine residues occurring in Asn-Xaa-(Ser/Thr) triplets are indicated (●). Those shown to be glycosylated have a branched structure added. Asn¹⁵⁴ forms a glycosylation consensus site because of a nearby substitution in the case of one patient with CGD (○) (see text for details).

membrane reduced protein synthesis markedly, and so a complete shift from 50 to 60 kDa was not observed.

gp91*phox* normally produces bands with a wider range of molecular mass (76–90 kDa) on SDS/PAGE. This is because, *in vivo*, simple glycosylation of nascent protein occurs within the endoplasmic reticulum and the complex, higher-mass forms are processed later within the Golgi. *In vitro*, only the first step occurs and thus glycosylation is incomplete [12]. A similar (65 kDa) biosynthetic precursor for gp91*phox* has been found in certain variant patients with CGD [13,14]. It was believed that when gp91*phox* was prevented from associating with p22*phox*, because of either the absence of p22*phox* or mutations in gp91*phox*, the biosynthetic precursor was retained within the endoplasmic reticulum and further glycosylation in the Golgi was prevented.

The cDNA sample was found, after sequencing, to be complete and similar results were observed when a different sample of gp91phox cDNA, kindly supplied by Dr. M. Dinanuer (Center for Pediatric Research, Indiana University Medical Center, Indianapolis, IN, U.S.A.), was used. p22phox cDNA produced a band of 21 kDa, and when p22phox and gp91phox cDNAs were introduced together into the same transcription-translation mix, no effect on the banding patterns were seen (results not shown). The bands of 53, 56 and 60 kDa were assumed to correspond to proteins glycosylated at one, two or three sites, and the band at 50 kDa to be the residual, unglycosylated form. The glycosylation inhibitor benzyl-Asn-Leu-Thr-N-methylamide (30 mM) completely abolished glycosylation (results not shown).

Lanes 4–11 (Figure 1A) show the transcription-translation products of the mutated gp91phox cDNA samples *in vitro*. The locations of the five substituted asparagine residues are indicated schematically in Figure 1(B) and in the topological model, Figure 1(C). Substitution of Asn⁹⁶ and/or Asn⁴²⁹ had no effect on glycosylation, and all four bands were seen. After substitution of any one of Asn¹³¹, Asn¹⁴⁸ or Asn²³⁹ only three bands, representing two glycosylation events, were seen. Substitution at a combination of any two of these three sites eliminated all but one glycosylation event, resulting in two bands. When Asn¹³¹, Asn¹⁴⁸ and Asn²³⁹ were substituted, glycosylation was abolished. This shows that Asn¹³¹, Asn¹⁴⁸ and Asn²³⁹ are positioned in extracellular loops. These results from analysis *in vitro* have been confirmed by fast atom bombardment-MS analysis (E. Stimson and A. Dell, unpublished work).

This work advances our understanding of the membrane topology of gp91phox. Histidine residues 101, 115, 209 and 222 have been implicated in co-ordinating the haems found in the complex [8]. In the model (Figure 1), they would be placed in helices III and V. From this, Asn¹³¹ together with Asn¹⁴⁸, and Asn²³⁹ must occupy two distinct loops. This conflicts with the results of a study of 250 membrane proteins, in which it was found that multiple N-glycosylations are almost always observed on a single hydrophilic loop *in vivo* [15]. This loop is normally the loop nearest the N-terminus, even when distal loops also contain consensus N-glycosylation site(s). The sole exception was a tandemly duplicated protein and, in this case, only the most proximal loops in each half were glycosylated. As gp91phox appears to be glycosylated on two such loops, it makes an interesting exception.

One way of reconciling this apparent inconsistency is by suggesting that, because gp91phox is a flavocytochrome, the glycosylation pattern is a reflection of the glycosylation patterns

of the two major functional domains of this molecule, the cytochrome and the flavin, suggesting that the natural division between these molecules could be between helix V and Asn²³⁹.

'Variant' CGD is a condition in which the patient develops similar clinical symptoms of infection to that of classical CGD, but small amounts of superoxide are produced by the neutrophils. One such X-linked patient has been described [16], where an Ala¹⁵⁶ → Thr substitution converted Asn¹⁵⁴ into a potential glycosylation site (indicated in Figure 1B). In this patient, Asn¹⁵⁴ was found to be glycosylated, and this observation fits with the proposed topology. p22phox was absent in this patient, suggesting that this site might be close to a point of interaction between gp91phox and p22phox, and that glycosylation prevents this interaction and renders p22phox unstable.

We thank the Wellcome Trust for financial support. Dr. J. Groves, Department of Biochemistry, Bristol University, U.K. kindly donated the benzyl-Asn-Leu-Thr-N-methylamide and gave advice on its use in the TNT® system.

REFERENCES

- 1 Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1994) *Biochim. Biophys. Acta* **1227**, 1–24
- 2 Roos, D., de Boer, M., Kuribayashi, F., Weening, R. S., Segal, A. W., Ahlin, A., Nemet, K., Hossle, J. P., Bernatowska-Matuszkiewicz, E. and Middleton-Price, H. (1996) *Blood* **87**, 1663–1681
- 3 Wallach, T. M. and Segal, A. W. (1996) *Biochem. J.* **320**, 33–38
- 4 Parkos, C. A., Dinanuer, M. C., Walker, L. E., Allen, R. A., Jesaitis, A. J. and Orkin, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3319–3323
- 5 Shatwell, K. P., Dancis, A., Cross, A. R., Klausner, R. D. and Segal, A. W. (1996) *J. Biol. Chem.* **271**, 14240–14244
- 6 Segal, A. W., West, I., Wientjes, F. B., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H. and Scrase, G. (1992) *Biochem. J.* **284**, 781–788
- 7 Taylor, W. R., Jones, D. T. and Segal, A. W. (1993) *Prot. Sci.* **2**, 1675–1685
- 8 Finegold, A. A., Shatwell, K. P., Segal, A. W., Klausner, R. D. and Dancis, A. (1996) *J. Biol. Chem.* **271**, 31021–31024
- 9 Harper, A. M., Chaplin, M. F. and Segal, A. W. (1985) *Biochem. J.* **227**, 783–788
- 10 Kleinberg, M. E., Rotrosen, D. and Malech, H. L. (1989) *J. Immunol.* **143**, 4152–4157
- 11 Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U.K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- 12 Titus, D. E. (1991) in *Protocols and Applications Guide* (Titus, D. E., ed.), pp. 156–173, Promega Corp., Madison
- 13 Porter, C. D., Kuribayashi, F., Parkar, M. H., Roos, D. and Kinnon, C. (1996) *Biochem. J.* **315**, 571–575
- 14 Porter, C. D., Parkar, M. H., Verhoeven, A. J., Levinsky, R. J., Collins, M. K. and Kinnon, C. (1994) *Blood* **84**, 2767–2775
- 15 Landolt Marticorena, C. and Reithmeier, R. A. (1994) *Biochem. J.* **302**, 253–260
- 16 Bolscher, B. G. J. M., de Boer, M., De Klein, A., Weening, R. S. and Roos, D. (1991) *Blood* **77**, 2482–2487