

# Chapitre 1

## Article 1 – Analyse fonctionnelle de la substitution de deux acides aminés de gp91<sup>phox</sup> chez un patient atteint de CGD X<sup>+</sup> à partir de cellules PLB-985 transgéniques

### Introduction

La granulomatose septique chronique (CGD) est une maladie génétique rare (fréquence 1/250 000), caractérisée par des infections bactériennes et fongiques récurrentes et sévères (Roos et al. 1996a). La forme variante CGD X<sup>+</sup>, caractérisée par une expression normale du cytochrome *b*<sub>558</sub> mais une activité oxydase abolie, est très intéressante car elle permet d'étudier la relation de la structure et la fonction de certaines régions protéiques de gp91<sup>phox</sup>. Un cas de CGD X<sup>+</sup>, diagnostiqué au laboratoire, est caractérisé par une double mutation faux-sens (C919A/C923G) dans l'exon 9 du gène *CYBB*, entraînant une double substitution (His303Asn/Pro304Arg) dans la séquence protéique de gp91<sup>phox</sup>, responsable d'un défaut d'assemblage du complexe oxydase (Stasia et al. 2002).

### Objectifs et méthodologie

L'objectif de ce travail est :

- De valider le modèle cellulaire X-CGD PLB-985 pour l'étude de cas de CGD X<sup>+</sup>. Le modèle cellulaire utilisé est une lignée myélomonoblastique humaine PLB-985 induite en granulocytes sous l'effet du DMF (diméthylformamide) et dont le gène *CYBB* a été inactivé par recombinaison homologue (X-CGD PLB-985) (Tucker et al. 1987 ; Zhen et al. 1993). La première étape a donc été de reconstituer une activité NADPH oxydase par transfection du cDNA de gp91<sup>phox</sup> sauvage (WT) dans la lignée PLB-985 KO. Les clones résistants à la généticine (1.5 mg/ml) ont été sélectionnés par dilution limite.
- De reconstituer le cas de CGD X<sup>+</sup> dans le modèle X-CGD PLB-985 par mutagenèse dirigée et transfection stable afin d'obtenir le phénotype exact des neutrophiles du patient.
- L'étude de 2 modèles cellulaires contenant chacun une mutation (His303Asn ou Pro304Arg) pour nous permettre d'étudier l'impact de chaque mutation sur l'activité oxydase et son mécanisme d'assemblage.

L'expression de Nox2 WT et mutée a été suivie par 3 méthodes : cytométrie en flux grâce à l'anticorps 7D5 (don du Dr. Nakamura, Nagasaki, Japon) qui reconnaît un épitope externe de Nox2, immuno-détection grâce à 2 anticorps monoclonaux reconnaissant Nox2 et p22<sup>phox</sup> (don du Dr. Roos, Amsterdam, Hollande), spectrophotométrie différentielle du cytochrome *b*<sub>558</sub>.

L'activité NADPH oxydase a été mesurée dans les cellules intactes par chemiluminescence et dans le système acellulaire reconstitué par réduction du cytochrome *c* sensible à la SOD (Stasia et al. 2002a).

L'assemblage du complexe oxydase a été évalué *in vitro* par une méthode classique (Stasia et al. 2002a), et *in vivo* par le suivi de la translocation de p47<sup>phox</sup> dans la membrane des phagosomes des cellules PLB-985 transfectées et activées par du zymosan opsonisé par microscopie confocale (Collaboration avec le Dr. Roos, Amsterdam, Hollande).

## Résultats

- Les taux d'expression de gp91<sup>phox</sup> dans les clones PLB-985 WT ou mutés rapportés à celui des cellules PLB-985 WT d'origine et différenciées, sont comparables pour les trois techniques utilisées.
- Les cellules PLB-985, exprimant la mutation His303Asn/Pro304Arg, miment exactement le phénotype des neutrophiles issus du patient, caractérisé par une expression normale du cytochrome *b*<sub>558</sub> associée à une activité oxydase totalement abolie en relation avec un défaut d'assemblage du complexe oxydase.
- La mutation His303Asn de gp91<sup>phox</sup> est suffisante pour abolir totalement l'activité oxydase ainsi que l'assemblage du complexe oxydase *in vivo* et *in vitro*.
- La mutation Pro304Arg n'inhibe pas totalement l'activité oxydase (activité résiduelle de 4 % *in vivo* et 67% *in vitro* par rapport aux cellules sauvages). Il semble que cette inhibition soit en relation avec un défaut partiel d'assemblage du complexe. Ainsi chacune de ces mutations ne peut pas être considérée comme un polymorphisme du gène *CYBB*.
- Ces résultats sont confirmés par la reconstitution d'un modèle en trois dimensions (3D) de la queue cytosolique C-terminale de gp91<sup>phox</sup> permettant de localiser l'His<sup>303</sup> et la Pro<sup>304</sup> (Collaboration Dr. Eppink, Oss, Hollande). Les deux acides aminés se situent à la surface de la protéine. Ces résidus ne forment pas de liaisons hydrogènes fortes avec les acides aminés alentour, et leur position n'est pas compatible avec une interaction avec le FAD ou le NADPH (distance minimale 6–7 Å). Par contre leur position pourrait leur permettre une interaction possible avec les facteurs cytosoliques p47<sup>phox</sup> et /ou p67<sup>phox</sup>.

# Chapter 1

## **Functional analysis of two-amino acid substitutions in gp91<sup>phox</sup> in a patient with X-linked flavocytochrome *b*<sub>558</sub>-positive chronic granulomatous disease by means of transgenic PLB-985 cells**

### **Background**

Chronic granulomatous disease (CGD) is a rare inherited disorder characterized by a greatly increased susceptibility to severe bacterial and fungal infections (Roos et al. 1996a). It results from any one of four genes that encode the subunits of phagocyte NADPH oxidase, the enzyme that generates microbicidal (and pro-inflammatory) oxygen radicals (Heyworth et al. 2003). The most common form (X<sup>0</sup>-CGD) is caused by mutations in the *CYBB* gene encoding gp91<sup>phox</sup> protein, the heavy chain of cytochrome *b*<sub>558</sub>, which is the catalytic center of NADPH oxidase. Rare few mutations in gp91<sup>phox</sup> lead a normal expression of mutated protein with nonfunctional cytochrome *b*<sub>558</sub>, referred as X<sup>+</sup>-CGD. This subtype of X-CGD provides an ideal model for studying the interaction between gp91<sup>phox</sup> and the oxidase cytosolic components. We have previously reported an X<sup>+</sup>-CGD case with a double-missense mutation in gp91<sup>phox</sup> (His303Asn/ Pro304Arg), resulted from two base substitutions (C919 to A and C923 to G) in genomic DNA (Stasia et al. 2002). This mutated gp91<sup>phox</sup> has no effect on the FAD-binding capacity in the patient's neutrophil membranes and its proton channel-function in EBV-transformed B lymphocytes. While a defective translocation of p47<sup>phox</sup> and p67<sup>phox</sup> to the neutrophil membranes from the patient was observed, suggesting that residues 303 and 304 are crucial for the stable assembly of the NADPH oxidase complex. Mutated gp91<sup>phox</sup> cDNA was transfected in X-CGD PLB-985 cells (Zhen et al. 1993) to study the impact of each single mutation on oxidase activity and assembly and to rule out a possible new polymorphism in the *CYBB* gene.

### **Construction of gp91<sup>phox</sup> transfected PLB-985 cells – Evaluation of WT and mutated gp91<sup>phox</sup> expression by flow cytometry, immuno-detection and reduced minus oxidized difference spectra of cytochrome *b*<sub>558</sub>**

His303Asn, Pro304Arg and the double mutation were generated from the WT gp91<sup>phox</sup> cDNA in pBluescript II KS (+) vector by QuikChange site-directed mutagenesis kit according to the manufacturer's instructions and subcloned into the mammalian expression vector pEF-PGKneo (Zhen et al. 1998; Yu et al. 1999). All the gp91<sup>phox</sup> cDNA constructs were sequenced to confirm the mutations and were transfected into X-CGD PLB-985 cells by electroporation at 250 V for 20 ms. Positive clones were selected by limited dilution in the presence of 1.5 mg/ml Geneticin. 7D5 mAb against gp91<sup>phox</sup>, which recognizes an external

epitope of gp91<sup>phox</sup> (Yamauchi et al. 2001), was used to select the clones expressing high levels of recombinant wild-type or mutated gp91<sup>phox</sup>.

Gp91<sup>phox</sup> expression was assessed in 10 clones for His303Asn gp91<sup>phox</sup> PLB-985 cells, 10 clones for Pro304Arg gp91<sup>phox</sup> PLB-985 cells, and 6 clones for His303Asn/Pro304Arg gp91<sup>phox</sup> PLB-985 cells. A high and stable expression of recombinant His303Asn/Pro304Arg, His303Asn and Pro304Arg gp91<sup>phox</sup> in X-CGD PLB-985 cells was observed by flow cytometry. The specificity of the 7D5 binding was assessed with an irrelevant monoclonal IgG1 (Fig. 1A). The high gp91<sup>phox</sup> expression in transgenic PLB-985 cells was confirmed by immunoblotting analysis by using mAb 48 (Fig. 1B). A correlated expression of p22<sup>phox</sup> was observed in the wild-type and in the mutant gp91<sup>phox</sup> transfected PLB-985 cells compared to the original WT PLB-985 cells. Reduced-minus-oxidized difference spectra were performed in 1% Triton-X100 soluble extracts from transfected PLB-985 cells. Identical spectra characteristic of flavocytochrome *b*<sub>558</sub> were observed for both mutated and WT gp91<sup>phox</sup> PLB-985 cells and the amount of cytochrome *b*<sub>558</sub> correlated with the level of gp91<sup>phox</sup> expression observed by flow cytometry and Western blotting (Fig. 1C, Table 1). While no gp91<sup>phox</sup> and cytochrome *b*<sub>558</sub> was detected in X-CGD PLB-985 cells or empty vector transfected cells. This is consistent with the previous observations that co-expression of both gp91<sup>phox</sup> and p22<sup>phox</sup> and subsequent heterodimer formation is important for the stable of each flavocytochrome *b*<sub>558</sub> subunit (Yu et al. 1997; Yu et al. 1999).

- *A high and stable expression of recombinant WT and mutated gp91<sup>phox</sup> was observed in transfected PLB-985 cells. Similar results were observed by flow cytometry, Western blot and spectrophotometry for the quantification of the level of gp91<sup>phox</sup> expression.*

### **Detection of NADPH oxidase activity in transfected PLB-985 cells**

The NADPH oxidase activity of intact mutant or wild-type gp91<sup>phox</sup> transfected PLB-985 cells was determined by luminol-amplified chemiluminescence. After 0.5% dimethylformamide (DMF)-induced granulocytic differentiation, H<sub>2</sub>O<sub>2</sub> production of activated cells by 80 ng/ml phorbol 12-myristate 13-acetate (PMA), was measured every 30 s for a total of 60 min at 37°C in a Luminoscan luminometer coupled to a computer. WT gp91<sup>phox</sup> transfected PLB-985 cells have a comparable H<sub>2</sub>O<sub>2</sub> generation to the original WT PLB-985 cells with a maximum activity at day 6 (Fig. 2). Even though the expression of His303Asn/Pro304Arg was normal, the mutant transfected cells were unable to generate H<sub>2</sub>O<sub>2</sub> as observed in X-CGD PLB-985 cells or empty vector transfected cells. To compare only the effect of mutations on gp91<sup>phox</sup> in the plasma membranes, cytosol from purified human neutrophils was used as a source of the oxidase cytosolic components. Residual NADPH oxidase activity was measured in the membranes from the double mutant-transfected cells (24% of the WT PLB-985 cell activity). This reconstituted activity could be due to the artificial NADPH oxidase assembly in presence of arachidonic acid which is probably absent in physiological conditions where phosphorylation steps occur to conduct the assembly. For example, superoxide-generating activity in a CFS assay could be reconstituted with higher concentration of p67<sup>phox</sup> and Rac without p47<sup>phox</sup> (Freeman et al. 1996; Palet et al. 2000). On the contrary, in AR47<sup>0</sup>-CGD neutrophils, NADPH oxidase activity is abolished even in the presence of p67<sup>phox</sup> and Rac.

The His303Asn mutation alone is sufficient to inhibit the oxidase activity in intact cells and in a broken cell system (Table 1, Fig. 2), mimicking the effect obtained with the double mutation on the oxidase activation. Whereas the Pro304Arg mutant restored 4% activity in intact transfected cells and 67% activity in cell-free-system, suggesting that the Pro304Arg substitution is less devastating to oxidase activity than the His303Asn mutation.

- *These data demonstrate that the double mutation gp91<sup>phox</sup>-transfected PLB-985 cells mimicked exactly the X<sup>+</sup>-CGD neutrophils from the patient. His303Asn and Pro304Arg mutations inhibit the NADPH oxidase activity ruling out the possibility of a polymorphism in the CYBB gene. However the His303Asn mutation has a more devastating effect on oxidase activity than the Pro304Arg.*

### Study of NADPH oxidase assembly in transfected PLB-985 cells

The membrane translocation of the oxidase cytosolic components p47<sup>phox</sup> and p67<sup>phox</sup> was determined *in vitro* and *in vivo* to investigate whether the inhibitory effect of each single mutation and the double mutation on NADPH oxidase activity is related to a defect of oxidase assembly process. The *in vitro* translocation of p47<sup>phox</sup> and p67<sup>phox</sup> to the plasma membranes purified from transfected PLB-985 cells, was determined using a classical method (Stasia et al. 2002). After stimulation by SDS and GTPγS, the translocation of p47<sup>phox</sup> and p67<sup>phox</sup> occurs normally as observed in original WT PLB-985 cells and in control human neutrophils (Fig. 3A). No translocation of the cytosolic factors was detected in the membranes from the double mutated gp91<sup>phox</sup> transfected cells. This result is in agreement with the defective translocation of the oxidase cytosolic factors observed in plasma membranes from the X<sup>+</sup>-CGD patient's neutrophils (Stasia et al. 2002a). For the single mutation, a faint cytosolic factor translocation was observed (more visible for p47<sup>phox</sup>), but the defect of translocation was more pronounced in the His303Asn. No *in vitro* cytosolic factor translocation to the plasma membrane occurred in the empty vector-transfected cells (data not shown) as seen in human neutrophils from an X<sup>0</sup> CGD patient (Fig. 3A). This confirmed that the membrane-translocation of p67<sup>phox</sup> and p47<sup>phox</sup> is dependent on the presence of cytochrome b<sub>558</sub> (Heyworth et al. 1994).

The *in vivo* p47<sup>phox</sup> translocation was checked in WT, X-CGD and transfected PLB-985 cells differentiated for 6 days with 0.5% DMF and stimulated with STZ (serum-treated zymosan) for 10 or 30 min at 37°C. The assembly of the oxidase complex was performed by confocal microscopy to follow the p47<sup>phox</sup> translocation to the phagosomal membranes. Undisturbed phagocytosis of STZ was checked by phase contrast microscopy (Fig. 3B). Phagocytosis of STZ occurred in WT, X-CGD and transgenic PLB-985 cells, independently of the oxidase activity. P47<sup>phox</sup> protein was present in cytosol from all the tested PLB-985 cells. In WT gp91<sup>phox</sup> PLB-985 cells, p47<sup>phox</sup> translocation occurred, as a strong red fluorescence was present surrounding the STZ particles. On the contrary, no p47<sup>phox</sup> translocation was observed in the double-mutant gp91<sup>phox</sup> PLB-985 cells or in His303Asn gp91<sup>phox</sup> PLB-985 cells. While, in Pro304Arg gp91<sup>phox</sup> PLB-985 cells, the p47<sup>phox</sup> translocation occurred but with a seemingly lower efficiency than in WT 91<sup>phox</sup> PLB-985 cells.

- *In the double and single mutated gp91<sup>phox</sup> transfected PLB-985 cells, the inhibition of NADPH oxidase activity results from a defect in oxidase complex assembly. However His303Asn mutation seems to have a higher inhibitory*

*effect than the Pro304Arg mutation.*

**Analysis of the 3D-model of the cytosolic C-terminal of gp91<sup>phox</sup>** (Taylor et al. 1993; Leusen et al. 2000)

The 3D-model of the C-terminal tail of gp91<sup>phox</sup> indicates that both His<sup>303</sup> and Pro<sup>304</sup> are located at the surface of the protein, at the N-terminus of the beta-sheet ( $\beta$ F2) in hydrophilic surroundings (Fig. 4). The charge changes induced by the mutations (His303Asn, more neutral; Pro304Arg, strongly positive) will therefore not strongly affect the positions of the surrounding residues. The Pro304Arg mutation is expected to have the strongest effect on the structure of the protein from the structural model, because this mutant affects the backbone of the loop between  $\beta$ F1 and  $\beta$ F2; While the position of His303 and Pro304 is compatible with a possible direct binding with cytosolic factors during the oxidase assembly process. Moreover, His<sup>303</sup> and Pro<sup>304</sup> do not form strong hydrogen bonds with nearby amino acids and do not directly interact with FAD or NADPH (the distance is minimally 6–7 Å). This model indicates that the distance to the FAD-binding site is not compatible with a direct interaction of these amino acids with the binding site. This confirms our previous result that FAD binding is not affected by the double mutation in the neutrophil membranes from the X<sup>+</sup>-CGD patient (Stasia et al. 2002a).

- *The His303Asn/Pro304Arg mutation in gp91<sup>phox</sup> may lead to a conformational change in cytochrome b<sub>558</sub> which result in a defective cytosolic factor assembly which can explain the NADPH oxidase activity inhibition.*