Conclusion et Perspectives

Les formes variantes CGD X^+ et X^- sont de loin les plus intéressantes puisqu'elles permettent l'étude directe du lien entre une région précise de gp91^{phox} ou Nox2 et sa fonction (CGD X^+) ou son mécanisme d'expression (CGD X^-). La modélisation cellulaire de ces mutations humaines est nécessaire pour l'obtention d'un matériel biologique suffisamment abondant afin de permettre des études biochimiques approfondies. Le rôle joué par certains domaines spécifiques de gp91^{phox} dans le transfert électronique, l'activation, l'assemblage et la désactivation du complexe oxydase, mais également dans les processus de maturation de gp91^{phox}, pourront alors être étudiés au niveau moléculaire.

Le modèle utilisé au laboratoire est une lignée cellulaire pro-myelocytaire PLB-985 induite en neutrophile sous l'effet du diméthylformamide et dont le gène *CYBB* a été inactivé par recombinaison homologue (PLB-985 KO) mais possédant tous les autres composants de l'oxydase (p47^{phox}, p67^{phox}, p40^{phox} et Rac2). A l'heure actuelle une seule CGD X^+ a été reconstituée et étudiée par l'équipe de Mary Dinauer dans le modèle cellulaire PLB-985 (Yu et al. 1999).

L'approche expérimentale consiste à reproduire les mutations humaines dans le cDNA de gp91^{phox} par mutagénèse dirigée. Le clonage du cDNA sauvage et muté de gp91^{phox} est ensuite effectué dans un vecteur d'expression, pEF-PGK-neo, puis les différentes constructions sont introduites dans les cellules PLB-985 KO pour le gène *CYBB*, par électroporation. La sélection des clones positifs se fait par la méthode des dilutions limites en présence d'un antibiotique (la généticine). Après trois semaines de culture, les clones positifs sont repérés, les cellules sont cultivées toujours avec une pression de sélection. Lorsque le nombre de cellules est suffisant, la correction d'activité oxydase par transfection du cDNA de gp91^{phox} sauvage ainsi que l'impact des différentes mutations mimant la CGD X^+ ou la CGD X^-, sont évalués par mesure des dérivés toxiques de l'oxygène par chimiluminescence et par spectrophotométrie. L'expression de la protéine gp91^{phox} mutée ou sauvage est révélée par immuno-détection à l'aide d'anticorps monoclonaux spécifiquement dirigés contre cette protéine (Don du Dr. D. Roos, CLB, Amsterdam) et par cytométrie en flux (collaboration avec le Dr. J. Boutonnat, laboratoire de cytologie, CHU Grenoble et le Dr. J. Mathieu, Département de radiobiologie, CRSSA, Grenoble) grâce à un anticorps monoclonal 7D5 dirigé contre un épitope externe de gp91^{phox} (Don du Dr. Nakamura, Nagasaki, Japon). Cette dernière technique permet également de contrôler l'insertion membranaire correcte de gp91^{phox} muté. L'activité NADPH oxydase dans les cellules intactes est mesurée par le suivi de la production de H_2O_2 par chimiluminescence. L'activité oxydase reconstituée est mesurée par réduction du cytochrome *c* sensible à la SOD, en présence de membranes plasmiques (ou de cytochrome b_{558} purifié), de facteurs cytosoliques (ou de protéines cytosoliques recombinantes purifiées), de NADPH, de GTPγS et d'acide arachidonique. L'assemblage du complexe oxydase *in vivo* est étudié par microscopie confocale en suivant la translocation du facteur cytosolique p47^{phox} à la membrane après stimulation des cellules PLB-985 transfectées par des particules de latex enrobées de PMA (Collaboration avec le Dr. D. Roos, CLB, Amsterdam, Hollande et le Dr. D. Grunwald, CIS, CEA, Grenoble).

⇨ La lignée PLB-985 KO pour le gène codant gp91^{phox} nous a déjà permis :

- De reconstituer une activité NADPH oxydase par transfection du cDNA de gp91^{phox} sauvage. Ceci constitue un essai de thérapie correctrice *ex-vivo.* L'activité NADPH oxydase générée est comparable à celle mesurée dans les cellules PLB-985 de type sauvage (100 % de restauration d'activité, expression de gp91^{phox} comparable à celle mesurée dans les PLB-985 sauvages).
- De reproduire par mutagénèse dirigée, les modèles de **CGD X^{+}** que nous avons diagnostiqués au laboratoire.
- De reproduire un modèle de **CGD X^{-}** (Ser193Phe) par mutagénèse dirigée.

Dans le cas de la **double mutation His303Asn/Pro304Arg** nous avons établi par transfection, des lignées cellulaires PLB-985 exprimant soit la mutation His303Asn, soit la seconde mutation Pro304Arg soit la double mutation. Nous avons pu vérifier que :

- la mutation His303Asn de gp91^{phox} est suffisante pour abolir totalement l'activité NADPH oxydase ainsi que l'assemblage du complexe oxydase *in vivo*.
- la mutation Pro304Arg inhibe en partie l'activité NADPH oxydase avec un effet partiel sur l'assemblage du complexe.
- Ainsi chacune de ces mutations ne constitue pas un polymorphisme mais seule la mutation His303Asn est responsable d'un défaut de translocation des facteurs cytosoliques et de leur assemblage au niveau du cytochrome b_{558} pour la constitution d'une oxydase.
- L'étude du modèle 3D de la queue cytosolique C-terminale de Nox2 nous montre une localisation des His^{303} et Pro^{304} à la surface de la protéine, dans un environnement hydrophile compatible avec une éventuelle interaction avec les facteurs cytosoliques de l'oxydase (collaboration Dr. Eppink, Oss, Hollande). Ces acides aminés ne forment pas de liaisons hydrogène fortes avec les acides aminés alentour, et leur localisation n'est pas compatible pour interagir avec les sites de fixation du FAD ou du NADPH (distance minimale 6-7 Å).

La même approche a été utilisée pour l'étude d'un autre cas de **CGD X^{+}** dû à une mutation faux-sens **Leu505Arg** sur Nox2. Nous avons pu montrer que le modèle cellulaire utilisé permet de reconstituer exactement le phénotype des neutrophiles du cas de CGD X^{+}, comme dans le cas précédemment étudié. Malgré une activité oxydase nulle, la translocation des facteurs cytosoliques semble exister mais n'est vraisemblablement pas optimale. Etonnamment une activité NADPH oxydase et INT réductase pour le mutant ont été reconstituées *in vitro* à 50% par rapport au cytochrome b_{558} WT, prouvant qu'un transfert électronique ainsi que l'assemblage de l'enzyme sont possibles dans certaines conditions.

Le K_m du NADPH et du NADH pour le cytochrome b_{558} muté purifié, en présence de protéines recombinantes p67^{phox}, p47^{phox} et Rac, est augmenté de 3 fois alors que le turnover de l'enzyme est diminué de moitié. La mutation Leu505Arg gêne la fixation du NADPH sur son site mais ne semble pas être directement impliquée dans cette reconnaissance. Enfin il faut 3 fois plus de p67^{phox} en présence du cytochrome b_{558} muté purifié que dans le cas du cytochrome b_{558} WT pour obtenir une activité maximale de la NADPH oxydase, mais qui reste inférieure à celle du cytochrome b_{558} WT. Le modèle 3D de la queue C-terminale cytosolique de Nox2 montre que la Leu^{505} possède une position stratégique pour l'accès au site de fixation du NADPH sans que cet acide aminé soit directement impliqué dans sa fixation (collaboration Dr. F. Fieschi, IBS, Grenoble). Cette mutation met l'accent sur

l'importance de la fonction de l'hélice α dans la liaison des facteurs cytosoliques, probablement p67^{phox}, et sur le rôle de cette liaison pour la fixation du NADPH à son site.

La même approche a été utilisée pour l'étude de la biosynthèse du cytochrome b_{558} dans le cas de la CGD X^{-} (Ser193Phe). La mutation a été reproduite dans les lignées cellulaires PLB-985 et COS-7. Les cellules PLB-985, exprimant la mutation Ser193Phe, miment exactement le phénotype des neutrophiles issus du patient (Roesler et al. 1999), avec une expression diminuée du cytochrome b_{558} associée à une activité oxydase abolie. Cette mutation inhibe la maturation de la protéine Nox2 dans les deux lignées cellulaires étudiées. D'autre part, dans les cellules PLB-985 transfectées, la mutation Ser193Phe inhibe l'expression de l'ARNm de Nox2 mutant.

Grâce à la mise en place de la méthodologie d'étude de l'impact des mutations humaines du gène *CYBB* sur le fonctionnement du complexe oxydase des phagocytes, nous avons pu explorer le rôle joué par **deux régions intra-cytosoliques de Nox2, la boucle D et une boucle formant une hélice α dans la partie C-terminale de Nox2** (Figure 7-Introduction).

L'impact des mutations générées dans la boucle D sur l'activité NADPH oxydase a été évalué par mesure des H_2O_2 totaux après activation par le PMA. Les cellules PLB-985 transfectées exprimant K195A-gp91^{phox}, K195E-gp91^{phox}, R198E-gp91^{phox} R199E-gp91^{phox} RR198199QQ-gp91^{phox} ont une activité NAPDH oxydase totalement abolie. Ceci prouve l'importance des charges positives des Lys et Arg de la boucle D dans le maintien de l'activité NADPH oxydase, et notamment pour la Lys^{195}, l'abolition (A) ou le changement de charge (E) est délétère pour l'activité oxydase. Etonnamment la double mutation RR198199EE permet d'obtenir une activité oxydase de l'ordre de 60% par rapport aux cellules PLB-985 WT alors que les simples mutations (R198E ou R199E) abolissent complètement cette activité. Ceci prouve que la charge globale de la boucle D est également importante pour l'activité oxydase. Nous avons également voulu remplacer la boucle D de Nox2 par celle de Nox1, Nox3 ou Nox4. La Lys^{195} est absente dans ces homologues de Nox2 alors que l'Arg^{198} est conservée. Cependant nous remarquons que non seulement l'activité oxydase des protéines chimères gp91^{phox}-boucle D_{Nox1}, gp91^{phox}-boucle D_{Nox3}, gp91^{phox}-boucle D_{Nox4} n'est pas abolie mais elle est augmentée, et ceci est particulièrement significatif pour les cellules mutantes exprimant gp91^{phox}-boucle D_{Nox4}. Afin de contrôler au mieux l'impact des mutations de gp91^{phox} sur l'activité du complexe oxydase, un autre agent stimulant, le fMLP, a été utilisé pour activer les cellules PLB-985 WT et "transgéniques". Globalement nous obtenons les mêmes effets inhibiteurs de certaines mutations sur l'activité oxydase, lorsque les cellules sont activées par le PMA et le fMLP. Par contre certaines mutations et notamment celles de la boucle D, qui n'avaient aucun effet sur l'activité oxydase lorsque les cellules étaient stimulées par le PMA, provoquent une suractivation de cette enzyme lorsque les cellules PLB-985 "transgéniques" sont activées par le fMLP. En effet nous remarquons deux super-mutants gp91^{phox}-R199Q et gp91^{phox}-boucle D_{Nox4} qui présentent respectivement une activité oxydase multipliée par 8 et par 4 par rapport aux cellules WT. Nous pouvons peut être penser que le PMA et le fMLP empruntent des voies de signalisation différentes impliquant l'activation de kinases et/ou de phosphatases permettant un niveau de phosphorylation différent des facteurs cytosoliques p47^{phox} et/ou p67^{phox} modulant ainsi l'activité NADPH oxydase et son assemblage. Il se peut également que ces deux agents stimulants provoquent une localisation du complexe oxydase différente.

L'assemblage du complexe oxydase des lignées PLB-985 transfectées a ensuite été étudié par microscopie confocale, en suivant la translocation du facteur cytosolique p47^{phox} à la membrane des phagosomes après activation par des billes de latex enrobées de PMA. Nous avons tout d'abord validé la qualité de cette technique en effectuant un triple marquage, p47^{phox}-Alexa 488, gp91^{phox}-Alexa 568, l'ADN nucléaire-TO-PRO 3. Une aquisition séquentielle des images nous a permis de visualiser une co-localisation de p47^{phox} et de gp91^{phox} au niveau de la membrane des phagosomes dans les cellules PLB-985 WT. Les résultats obtenus avec la lignée Asp500Gly-Nox2 PLB-985 mimant le cas de CGD X^{+} décrit par l'équipe de D. Roos (Leusen et al. 1994) montrent une parfaite adéquation avec le comportement des neutrophiles du patient. En effet lors de l'activation de ces cellules par les billes de latex, gp91^{phox} se retrouve au niveau de la membrane du phagosome alors que p47^{phox} a une localisation strictement cytosolique, montrant un défaut d'assemblage du complexe oxydase. Pour les mutants de la boucle D nous avons remarqué une translocation correcte de p47^{phox} à la membrane, montrant que l'abolition de l'activité NADPH oxydase ne provenait pas d'un défaut d'assemblage du complexe. Les résultats de translocation *in vivo* de p47^{phox} ont été confirmés par la méthode plus classique d'activation de l'assemblage du complexe oxydase *in vitro*.

Dans un dernier volet, l'activité INT réductase, reflet du transfert électronique du NADPH vers le FAD au niveau de la partie cytosolique C-terminale de gp91^{phox}, a été évaluée. Clairement les mutants de la boucle D de gp91^{phox} ont une activité INT réductase équivalente à celle mesurée dans les cellules PLB-985 WT ou KO transfectées par l'ADNc de gp91^{phox} WT.

En ce qui concerne **les mutants de la queue cytosolique C-terminale de Nox2,** les mutations D484T, H490D, mais pas D484H et H490A, abolissent totalement l'activité NADPH oxydase. L'acide aminé Asp^{500} comme nous l'avons vu plus haut pour la Lys^{195}, semble essentiel au maintien de l'activité NADPH oxydase. En effet les charges négatives sont ici primordiales pour cette activité puisque les mutations D500A, D500R et D500G sont toutes délétères pour cette enzyme.

A la différence des mutants de la boucle D de Nox2, les mutants de la queue cytosolique C-terminale ne possédant pas d'activité oxydase (mutations D484T, D500A, D500R) montrent un défaut de translocation de p47^{phox}. Les résultats obtenus par l'équipe de M. Dinauer avec le double mutant RR9192EE de la boucle B de gp91^{phox} connu pour induire ce même défaut, ont été confirmés. L'activité INT réductase pour les mutants ne possédant pas d'activité oxydase, est totalement abolie signifiant qu'il n'y a pas de passage électronique du NADPH vers le FAD.

Finalement nous pouvons penser que les charges positives de la boucle D (Lys^{195}, Arg^{198}, Arg^{199}) ainsi que les charges négatives de la queue cytosolique C-terminale de gp91^{phox} formant une hélice α, sont indispensables au maintien de l'activité NADPH oxydase des phagocytes. Cependant seule l'hélice α semble impliquée dans les processus d'assemblage du complexe oxydase. La séquence C-terminale de gp91^{phox} centrée autour de l'Asp^{500} est essentielle pour l'assemblage du complexe oxydase et probablement pour une conformation adaptée de gp91^{phox}, permettant un transfert électronique correct du NADPH vers le FAD. Nous pouvons remarquer également que la boucle D est localisée dans une région très proche du 5ème passage transmembranaire de Nox2. Elle pourrait

ainsi permettre un contrôle du passage électronique du FAD vers l'oxygène *via* les hèmes. La mutation Ser193Phe à l'origine d'une CGD X^- en cours d'étude, met clairement en évidence que la boucle D, cruciale pour l'activité NADPH oxydase, est probablement impliquée dans le maintien d'une structure correcte du cytochrome b_{558}. Ces hypothèses restent à confirmer.

Perspectives

Pour les mutants dans la boucle-D

1. Etudier le mécanisme d'activation de l'oxydase dans les deux super-mutants (R199Q et gp91^{phox}-boucle D_{Nox4}).
2. Essayer de comprendre le défaut de la maturation de Nox2 dans le cas de CGD X^- (Ser193Phe).
   1) Abandonner le système COS-7.
   2) Transfecter Nox2 WT et mutée dans les cellules HEK 293 humaines pour verifier le défaut de glycosylation observée dans les cellules PLB-985.
   3) Vérifier l'expression de ARNm de Nox2 WT et mutée dans le système PLB-985 et HEK 293 par Northern blot.
   4) Etudier la localisation sub-cellulaire des différentes formes de Nox2 WT et mutée et p22^{phox} dans ces deux lignées cellulaires.
   5) Analyser l'incorporation des hèmes dans Nox2 muté dans les deux types cellulaires par spectrophotométrie différentielle du cytochrome b_{558}.
   6) Etudier la synthèse de Nox2 muté à partir d'ARNm purifié en présence du système de synthèse protéique *in vitro* de réticulocytes de globules rouges de lapin.

Pour l'helice α C-terminale de Nox2

3. Determiner le rôle de l'hélice α C-terminale (acides aminés 484-504) sur l'activation du complexe oxydase. Un mutant delété de cette hélice pourra être étudié. En effet une grande partie des réductases de la famille FNR ne posséde pas cette séquence en C-terminal et sont activables uniquement en présence de leur substrat sans assemblage préalable.

Les autres idées

4. Essayer de comprendre la relation entre le taux d'activité oxydase des cellules exprimant Nox2 muté, et la bactéricidie. Ceci permettra de determiner le taux minimal d'ions superoxyde nécessaires pour tuer les bactéries.
5. Analyser des mutations localisées dans le site potentiel de fixation du NADPH, ou du FAD (Thr341Lys, Gly408Glu, Pro415His, Cys537Arg).

Conclusion and Perspectives

Mutagenesis and stable transfection approaches in the X-CGD PLB-985 cellular model were used to study the defective molecular mechanisms of three X^+-CGD mutations, one X^--CGD substitution and the structure-function analysis of two important cytosolic domains of $gp91^{phox}$ or Nox2. The X-CGD PLB-985 cells produced by *CYBB* gene targeting (Zhen et al. 1993) were a generous gift of Dr. M. Dinauer (University of Indianapolis, U.S.A).

Study of the defective molecular mechanism of three X^+-CGD mutations in the transfected PLB-985 cells

CGD results from a defect in the NADPH oxidase system, composed of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac. $Gp91^{phox}$, harboring the binding sites for haems, FAD, and NADPH (Vignais 2002), is the catalytic center of the NADPH oxidase enzyme, and mutations in *CYBB* gene cause most of the CGD cases (Heyworth et al. 2003). X^+-CGD is characterized by a normal level of a nonfunctional $gp91^{phox}$ in phagocytes, providing an ideal system for studying functional domains in $gp91^{phox}$ (Leusen et al. 1994; Cross et al. 1995*a*; Cross et al. 1995*b*; Yu et al. 1999; Stasia et al. 2002). Three X^+-CGD mutations (the His303Asn/Pro304Arg and Leu505Arg mutations identified in our laboratory, and the Asp500Gly mutation found by Leusen *et al.*) were reproduced in the X-CGD PLB-985 cells harboring the same phenotype as the neutrophils from the X^+ CGD patients (Leusen et al. 1994; Stasia et al. 2002; Stasia et al. 2005). Although these $gp91^{phox}$ mutations have no effect on the expression and maturation of cytochrome b_{558}, no superoxide was generated in transfected PLB-985 cells.

His303Asn/Pro304Arg totally abolished the oxidase activity *in vivo* and *in vitro*. This inhibition is associated with a defective translocation of $p47^{phox}$ and $p67^{phox}$ to the plasma membranes of mutated cells. Separate analysis of His303Asn and Pro304Arg mutations showed that His303Asn is sufficient to abolish the NADPH oxidase activity, associated with a defective oxidase assembly, while Pro304Arg substitution has a less drastic effect. Meanwhile neither mutation could be considered as a polymorphism in the *CYBB* gene. The 3D-model study according to Taylor *et al.*, shows that these two residues are localized at the surface of $gp91^{phox}$, suggesting that these residues may participate to the oxidase assembly process directly or indirectly by maintaining a correct conformation of $gp91^{phox}$ to bind cytosolic factors. The distance of His^{303} and Pro^{304} from the FAD and NADPH potential binding sites is not compatible with a possible interaction with them.

Asp500Gly mutation in $gp91^{phox}$ totally inhibited the oxidase activity and assembly process in whole cells and in a cell-free system assay using membranes from Asp500Gly $gp91^{phox}$ transfected PLB-985 cells. This X^+-CGD mutation disturbed the electron transfer from NADPH to FAD, confirming the 3D model of Taylor and Segal (Taylor et al. 1993). Indeed Asp^{500} is located in an α-helical loop proposed to be a potential binding site for the cytosolic factors. In resting state this loop should covers the NADPH binding site and upon activation it should move aside, leaving free access for NADPH to the cleft containing its binding site.

Unlike both X^+-CGD mutations described above, the NADPH oxidase inhibition

resulting from the Leu505Arg substitution is not related to a defect on oxidase cytosolic components translocation. According to the sequence alignment of Nox2 with members of FNR family, this Leu^{505} was proposed to be located in the adenine of NADPH binding site of Nox2 (Rotrosen et al. 1992; Vignais 2002). Interestingly in a CFS assay, 50% of the oxidase activity found in WT PLB-985 membranes could be restored using plasma membranes from Leu505Arg Nox2 PLB-985 cells. This was also demonstrated for the INT reductase activity suggesting that a partial electron transfer from NADPH to the molecular oxygen via FAD can occur. The K_m for NADPH and NADH of the purified cytochrome b_{558} from this mutant transfected cells is approximately 3-time higher than that of wild type, suggesting that this mutation slightly inhibits the affinity for NADPH and NADH. However, this inhibition is probably not compatible with a direct interaction of Leu^{505} with the adenine of NADPH. In addition the α helical loop insertion, only found in the members of Nox/Duox family and FRE1, covers the potential binding site of adenine of NADPH (^{504}GLKQ^{507}) shown in FNR members. An increasing amount of recombinant $p67^{phox}$ in a CFS containing the Leu505Arg mutated cytochrome b_{558} could restore most of the oxidase activity measured in the wild type cytochrome b_{558}, indicating that the Leu505Arg mutation disturbs the affinity of $p67^{phox}$ for the cytochrome b_{558}. The 3D model of the cytosolic C-terminus of $gp91^{phox}$ demonstrates that Leu^{505} is located at the end of the α-helical loop at the surface of Nox2 just at the entry of the cleft containing the NADPH binding site. We suppose that this mutation can disturb efficient movement of α-helical loop resulting to a defective $p67^{phox}$ binding to the α helical loop and a reduced access of NADPH to its binding site. This defect is partially restored *in vitro* with an artificial forcing on the interactions between the oxidase components.

Study of the cytochrome b_{558} biosynthesis defect in an X^--CGD case (Ser193Phe) reproduced in X-CGD PLB-985 and COS-7 cell lines

Finally the Ser193Phe Nox2 transfected PLB-985 cells mimic the phenotype of neutrophils from the X^--CGD patient (Roesler et al. 1999), characterized by low expression of mutant $gp91^{phox}$ with a correlated $p22^{phox}$ expression. This result provides further evidence that the X-CGD PLB-985 cell line is an ideal model for study X^+-CGD and X^--CGD mutations (Yu et al. 1999; Bionda et al. 2004; Li et al. 2005). Ser^{193} is located in the D loop which was demonstrated that it had no impact on the oxidase complex assembly (Li et al. 2005). This mutation disturbs the mRNA expression and/or stability in transfected PLB-985 cells, but not in the transfected COS-7 cells. The low expression of $gp91^{phox}$ protein in these two cell lines was associated with a defective maturation of $gp91^{phox}$. In PLB-985 cellular model, this could be due to a defective interaction between the mutant gp65 precursor and $p22^{phox}$, or to a defect in glycosylation and/or haems incorporation within the mutant gp65. Reduced minus oxidized difference spectrum of a soluble extract from the S193F Nox2 transfected PLB-985 cells will be done to know if the haems are indeed incorporated in the gp65 precursor. In COS-7 cellular model, the low expression of $gp91^{phox}$ is also due to a maturation defect but it seems not only to be a consequence of the Ser193Phe mutation but also seems to be a cell line dependent defect as recently described (Murillo et al. 2005).

Analysis of the second intra-cytoplamic loop of gp91*phox*

$Gp91^{phox}$ contains four cytosolic regions, ^{1}MGNWVAVNEGL^{11} sequence, the B loop

^{70}PVCRNLLSFLRGSSACCSTRIRRQLDRNLTFHK^{102}, the D loop ^{191}TSSTKTIRRS^{200}, and a C-terminal region containing binding sites for FAD and NADPH (Vignais 2002). Four cytosplasmic regions (residues 87-94, 451-458, 491-504, and 555-564) in gp91^{phox} have been suggested to interact with p47^{phox} and/or p67^{phox}. The X-CGD PLB-985 cell line has been used to investigate the D loop, which had never been studied, on the oxidase activity and assembly. The transfected PLB-985 cells expressed similar amounts of recombinant WT or mutant Nox2, which was crucial for comparing the impact of each mutation on the oxidase function in such cells. K195A/E mutant completely inhibited the oxidase activity in intact cells and CFS assay, indicating the Lys^{195} is indispensable for the oxidase activity. The R198Q and RR198199EE mutations had little inhibitory effect on the oxidase activity, while R198E, R199E, RR198199QQ/AA mutations abolished the O_2^- generation *in vivo* and *in vitro*, suggesting that the total charge of Arg^{198} and Arg^{199} is important for maintaining the oxidase activity. However, the R199Q mutation had a "super" oxidase activity. Interestingly, the replacement of the D loop by the same region of the Nox2 homologues (Nox1, 3, 4) had a higher oxidase activity than that of Nox2, demonstrating that the D loop of Nox homologues is functional for the Nox2 activation process. We could conclude that the D loop has a similar role in human Nox family.

The translocation of the oxidase cytosolic components to the plasma membranes occurred normally in all the mutants of the D loop of Nox2, suggesting that this domain is not involved in the oxidase assembly process. The INT reductase activity of the mutants, which had no NADPH oxidase activity, could be totally reconstituted in a CFS assay, indicating that this region is not implicated in the electron transfer from NADPH to FAD.

In the two super-mutants (D loop$_{Nox4}$-Nox2 and R199Q) cells, the total H_2O_2 production was about 1.3-1.6 higher than in the WT PLB-985 cells after stimulation by PMA, and 5-8 times after fMLP activation. Higher H_2O_2 production was also observed in these super-mutants and D loop$_{Nox1/3}$-Nox2 after stimulation by ionomycin (Ca^{2+} ionophore) (data not shown). However, the over H_2O_2 production in intact cells was not reconstituted *in vitro*, suggesting that the cell-free system does not reproduce exactly what occurs *in vivo* (absence of phosphorylation).

Analysis of the α-helical loop (residues 484-500) of gp91*phox*

As it was discussed before, the residues 484-504 form an α-helical loop in the C-terminal tail of gp91^{phox} (Taylor et al. 1993). This loop is only found in Nox/Duox family and FRE1, but not in the other members of FNR family. It covers the cleft in which NADPH binds, upon activation of the enzyme, it is believed to move aside to allow NADPH to access its binding site and fulfill its electron transfer to FAD. In this charged region, Asp^{484}, His^{490}, Asp^{496}, and Asp^{500} are heavily conserved in the FNR family. D484T-Nox2 mutant completely inhibited NADPH oxidase activity *in vivo* and *in vitro*, while D484H had no significant inhibition on oxidase activity, suggesting that the charge in the residue 484 is absolutely necessary to maintain the oxidase activity. H490A-Nox2 transfected cells exhibited normal H_2O_2 production, while H490D lost most of the oxidase activity, indicating that the positive charge or neutral residue at 490 could also maintain the oxidase activity. D496H mutant had no effect on oxidase activity, suggesting that the charged residue is necessary for the H_2O_2 production.

However, D500A/R/G mutations destroy the oxidase activity in intact cells and in the CFS assay, demonstrating that the oxidase activity is absolutely dependent on the Asp^{500}. Unfortunately the D500E mutation was not performed to know if the negative charge in the residue 500 was efficient to maintain the oxidase activity. Yet these data suggest that the charges in this α-helical loop are essential for the oxidase function.

D484T and D500A/R/G mutations led to a defective translocation of the oxidase cytosolic components *in vivo* and *in vitro* and an inhibition of INT reductase activity. This suggests that the dysfunction of oxidase activity is associated with a defect of the assembly process leading to a defective electron transfer from NADPH to FAD.

The main conclusion of the work is that X-CGD PLB-985 cellular model is useful and efficient for studying functional consequences of X^+-CGD and X^--CGD or *de novo* mutations on oxidase activation process. His^{303} and Pro^{304} residues are essential to maintain a functional conformation of Nox2 for the oxidase activity and assembly. The D-loop of Nox2 is important for the oxidase activity, but not involved in the oxidase assembly and the electron transfer from NADPH to FAD. This loop is localized near the V transmembrane domain supporting haem- binding, it maybe involved in the electron transfer from FAD to molecular oxygen. The X^- CGD case with a Ser193Phe mutation in the D-loop, pointed out that this region maybe essential for the cytochrome b_{558} structure (Roesler et al. 1999). This X^--CGD mutation (Ser193Phe) is associated with a defective maturation of Nox2 in the transfected PLB-985 cells and COS-7 cells. The α-helical loop (residues 484-504) is essential for NADPH activation and is also implicated in the oxidase assembly, which is associated with the electron transfer from NADPH to FAD. Finally Leu^{505} has a strategic position in Nox2, which is indispensable for an efficient interaction between Nox2 and $p67^{phox}$, leading to a correct NADPH binding.

Perspectives

For the D-loop mutants

1. Understand the molecular mechanism of the increased "respiratory burst" observed in the D $loop_{Nox4}$-Nox2 and R199Q super-mutants.
2. Determination of the sub-cellular localization of Nox2, its precursors p58 and gp65, of $p22^{phox}$ in Ser193Phe-Nox2 transfected PLB-985 to find the limiting step for a correct cytochrome b_{558} maturation. We will leave the COS-7 model for studying the $gp91^{phox}$ maturation because of its special defect in the glycosylation system. Transfection of Ser193Phe-Nox2 cDNA in HEK293 human cells to verify the glycosylation defect observed in transfected PLB-985 cells. Study of the Nox2 synthesis from purified mRNA in an *in vitro* system using reticulocytes from rabbit red blood cells. Northern blot analysis of mRNA from the transfected PLB-985 and the HEK293 cells.

For the C-terminal α-helical loop

3. Clarify the role of the total α-helical loop (residues 484-504) on oxidase activation.
   This region will be totally deleted to produce a mutant which is supposed to have a constitutive oxidase activity devoid of cytosolic factors assembly. In addition FRE1 is the unique FNR reductase from *Saccharomyces cerevisiae* (Shatwell et al. 1996) proposed to be a flavocytochrome *b* which possesses a charged α-helical loop as Nox2. The idea is to transfect the WT cDNA FRE1 and a mutant cDNA devoid of the α-helical loop in X-CGD PLB-985 cell, to study the oxidase activity and the assembly of the complex under activation.

Other ideas…

4. We possess a series of Nox2 PLB-985 mutant cells which are able to produce different superoxide production level. The aim is to study the bactericidal power of these mutants to determine the minimal superoxide production level needed to kill bacteria. We would like to study in parallel, the membrane potential to verify the Dr. Segal's hypothesis that superoxide is not directly bactericidal, but leads to create a K^+ influx inside the phagosome to liberate cationic proteases which are the real bactericidal agents (Reeves et al. 2002).
5. Investigation of other published X^+-CGD mutations, which are located in the potential FAD- or NADPH-binding sites (Thr341Lys, Gly408Glu, Pro415His, Cys537Arg) to investigate their dysfunctional mechanism.

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