Relació estructura-funció en la família de transportadors d'aminoàcids heteromultimèrics. Identificació d'una nova família de transportadors lisosomals

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RELACIÓ ESTRUCTURA-FUNCIÓ EN LA FAMÍLIA DE TRANSPORTADORS D'AMINOÀCIDS HETEROMULTIMÈRICS IDENTIFICACIÓ D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS

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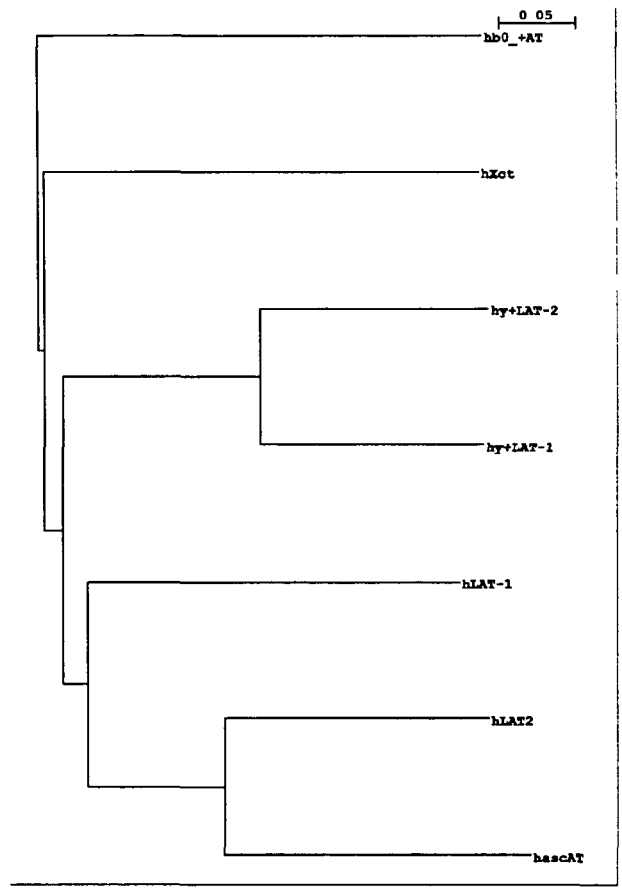

Figura 22. Arbre filogenètic dels membres coneguts de la família HAT.

5. INTERACCIONS CREUADES ENTRE SUBUNITATS. PAPER DE LA CADENA PESADA EN LA FUNCIÓ DEL TRANSPORTADOR (TREBALL 5)

En l'apartat 3 de "Resultats i discussió" he comentat que una de les evidències indirectes que les proteïnes 4F2hc i rBAT no constituïen per si soles un transportador era que s'havia observat que, en expressar-les en oòcits, induïen diferents activitats de transport. En el cas de 4F2hc podem entendre ara aquest resultat: com que hi ha diferents subunitats lleugeres que comparteixen la mateixa subunitat pesada, les diferents activitats associades a 4F2hc es deuen a l'expressió en l'oòcit de la subunitat lleugera corresponent. Com podem interpretar els resultats de les diferents activitats associats a l'expressió de la proteïna rBAT? En el nostre laboratori sempre hem detectat una lleugera component dependent de sodi, més visible a concentracions altes de substrat.

Per comprovar si podien haver-hi interaccions creuades entre subunitats vaig coexpressar en els oòcits les proteïnes rBAT i y^+LAT-1 i vaig mesurar el transport induït d'arginina i leucina en presència o no de sodi, i de cistina en absència de sodi. Vaig observar dos fenòmens: una reducció del total de substrat transportat, però, molt més important, un increment notable de la dependència de sodi per a la leucina. És a dir, podríem dir que havíem expressat dos sistemes de transport: el sistema $b^{0,+}$, ja que

observem una inducció de transport de cistina (la cistina no es transporta pel sistema y^+L), i el sistema y^+L, ja que observem una dependència de sodi pel transport de leucina.

Per estar segurs que estàvem expressant el sistema y^+L van mesurar el transport d'arginina en presència de diferents concentracions de leucina en presència o en absència de sodi. L'experiment es mostra en la **figura 23.**

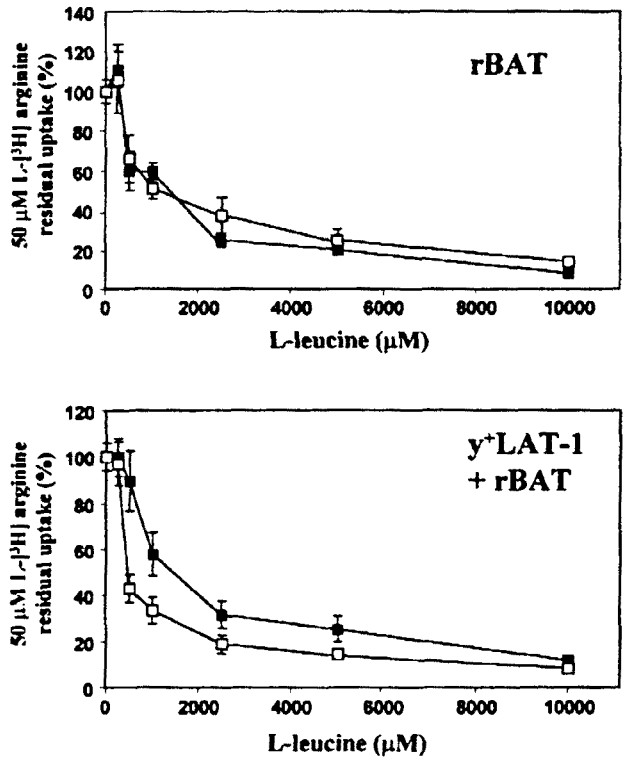

Figura 23. Expressió del sistema y^+L en coexpressar rBAT i y^+LAT-1 en oòcits de *Xenopus*. Els quadrats negres són en absència de sodi i els blancs, en presència.

Com es pot veure en aquesta figura la corba d'inhibició per leucina en el cas d'rBAT és independent de si és present o no l'ió de sodi, mentre que en coexpressar amb y^+LAT-1 observem que la leucina inhibeix més en presència de sodi.

Vam voler estudiar llavors si les dues proteïnes interaccionaven físicament. Per això vaig fer un experiment d'immunocitoquímica expressant N-*myc*-y^+LAT-1 sol, amb 4F2hc o amb rBAT. Els experiments van mostrar que igual que 4F2hc, rBAT porta y^+LAT-1 a la membrana plasmàtica (vegeu **figura 25**). Com interaccionen aquestes dues proteïnes? El més lògic és pensar que la interacció es donaria a través de la cisteïna homóloga a la proteïna 4F2hc. Vaig construir el mutant de rBAT cisteïna114serina i, en primer lloc, el vam voler caracteritzar. Carsten A. Wagner, del grup del professor Florian Lang, a Tübingen, va analitzar el mutant mitjançant tècniques d'electrofisiologia. Va analitzar la dosi de cRNA injectat-funció, la dependència de voltatge de la intensitat de corrent, l'afinitat del mutant per arginina i leucina en presència o no de sodi, i les propietats funcionals de l'intercanvi. Només va detectar un canvi en comparar les cinètiques en presència o no de sodi, experiment que es mostra en la **figura 24.** Es pot observar que el mutant C114S-hrBAT té una activitat que és 100%

$b^{0,+}$, és a dir, el transport d'aminoàcids bàsics i neutres és completament independent de sodi.

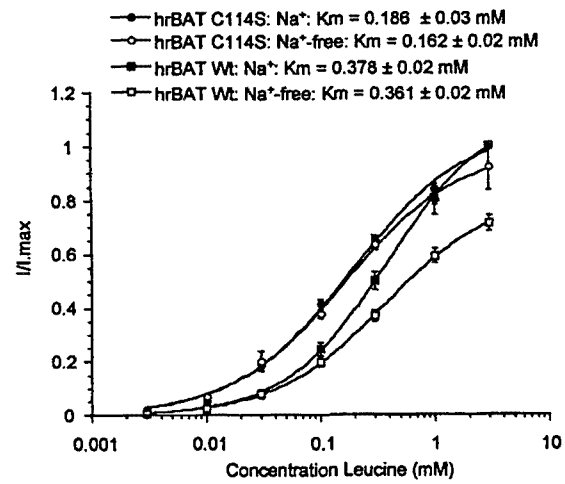

Figura 24. El mutant C114S-hrBAT funciona com un transportador "pur" $b^{0,+}$ en oòcits de *Xenopus*.

Aquest resultat suggeria que aquest mutant seria incapaç d'interaccionar amb y^+LAT-1, ja que suposàvem que aquesta dependència de sodi que presentava rBAT es devia a la interacció amb altres subunitats que no serien la subunitat lleugera $b^{0,+}$.

Vaig coexpressar llavors el mutant C114S-hrBAT amb la proteïna y^+LAT-1 en oòcits, ho vaig analitzar funcionalment i per immunocitoquímica, i ho vaig comparar amb el mutant CS1 de 4F2hc. Vam observar que el mutant C114S no presentava cap tipus de dependència de sodi en el transport dels aminoàcids neutres, encara que es coexpressés amb y^+LAT-1. En analitzar per immunocitoquímica veiem que aquest mutant és incapaç de portar y^+LAT-1 a la membrana plasmàtica, a diferència del mutant CS1 de 4F2hc **(figura 25).**

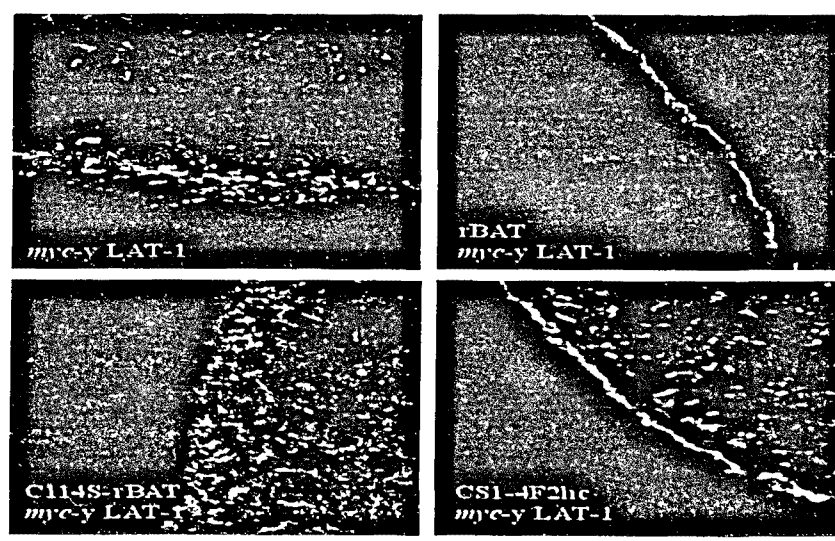

Figura 25. El mutant C114S-hrBAT no porta y^+LAT-1 a la membrana plasmàtica a diferència del mutant CS1 de 4F2hc.

Amb aquest resultat podem extreure la conclusió que la cisteïna 114, en el cas d'rBAT o la cisteïna 109, en el cas de 4F2hc no tenen cap paper per determinar l'especificitat de la subunitat. El fet que el mutant CS1 de 4F2hc però no el mutant C114S de rBAT, pugui portar y^+LAT-1 a la superfície indica que 4F2hc presenta un domini no present en rBAT que pot interaccionar (probablement de forma no covalent) amb y^+LAT-1. La formació d'aquest pont disulfur entre 4F2hc i y^+LAT-1 no és imprescindible però és necessària per tenir la mateixa funció que la proteïna normal. En canvi, el fet que no observem cap tipus d'inhibició en el transport induït pel mutant C114S d'rBAT respecte a la proteïna salvatge, indica que la formació del pont disulfur entre rBAT i $b^{0,+}$AT té un paper poc important respecte a altres interaccions entre altres dominis d'ambdues proteïnes.

Quins són aquests dominis? Quan es va identificar el cDNA d'rBAT, es va suggerir que l'extrem C-terminal tindria un possible paper com a domini que permetria la interacció amb altres proteïnes (Wells *et al.*, 1992), ja que semblava que hi hagués un domini conegut com *cremallera de leucina.* El grup de Takeda va construir i expressar constructes en oòcits on havia suprimit alguns aminoàcids de l'extrem C-terminal (Miyamoto *et al.*, 1996). En concret, el mutant Δ(511-685)hrBAT induïa una activitat en oòcits que era compatible amb l'activitat y^+L excepte per una característica: podia detectar corrents en afegir aminoàcids. Únicament es detecten corrents de sortida en expressar 4F2hc en oòcits quan s'afegien aminoàcids neutres en absència de sodi, perquè funciona com un intercanviador asimètric (veure apartat 2 de "Resultats i discussió"). De totes formes aquest treball suggeria que l'extrem C-terminal podria jugar un paper en la interacció amb la subunitat endògena. El grup de Tate va aportar una altra peça d'informació. També va expressar diferents delecions de l'extrem C-terminal. Les primeres deleccions (625-683) provocaven una pèrdua total de la funció. En canvi, la delecció Δ(588-683)rBAT no presentava cap defecte respecte a la proteïna salvatge, però era inactivat per la mutació de la cisteïna homòloga a 4F2hc. Igual que els nostres resultats, la mutació d'aquesta cisteïna per si sola no afectava la funció de la proteïna, encara que feia desaparèixer els complexos de pes molecular alt que es detectaven en els oòcits, i que podrien correspondre a la unió d'rBAT amb la subunitat endògena.

Per entendre millor la importància del domini C terminal vaig construir una quimera (kc4F2) on intercanviava els dominis C-terminal (**figura 26**).

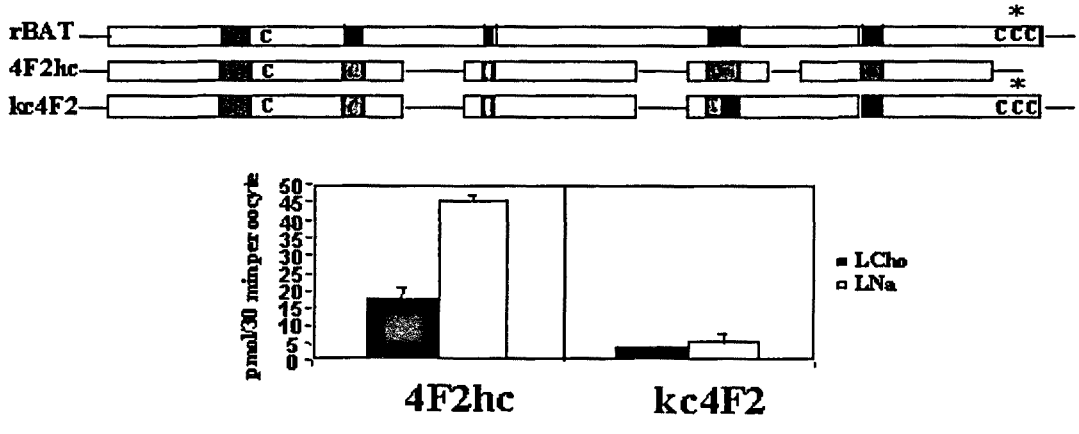

Figura 26. El domini C-terminal d'rBAT és important per a la interacció amb la subunitat lleugera $b^{0,+}$. El (*) representa la cisteïna 673, la qual hem vist que té un paper funcional important (vegeu més endavant).

El constructe kc4F2, com es pot veure en la **figura 26**, s'expressa a nivells inferiors que la proteïna 4F2hc. En canvi, l'activitat induïda és completament diferent; mentre que el transport de leucina depèn de l'ió de sodi en 4F2hc, en el cas de kc4F2, aquest transport és independent de sodi. Aquest resultat, juntament amb els abans citats suggereix que l'extrem C-terminal d'rBAT és necessari per interaccionar amb la seva subunitat.

Si analitzem en detall l'extrem C-terminal de rBAT observem que en l'extrem final hi ha 3 residus de cisteïna molt propers, i l'últim residu de la proteïna és una cisteïna (vegeu **figura 24**). Marta Pineda, del nostre grup, va mutagenitzar aquests residus (cisteïna 666, cisteïna 673 i cisteïna 685) a serina, per intentar comprendre quina seria la seva funció. Els mutants C666S i C673S són mutants de tràfic (vegeu apartat 1 "Resultats i discussió"), ja que es recuperen en expressar més proteïna, mentre que el mutant C685S és completament funcional. El mutant C673S es recupera totalment respecte a la proteïna salvatge, mentre que el mutant C666S no presenta uns nivells d'expressió alts.

C. Wagner, el nostre col·laborador a la Universitat de Tübingen, va analitzar el mutant C673S, ja que era l'únic que presentava defecte i per això tenia suficient sensibilitat per estudiar-lo. Va comprovar que la dependència de voltatge era la mateixa que la de la proteïna salvatge, però sorprenentment, en fer cinètiques d'arginina i leucina, va observar que mentre la K_m per arginina es mantenia constant, la K_m de leucina disminuïa clarament en el mutant. Va fer llavors cinètiques per altres aminoàcids: lisina, ornitina, histidina, fenilalanina, alanina i metionina. En tots ells va trobar que el mutant presentava clarament una K_m disminuïda per aminoàcids neutres.

Per comprovar que aquest defecte era específic del sistema $b^{0,+}$ i no d'altres possibles interaccions amb altres subunitats presents en l'oòcit, vaig construir el doble mutant C114S-C673S, ja que el mutant C114S no pot interaccionar amb les altres subunitats. Aquest doble mutant perd la dependència de sodi per aminoàcids neutres i bàsics i continua tenint una Km reduïda respecte a la proteïna salvatge.

Per tant, tenim un mutant que afecta propietats funcionals del transportador. Així, la proteïna rBAT no és únicament un activador que portaria la subunitat lleugera a la membrana, sinó que a més pot estar modulant propietats intrínsiques del transport. Quin és el sentit fisiològic d'aquesta modificació? El transportador $b^{0,+}$ tindria la funció de reabsorbir aminoàcids bàsics i cistina, encara que també transporta aminoàcids neutres. A més de l'asimetria creada pel potencial de membrana i la diferent composició d'aminoàcids neutres a l'interior de la cèl·lula (vegeu apartat 2 de "Resultats i discussió"), la proteïna rBAT podria provocar canvis conformacionals en el transportador, que farien que presentés una K_m superior pels aminoàcids neutres. Evidentment, són necessaris altres tipus d'experiments que estudiïn les dues proteïnes aïllades i juntes mitjançant purificació i reconstitució.

Amb aquest treball també expliquem els resultats anteriors on s'observaba que la proteïna rBAT induïa múltiples sistemes de transport en oòcits. Creiem que aquestes interaccions no són específiques, sinò que depenen del sistema d'expressió. Amb aquesta línia, Bröer va observar que la proteïna 4F2hc també induïa activitat $b^{0,+}$ en els oòcits (Bröer, *et al.*, 1998). Més tard, Rajan va obtenir resultats similars expressant $b^{0,+}$AT amb el sistema d'expressió del virus *vaccinia*. (Rajan *et al.*, 1999). En canvi, en el ronyó 4F2hc presenta una localització basolateral mentre que $b^{0,+}$AT és apical. Per

tant, s'han de demostrar que les interaccions són específiques en el teixit on trobem aquestes proteïnes per immunoprecipitació. Això ja s'ha fet per a rBAT i $b^{0,+}$AT, 4F2hc i LAT-1 (Mannion *et al.,* 1998). Podríem postular també que el mutant de la primera cisteïna de 4F2hc (C109S) seria incapaç d'induir l'activitat $b^{0,+}$ o interaccionar amb $b^{0,+}$AT en el sistema del virus *vaccinia.*

6. BUSCANT RELACIONS ESTRUCTURA-FUNCIÓ: MUTAGÈNESI DE LES CISTEÏNES DE LAT-1 (TREBALL EN PREPARACIÓ)

En l'apartat 3 de "Resultats i discussió" he explicat com a partir de l'efecte dels reactius sulfhidril sobre l'activitat y^+L induïda en oòcits per 4F2hc, deduïem que la cisteïna C109 formava un pont disulfur amb la subunitat lleugera que ja seria present en l'oòcit. Com que ara tenim les subunitats lleugeres, podem intentar trobar quina és la cisteïna diana d'aquests agents mitjançant mutagènesi dirigida de les cisteïnes presents en la subunitat.

Però, és aquest l'únic objectiu? Diferents grups d'investigació en el camp de les proteïnes de membrana (revisió de Frillingos *et al.,* 1998) fan servir l'estratègia de la mutagènesi dirigida en cisteïnes combinat després amb *cysteine-scanning mutagenesis* i la utilització de diferents reactius específics de cisteïnes, per obtenir informació estructural de la proteïna com ara topologia, lloc d'unió de substrat, zones de la proteïna conformacionalment actives, distàncies entre parts de la proteïna,etc. El fet de que sigui quasi impossible obtenir cristalls de proteïnes de membrana impedeix l'anàlisi detallada per difracció de raigs X, cosa que fa que siguin necessàries estratègies indirectes com ara la mutagènesi dirigida. De manera relativament més fàcil (encara que requereix molt esforç) es poden obtenir cristalls en dues dimensions (Wang *et al.,* 1993), que permeten l'anàlisi mitjançant criomicroscòpia electrònica, encara que la resolució no és tan elevada com en el cas de la difracció de raigs X.

Per totes aquestes raons vaig decidir estudiar una d'aquestes subunitats mitjançant mutagènesi dirigida en residus de cisteïna. Vaig escollir la proteïna xLAT-1 (IU12) per dues raons: *a*) a diferència de les altres subunitats, el nivell de transport d'aminoàcids neutres en absència de sodi és quasi zero en els oòcits injectats amb 4F2hc; pensem que aquesta subunitat no seria present en els oòcits com les subunitats y^+L o $b^{0,+}$; *b*) La proteïna s'expressa sempre a nivells alts, probablement pel fet de ser un gen de *Xenopus laevis*.

El primer que van comprovar va ser si l'activitat L induïda era sensible a reactius sulfhidril. Vam comprovar que l'activitat era sensible a pCMBS (reactiu poc permeable, vegeu apartat 3 "Resultats i discussió") i que aquesta activitat era reversible en tractar-se després amb β-mercaptoetanol, fet que indicava que la inhibició es devia a inhibició sobre residus de cisteïna. Si coexpressem el mutant CS1 de 4F2hc amb LAT-1, l'activitat és més sensible a reactius sulfhidril que si el coexpressem amb 4F2hc, igual que passava amb 4F2hc i la subunitat endògena. Això suggereix que aquest efecte dels reactius sulfhidril es conserva en tots els membres de la família.

Vam començar llavors a mutagenitzar cada residu de cisteïna de la proteïna LAT-1. Així vaig canviar cadascuna de les 11 cisteïnes a serina (excepte les mutacions C164S i C187S, que les va fer Marta Pineda) i vaig analitzar com era la funció induïda i la

sensibilitat a reactius mercurials (pCMBS) respecte a la proteïna salvatge. Vaig observar que únicament les mutacions C164S (la implicada en la formació del pont disulfur) i C443S afectaven a la funció, però no de forma total, cosa que indicava que no eren residus imprescindibles per a la funció.

Vaig observar també que la mutació C178S era més resistent als reactius sulfhidril que la proteïna salvatge, però sense arribar a tenir una protecció total. Això indica que hi deuen haver altres residus que també han de conferir resistència, o que la inhibició parcial es deu a una inhibició de l'activitat endògena. Podríem postular que la formació del pont disulfur provoca un canvi conformacional en el domini transmembrana on es troba aquesta cisteïna (vegeu **figura 27**). En la **figura 28** mostro el resultat de l'experiment de l'anàlisi de cada mutant únic de cisteïna respecte a la funció i la sensibilitat a reactius sulfhidril i en la **figura 27** mostro un esquema dels possibles dominis transmembrana de la proteïna LAT-1, on es marquen en negre els residus conservats en tota la famíla de proteïnes HAT i en vermell, la localització de les cisteïnes.

Per saber llavors quins eren els residus implicats vaig construir el mutant *Cysless* (sense cisteïnes) de LAT-1. Aquest mutant funciona entre un 40-50% respecte a la proteïna salvatge. Aquest reactiu que he construït servirà com a base per a estudis futurs en el camp de l'estructura d'aquesta família de proteïnes. En primer lloc, seria interesant comprovar que aquest mutant és completament resistent a agents mercurials. Un dels experiments interesants a realitzar seria fer *cysteine-scanning mutagenesis* (substitució de residus individuals per cisteïnes) en la proteïna sense cisteïnes o en la proteïna que pugui formar el pont disulfur. D'aquesta forma podríem entendre els canvis topològics que indueix la formació del pont disulfur sobre la proteïna LAT-1.

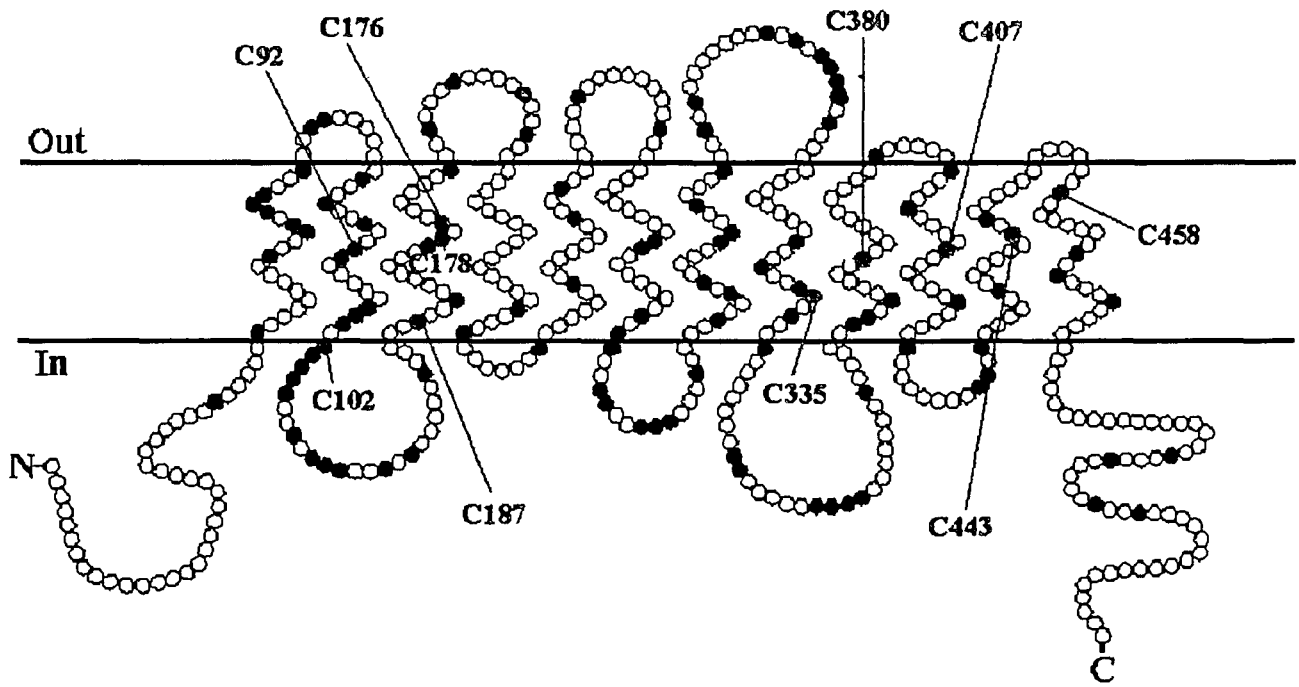

Figura 27. Esquema de la localització de les cisteïnes en la proteïna LAT-1 (vermell) i dels residus conservats en tota la família.

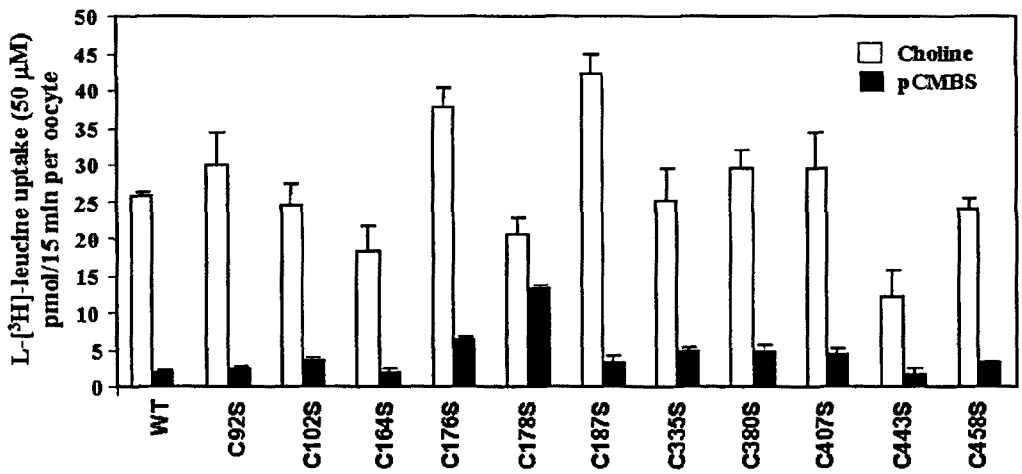

Figura 28. Anàlisi de la funció i de la sensibilitat a pCMBS dels diferents mutants individuals de la proteïna LAT-1.

7. DESCOBRIMENT D'UNA NOVA FAMÍLIA DE TRANSPORTADORS LISOSOMALS (TREBALL EN PREPARACIÓ)

En l'apartat 4 de "Resultats i discussió" explicava que una de les estratègies que vaig dur a terme per intentar aïllar la subunitat y^+L de 4F2hc va ser la coexpressió funcional de 4F2hc amb mRNA de pulmó. Així teníem un grup de 300 clons que era positiu en el nostre assaig funcional (vegeu **figura 14**). En seguir subfraccionant aquest conjunt de clons teníem grans problemes per seguir el senyal funcional de coexpressió, ja que el nivell basal induït per 4F2hc era molt variable i l'increment no era gaire consistent. Finalment, després de molts intents sense resultats, vaig decidir analitzar clons aïllats que continguéssin fragments de cDNA. Vam ser molt afortunats, ja que únicament vaig haver de coexpressar 23 clons fins a aïllar un clon, que vam anomenar posteriorment LyCAT, que incrementava la funció induïda per 4F2hc en els oòcits.

No podem saber si aquest és l'únic gen responsable de l'increment que vèiem en coexpressar-lo amb mRNA de pulmó, però almenys és un resultat positiu del cribellatge. En aquest punt vaig decidir que caracteritzaria aquest gen i no seguiria buscant altres possibles candidats. Val a dir que en aquell moment també estàvem caracteritzant els gens y^+LAT-1 i y^+LAT-2, qué també provocaven un increment en la funció induïda per 4F2hc, igual que LyCAT: no calia buscar gens que ja teníem.

Quina és l'activitat coexpressada per LyCAT? L'activitat coexpressada era també y^+L, cosa que estava d'acord amb tots els passos que havíem seguit fins a aquell moment durant l'*screening* funcional. Vaig seqüenciar llavors tot el cDNA i vaig comprovar que es tractava de l'ortòleg d'un gen que ja s'havia clonat en humans i en ratolí en un procés d'hibridació substractiva. En la **figura 29** presento un esquema d'aquesta proteïna.

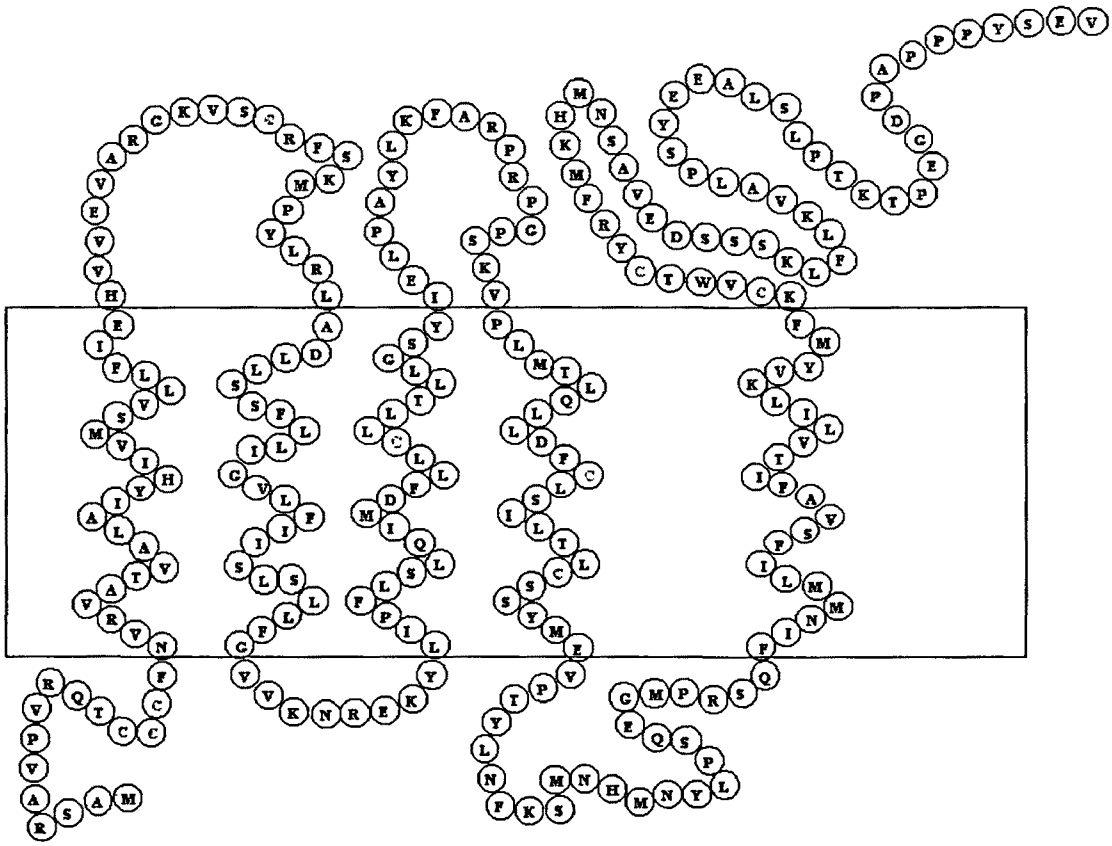

Figura 29. Possible esquema en 2-D de la proteïna LyCAT. En color apareixen marcats alguns aminoàcids conservats en aquesta família (vegeu més endavant).

A simple vista, veiem que no hi ha cap similitud entre aquesta proteïna i les subunitats lleugeres que he descriit en els capítols anteriors. Què és el que se sabia d'aquest gen? Va ser clonat per dos grups independents i de dues formes diferents: mitjançant hibridació subtractiva entre cèl·lules hematopoètiques i no hematopoètiques (Adra *et al.*, 1996) i com un gen induïble ràpidament per àcid retinoic en cèl·lules mieloides (Scott *et al.*, 1996). S'havia analitzat la seva expressió i s'havia detectat en línies cèl·lulars de llinatge eritroide, limfoide i mieloide, amb nivells alts en melsa, medul·la òssia, timus i nòduls limfoides i també en pulmó, placenta, fetge i ronyó. També es va analitzar la seva expressió en teixits embrionaris, i es va detectar en tots els teixits a nivells similars, però amb nivells superiors en cèl·lules ES. En el promotor del gen hi ha múltiples elements reguladors: E2A, TCF2α, AP2, IRF1, IRF2 i la família de factors de transcripció GATA en la regió proximal, i PEBP2, NFκB i SRF localitzats més cap a 5'. El promotor respon a àcid retinoic.

El grup dirigit per Lim (Adra *et al.*, 1996) va produir un anticòs policlonal dirigit contra l'extrem C-terminal, i va detectar una proteïna de 29 kD. En fer servir fraccions cel·lulars van detectar la proteïna en fraccions lisosomals i, en quantitats més petites en l'homogenat i en les fraccions microsomals. En fer immunocitoquímica van observar que estava distribuïda per l'aparell de Golgi i els lisosomes, però ells mateixos van afirmar no estar molt segurs de la seva localització. Van observar, fent transferència

Southern que era un gen que estava altament conservat durant l'evolució, ja que fins i tot es detectava en espècies inferiors com ara peixos.

En l'extrem C-terminal hi ha molts residus de prolina (vegeu **figura 26**), fet poc inusual per a una proteïna. Aquest mateix grup comenta en l'article com a dades no publicades que havien fet servir l'extrem C-terminal en un assaig de doble híbrid i van observar que interaccionava amb ubiqüitina. Això els va fer suggerir que aquesta proteïna tindria una funció en la degradació de proteïnes en el lisosoma.

Hi ha algun gen homòleg? El mateix any, però uns mesos després va aparèixer publicat a *Journal of Biological Chemistry* pel grup de C. Cass (Hogue *et al.*, 1996) la identificació d'un gen que presenta un cert grau d'homologia amb LyCAT. Feien servir una soca del llevat *S. cerevisiae,* on van inhibir l'enzim timidilat-sintasa per metotrexat (amb la qual cosa no pot sintetitzar desoxitimidina-fosfat i no pot créixer), com una eina per trobar transportadors de nucleòsids: en transfectar-se amb una genoteca, únicament aquelles soques que poguessin transportar nucleòsids sobreviurien en un medi amb metotrexat. D'aquesta forma van aïllar un cDNA que oferia resistència al metotrexat. Sorprenentment, en sequenciar-lo van veure que es tractava d'una proteïna truncada sense uns 80 aminoàcids de l'extrem N-terminal. Finalment, van aïllar el cDNA complet; era una proteïna de 233 aminoàcids amb una predicció d'estructura de 4 dominis transmembrana, però, en expressar-lo en llevat veien que no rescatava el fenotip.

Quina era la raó? Van observar que la proteïna era present en membranes intracel·lulars enriquides en membranes del complex de Golgi, mitjançant fraccionament subcel·lular i detecció amb un anticòs. En estudiar la seqüència de la proteïna van construir delecions més petites de l'extrem N i C-terminal i van observar en expressar-les en oòcits de *Xenopus*, que la delecció de l'extrem C-terminal (33 aminoàcids) induïa transport de timidina a nivells més alts que la delecció de 80 aminoàcids trobada inicialment. En canvi, ni la proteïna salvatge (que van anomenar MTP, per *mouse transporter protein*) ni la deleció de l'extrem N-terminal (22 aminoàcids) induïen cap tipus de transport.

No van poder caracteritzar en detall l'activitat induïda pel constructe MTPΔC (MTP amb l'extrem C-terminal delecionat), ja que els oòcits que expressaven aquesta proteïna eren especialment fràgils, però el transport induït semblava altament selectiu per nucleòsids. Posteriorment, per estudis d'immunocitoquímica en cèl·lules transfectades s'ha confirmat que la proteïna es localitza en lisosomes i en endosomes tardans (Cabrita *et al.*, 1999).

Hi ha altres gens homòlegs en les bases de seqüències? Vaig trobar l'homòleg humà del gen MTP que s'anomenava KIAA0108 i un grup d'EST que presentava alta homologia amb MTP. Vaig demanar diferents clons a IMAGE però cap d'ells tenia l'extrem N-terminal complet. Fent servir el clon 546789 vaig cribellar una genoteca de cor (ja que aquest nou gen s'expressava en aquest teixit a nivells alts, vegeu més endavant) i amb això vaig poder aïllar el cDNA complet. Aquest nou gen, LyMAT, es va seqüenciar i en la **figura 30** presento un multialineament d'aquestes 3 proteïnes.

```
mMTP          MVSMTFKRSRS-DRFYSTRCCGCFHVRTGTIILGTWYMVVNLLMAILLTVEVTHPNSMPA 59
hMTP          MVSMSFKRNRS-DRFYSTRCCGCCHVRTGTIILGTWYMVVNLLMAILLTVEVTHPNSMPA 59
hLyMAT        -----MNMVAPWTRFYSNSCCLCCHVRTGTILLGVWYLIINAVVLLILLSALADPDQY-- 53
hLyCAT        MDPRLSTVRQT--------CC-CFNVRIATTALAIYHVIMSVLLFIEHSVEVAHG----- 46
mLyCAT        MASRAAPVRQT--------CC-CFNIRVATIALAIYHIVMSVLLFIEHVVEVARG----- 46

mMTP          VNIQYEVIGNYYSSERMAD-NACVLFAVSVLMFIISSMLVYGAISYQVGWLIPFFCYRLF 118
hMTP          VNIQYEVIGNYYSSERMAD-NACVLFAVSVLMFIISSMLVYGAISYQVGWLIPFFCYRLF 118
hLyMAT        NFSSSELGGDF---EFMDDANMCIAIAISLLMILICAMATYGAYKQRAAWIIPFFCYQIF 110
hLyCAT        KASCKLSQMGY---LRIAD--LISSFLLITMLFIISLSLLIGVVKNREKYLLPFLSLQIM 101
mLyCAT        KVSCRFFKMPY---LRMAD--LLSSFLLIGVLFIISISLLFGVVKNREKYLIPFLSLQIM 101

mMTP          DFVLSCLVAISSLTYLPRIKEYL---DQLP-DFPYK-----DDLLALDSSC--------- 160
hMTP          DFVLSCLVAISSLTYLPRIKEYL---DQLP-DFPYK-----DDLLALDSSC--------- 160
hLyMAT        DFALNMLVAITVLIYPNSIQEYI---RQLPPNFPYR-----DDVMSVNPTC--------- 153
hLyCAT        DYLLCLLTLLGSYIELPAYLKLASRSRASSSKFPLMTLQLLDFCLSILTLCSSYMEVPTY 161
mLyCAT        DFLLCLLTLLGSYIELPAYLKLA-RPRPGPSKVPLMTLQLLDFCLSILTLCSSYMEVPTY 160

mmtp          -------------------------LLFIVLVFFVVFIIFKAYLINCVWNCYKYINNRNV 195
hMTP          -------------------------LLFIVLVFFALFIIFKAYLINCVWNCYKYINNRNV 195
hLyMAT        -------------------------LVLIILLFISIILTFKGYLISCVWNCYRYINGRNS 188
hLyCAT        LNFKSMNHMNYLPSQEDMPHNQFIKMMIIFSIAFITVLIFKVVMFKCVWRCYRLIKCMNS 221
mLyCAT        LNFKSMNHMNYLPSQEGVPHSQFINMMLIFSVAFITVLILKVVMFKCVYTCYKFLKHMNS 220

mmtp          PEIAVYPAFETPPQYVLPTYEMA-VKIPEKEP-----PPPYLPA 233
hMTP          PEIAVYPAFEAPPQYVLPTYEMA-VKMPEKEP-----PPPYLPA 233
hLyMAT        SDVLVY-VTSNDTTVLLPPYDDATVNGAAKEP-----PPPYVSA 226
hLyCAT        VEEKRN--SKMLQKVVLPSYEEA-LSLPSKTPEGGPAPPPYSEV 262
mLyCAT        AMEDSS--SKMFLKVALPSYEEA-LSLPPKTPEGDPAPPPYSEV 261
```

Figura 30. Multialineament d'MTP humà i de ratolí, LyCAT humà i de ratolí i LyMAT humà.

Amb totes aquestes dades vaig plantejar la hipòtesi següent: LyCAT i LyMAT serien també transportadors intracel·lulars d'algun substrat, i l'extrem C-terminal seria el domini que determinaria la localització subcel·lular. En primer lloc vaig construir la proteïna LyCAT amb l'extrem C-terminal delecionat (LyCATΔC) i vaig posar un tag *myc* a l'extrem N-terminal, tant a LyCAT com a LyCATΔC. Vaig expressar aquests constructes en cèl·lules COS i en oòcits de *Xenopus*, i vaig visualitzar la localització subcel·lular mitjançant immunocitoquímica. En el cas dels oòcits vaig també coexpressar-los amb la proteïna 4F2hc, per veure si hi havia algun canvi en la localització subcel·lular, com succeïa en el cas de y^+LAT-1 (vegeu **figura 16**). En la **figura 31** i en la **figura 32** mostro el resultat d'aquests experiments.

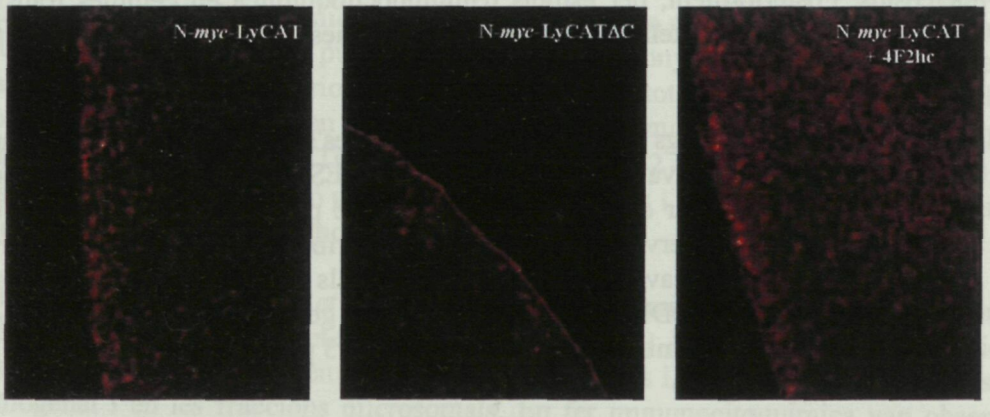

Figura 31. Immunocitoquímica de la proteïna N-*myc*-LyCAT i N-*myc*-LyCATΔC en oòcits de *Xenopus*. Efecte de l'expressió de 4F2hc sobre la localització subcel·lular de N-*myc*-LyCAT.

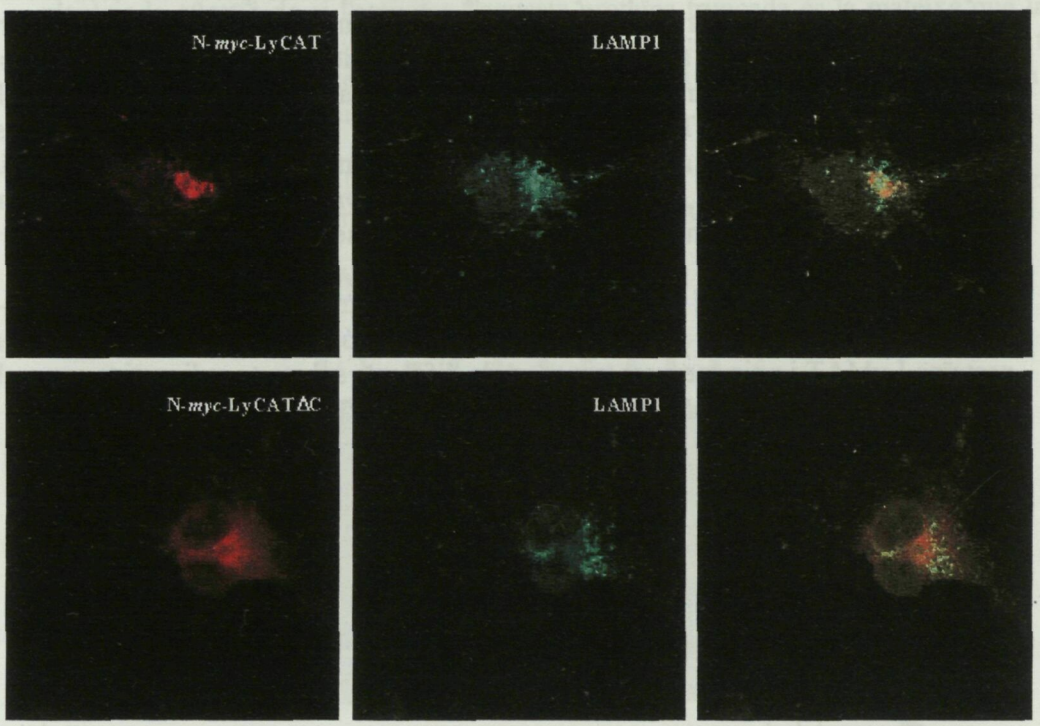

Figura 32. Immunocitoquímica de la proteïna N-*myc*-LyCAT i N-*myc*-LyCATΔC en cèl·lules COS transfectades. Col·localització amb LAMP-1. Efecte de la deleció C-terminal.

En expressar-la en oòcits (**Figura 31**) podem constatar que la proteïna presenta una localització intracel·lular. No podem afirmar que aquest marcatge correspongui als lisosomes de l'oòcit, ja que no disposem d'altres marcadors lisosomals. També veiem que en coexpressar-la amb la proteïna 4F2hc, a diferència del que passava amb y^+LAT-1, la proteïna N-*myc*-LyCAT no modifica la seva localització intracel·lular. Experiments de marcatge metabòlic i immunoprecipitació fent servir un anticòs dirigit contra 4F2hc mostren que no hi ha cap grau d'immunoprecipitació. Quin és el mecanisme pel qual LyCAT incrementa l'activitat y^+L? Més endavant intentaré donar una possible explicació.

Com es pot veure en la **figura 32**, la proteïna N-*myc*-LyCAT presenta una localització subcel·lular en el compartiment lisosomal, ja que es col·localitza completament amb la proteïna LAMP-1. Observem també que en delecionar l'extrem C-terminal la proteïna perd aquesta localitació, probablement queda en el reticle endoplasmàtic. Podem suggerir llavors que l'extrem C-terminal estaria implicat en la determinació de la localització subcel·lular de la proteïna LyCAT.

De forma molt important, en expressar en oòcits la proteïna N-*myc*-LyCATΔC, veiem que es localitza a la membrana plasmàtica. Com podem explicar aquest fet? Els oòcits són cèl·lules que es conserven a 17ºC on el control de qualitat del reticle no funciona de forma tan eficient. A més, estem expressant una gran quantitat de proteïna, cosa que pot saturar aquests mecanismes de retenció. Seria una situació similar a la descrita en l'apartat 1 de "Resultats i discussió" per als mutants d'rBAT M467T i M467K.

Disposem llavors d'una eina per poder expressar aquestes proteïnes a la membrana plasmàtica si suprimim una part de l'extrem C-terminal. En aquest domini apareixen dues senyals de tràfic cap a lisosomes: tirosina-X-X-hidrofòbic, concretament un senyal apareix en els 4 últims aminoàcids de la proteïna; també hi ha una quantitat alta de prolines, fet poc usual. Tots aquests residus estan altament conservats en els tres membres de la família aquí descrits. Desconeixem quin és el senyal implicat a determinar la localització: estudis com *alanine-scanning mutagenesis*, on es canvia cada residu per alanina, serien necessaris per trobar aquest senyal de localització.

Indueixen alguna activitat de transport aquestes proteïnes lisosomals en expressar-se en la membrana plasmàtica? Vaig fer la mateixa deleció en l'extrem C-terminal per a la proteïna LyMAT i vaig mesurar transport de diferents substrats radioactius per ambdues proteïnes (LyCATΔC i LyMATΔC) com es veu en la **figura 33.**

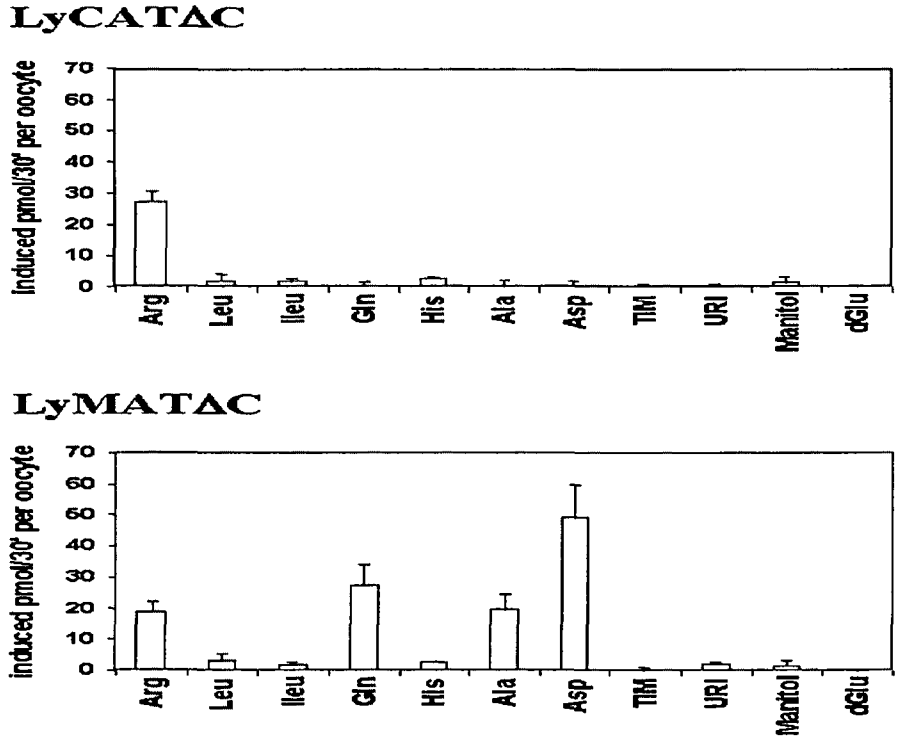

Figura 33. LyCATΔC i LyMATΔC indueixen diferents activitats de transport d'aminoàcids.

Podem observar que LyCATΔC indueix transport d'aminoàcids bàsics (arginina) i LyMATΔC indueix transport de diferents aminoàcids sense tenir en compte la càrrega. Podem assumir llavors que aquestes dues proteïnes funcionaran com a transportadors lisosomals d'aquests aminoàcids. D'aquest fet ve el nom d'aquestes dues proteïnes: LyCAT, de *lysosomal cationic amino acid transporter* i LyMAT, de *Lysosomal multiple amino acid transporter.*

Van voler caracteritzar més en detall el transport induït per LyCATΔC en els oòcits de *Xenopus.* Així, vam veure que presentava una afinitat molt alta per arginina; la K_m era de 13 μM. El transportador era molt específic per aminoàcids similars a l'arginina (evidenciat per experiments de competició amb altres aminoàcids bàsics). El transport era independent del pH, i per experiments d'acumulació veiem que acumula en els mateixos nivells que la proteïna MCAT-1 (sistema de transport de difusió facilitada).

Podem postular que la direcció de transport dependrà del gradient electroquímic. Es planteja la qüestió de si l'activitat d'aquesta proteïna truncada és la mateixa que la que tindria la proteïna nativa: totes les proteïnes que funcionen com a transportadors tenen els residus que determinen la selectivitat en dominis transmembrana o propers a ells, dominis que no s'han alterat en aquests constructes.

Quina és la funció fisiològica d'aquests dos gens?. En primer lloc vam analitzar-ne l'expressió mitjançant transferència Northern (**figura 34**).

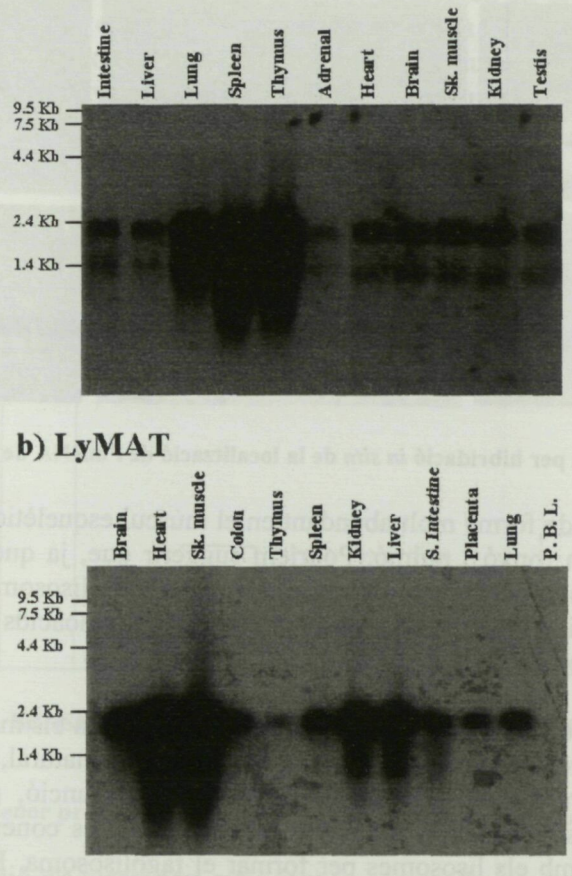

Figura 34. Anàlisi per transferència Northern de l'expressió tissular de LyCAT i LyMAT.

L'expressió de LyCAT és ubiqua però amb nivells més alts en melsa, pulmó i leucòcits. Aquest fet feia sospitar que l'expressió del gen estaria restringida a cèl·lules del sistema immune. Urs Berger, del grup del professor Hediger, va fer experiments d'hibridació *in situ* (**figura 35**). Tres dades experimentals apunten cap a aquesta idea: 1) els llocs d'expressió en els diferents teixits analitzats són patrons clars de cèl·lules immunes (**figura 35ª**, pulmó; **figura 35c,** timus; **figura 35b** i **35d,** controls amb la sonda amb sentit); 2) el marcatge en cervell s'incrementa en els lloc on s'ha produït una lesió amb quisquelat. (compareu **figura 35f** amb **figura 35e**); 3) el marcatge de LyCAT es col·localitza amb el marcatge d'M-CSF-R (*macrophage-colony stimulating factor-receptor*), un marcador típic dels macròfags (**figura 35g** amb **figura 35i**; **figura 35h** mostra la col·localització en melsa). Aquest fet suggeria que LyCAT s'expressava majoritàriament en cèl·lules del sistema immune, però especialment en els macròfags.

De fet, es va analitzar l'expressió en la línia cel·lular RAW 264.7 de macròfags i es van detectanr nivells alts d'expressió del missatger.

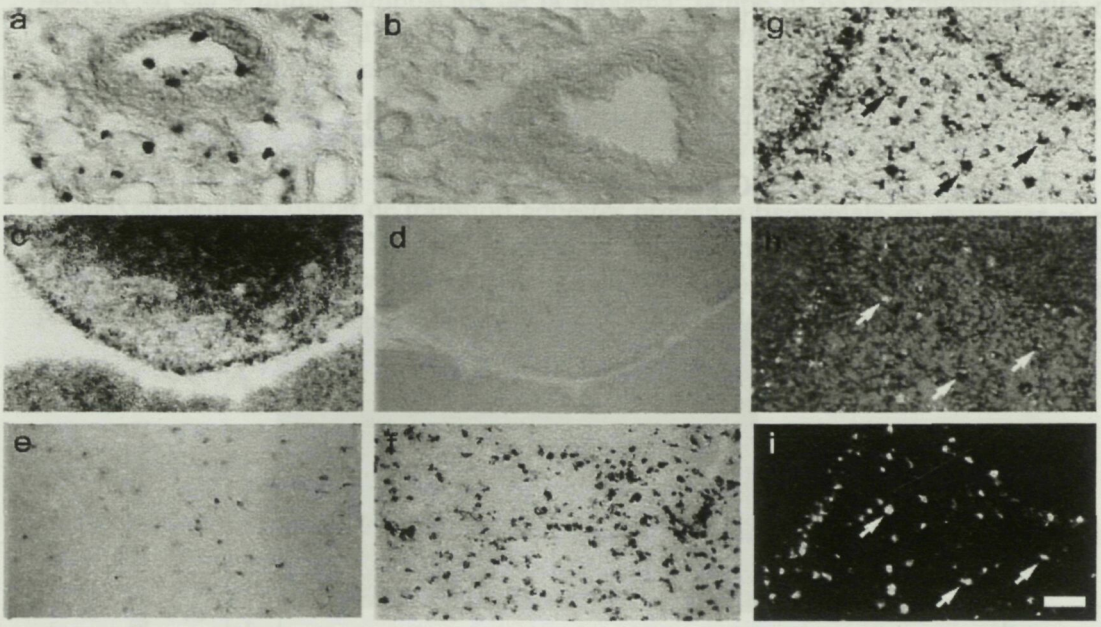

Figura 35. Anàlisi per hibridació *in situ* de la localització de l'mRNA de LyCAT.

LyMAT s'expressa de forma molt abundant en el múscul esquelètic i el cor, encara que també és present en ronyó i pulmó. Podríem suggerir que, ja que aquests teixits són teixits on la proteòlisi de proteïnes en el compartiment lisosomal és molt elevada, LyMAT podria tenir la funció de transportar aquests aminoàcids cap a l'exterior del compartiment lisosomal.

Quina seria la funció fisiològica del transportador LyCAT en els macròfags? Una de les funcions dels macròfags, en el context de la immunitat natural, és la destrucció de bacteris, virus, paràsits, etc. Per dur a terme aquesta funció, el primer pas és la fagocitosi d'aquests organismes extranys formant el que es coneix com fagosoma, el qual es fusionarà amb els lisosomes per formar el fagolisosoma. En aquesta estructura vesícular es produeix la destrucció del patògen, principalment per dos mecanismes: *a*) la producció de radicals derivats de l'oxigen (anió superòxid, radicals hidroxil, etc) generats a partir de l'acció de l'enzim NADPH-oxidasa sobre l'oxigen; *b*) la producció d'òxid nítric obtingut per l'acció de l'enzim òxid nítric-sintasa sobre l'L-arginina. Podríem hipotetitzar que la funció del transportador LyCAT seria la de regular les concentracions d'arginina en el compartiment lisosomal per mitjà del'enzim NO-sintasa.

Ja s'ha descrit que el transportador CAT2 (de la familia CAT, sistema y^+) està implicat en la introducció de l'arginina des de l'exterior de la cèl·lula per la síntesi de NO. Així, un ratolí *knockout* per CAT2 presenta una reducció dràstica dels nivells de NO produïts (Carol MacLeod, treball no publicat, Congrés de Colorado sobre transport d'aminoàcids). Encara no és clar en quin compartiment té lloc la síntesi de l'òxid nítric. Hi ha autors que diuen que la síntesi es produeix en el citosol, mentre que altres diuen

que l'enzim NO-sintasa estaria associat a unes vesícules de membrana que es fusionarien amb el compartiment lisosomal.

De totes formes, per provar aquesta hipòtesi vaig construir clons estables de la línia cel·lular de macròfags que expressaven establement una part de l'extrem 5' del gen en la orientació amb sentit i altres en la orientació sense sentit, per reduïr la concentració del gen endogen (vegeu "Materials i mètodes"). Amb quatre d'aquests clons *antisense* i dos *sense* vaig estudiar la producció d'òxid nítric davant d'estímuls que n'incrementen la seva producció, l'interferó γ (IFN-γ) i el lipopolisacàrid (LPS), de dues formes: mesurant la producció de nitrats i nitrits que apareixen en el medi de cultiu i mesurant per citometria de fluxe la fluorescència associada a la molècula DAF-2DA, que ens dóna una idea de la producció de NO intracel·lular durant una hora (vegeu "Materials i mètodes"). Vam observar una disminució de la producció de NO, com es mostra en la **figura 36.**

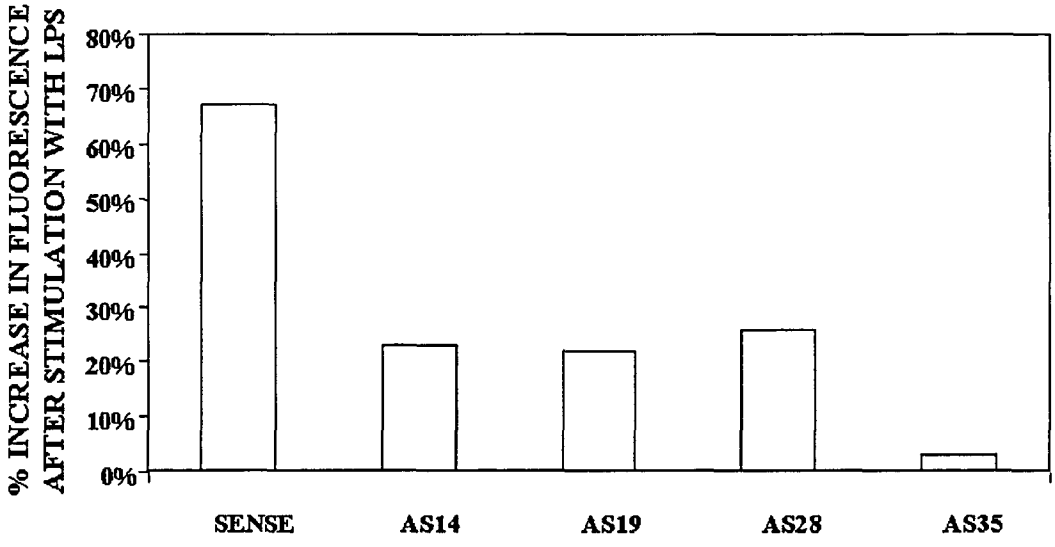

Figura 36. Menor producció de nitrats i nitrits en els macròfags *antisense* per LyCAT.

Durant la redacció d'aquesta tesi estic fent tres tipus d'experiments: 1) comprovar que la menor producció de NO no es deu a canvis en la síntesi o en l'activitat de l'enzim que produeix el NO (òxid nítric-sintasa induïble, iNOS); 2) demostrar que els clons *antisense* degraden en menor proporció microorganismes com ara la *leishmània major*, degradació que depèn sobretot de la producció de NO (així podríen donar un paper fisiològic a aquest transportador); 3) comprovar que els clons *antisense* presenten un defecte en el transport d'arginina en el compartiment fagolisosomal mitjançant la purificació d'aquests. 4) Determinar mitjançant la purificació de fagolisosomes o per immunocitoquímica de macròfags que estan fagocitant quina és la localització de l'enzim iNOS, informació que encara es desconeix.

Si aquests resultats fossin positius podrien plantejar un model en el que davant d'un estímul exterior el macròfag produiria òxid nítric amb la síntesi de l'enzim iNOS. Aquest NO es produiria de forma majoritària dins del fagolisosoma, que és el compartiment on té lloc la degradació dels agents exteriors. L'arginina, necessària per la síntesi de NO, s'obtindria desde l' exterior a través del transportador MCAT-2 (veure

"Introducció") i es transportaria cap al lisosoma a través del transportador LyCAT, el qual funcionaria en una direcció o un altre en funció del gradient de concentracions d'arginina.

Finalment, voldria proposar un mecanisme que expliqués com l'expressió de la proteïna LyCAT pot incrementar l'activitat y^+L induïda per 4F2hc. Com ja vaig explicar en l'apartat 2 de "Resultats i discussió", el transportador y^+L funciona com un intercanviador asimètric: permet la sortida d'aminoàcids bàsics mitjançant l'intercanvi amb aminoàcids neutres més sodi. A causa d'aquest mecanisme, una conseqüència que en podem extreure és que si no hi ha aminoàcids bàsics a l'interior el transportador no podrà funcionar.

Això és realment així; si es fa una preincubació amb leucina 1 mM més sodi durant 6 hores, i després es mesura el transport pel sistema y^+L, no s'observa cap inducció. Per tant, podem concloure que si modulem la concentració d'aminoàcids bàsics interna podem també modular el transportador y^+L. Podem hipotetitzar llavors que el transportador LyCAT estaria incrementaria la concentració d'aminoàcids bàsics assequible pel transportador, amb la qual cosa incrementaria la seva activitat. Aquest model està esquematitzat en la **figura 37**.

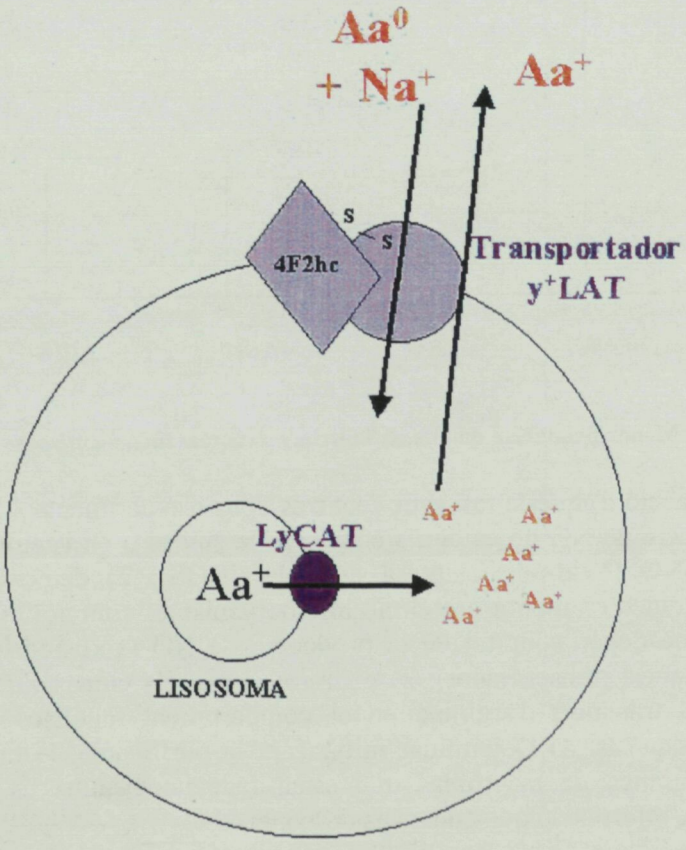

Figura 37. Model especulatiu que intenta explicar per què la coexpressió de LyCAT i 4F2hc incrementa l'activitat y^+L en oòcits de *Xenopus*.

Conclusions

CONCLUSIONS

1. Es reafirma la implicació del gen rBAT en la cistinúria de tipus I. Dues de les mutacions trobades en pacients cistinúrics, Met467Lys i Met467Thr, no són completament funcionals a causa d'un problema en el trànsit cap a la membrana plasmàtica.

2. El transportador $b^{0,+}$ funciona com un intercanviador obligatori d'aminoàcids amb estequiometria 1:1. En aquesta tesi, hem vist que el potencial de membrana i l'intercanvi amb aminoàcids neutres són les principals forçes que permeten acumular aminoàcids. Aquest mecanisme explica completament el fenotip de la cistinúria de tipus I. El transportador y^+L també funciona com un intercanviador d'aminoàcids, però de forma asimètrica ja que únicament permet la sortida d'aminoàcids bàsics, a causa probablement de les baixes concentracions de sodi intracel·lulars.

3. Els transportadors $b^{0,+}$ i y^+L formen part d'una gran família de transportadors heteromultimèrics constituïts per dues subunitats: una subunitat lleugera (LAT-1, LAT-2, ascAT, y^+LAT-1, y^+LAT-2, XCAT, $b^{0,+}$AT, etc) responsable de l'especificitat de substracte, i una subunitat pesada (4F2hc, rBAT), necessària per a l'expressió en superfície de la subunitat lleugera. Aquestes dues proteïnes interaccionen a través d'un pont disulfur entre 2 residus de cisteïna que estan altament conservats. A part d'aquesta interacció covalent, són necessàries altres interaccions entre altres dominis com ara l'extrem C-terminal d'rBAT. Així, rBAT determina propietats funcionals del transportador $b^{0,+}$.

4. La mutació L334R trobada en un pacient espanyol amb LPI i la mutació V170M trobada en pacients joeus amb cistinúria de tipus no-I, provoquen un defecte en la funció de la proteïna y^+LAT-1 i $b^{0,+}$AT, respectivament

5. S'ha identificat una nova família de transportadors lisosomals formada per 3 membres, MTP, LyCAT i LyMAT. LyCAT funciona com un transportador lisosomal d'aminoàcids bàsics, mentre que LyMAT funciona com un transportador lisosomal de múltiples aminoàcids. El domini C-terminal d'aquestes proteïnes en determina la localització subcel·lular. Estudis posteriors permetran implicar el transportador lisosomal LyCAT en la regulació de la síntesi de NO en macròfags, com permeten suggerir les dades obtingudes en la present tesi.

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Articles

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An Intracellular Trafficking Defect in Type I Cystinuria rBAT Mutants M467T and M467K*

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From the ‡Department of Biochemistry and Molecular Biology, Faculty of Biology, Universitat de Barcelona, Avda. Diagonal 645, Barcelona 08028, Spain and the §Institute of Physiology, Eberhard-Karls-Universität Tübingen, Gmelinstrasse 5, D-72076 Tübingen, Germany

The human rBAT protein elicits sodium-independent, high affinity obligatory exchange of cystine, dibasic amino acids, and some neutral amino acids in *Xenopus* oocytes (Chillarón, J., Estévez, R., Mora, C., Wagner, C. A., Suessbrich, H., Lang, F., Gelpí, J. L., Testar, X., Busch, A. E., Zorzano, A., and Palacín, M. (1996) *J. Biol. Chem.* 271, 17761–17770). Mutations in rBAT have been found to cause cystinuria (Calonge, M. J., Gasparini, P., Chillarón, J., Chillón, M., Galluci, M., Rousaud, F., Zelante, L., Testar, X., Dallapiccola, B., Di Silverio, F., Barceló, P., Estivill, X., Zorzano, A., Nunes, V., and Palacín, M. (1994) *Nat. Genet.* 6, 420–426). We have performed functional studies with the most common point mutation, M467T, and its relative, M467K, using the oocyte system. The K_m and the voltage dependence for transport of the different substrates were the same in both M467T and wild type-injected oocytes. However, the time course of transport was delayed in the M467T mutant: maximal activity was accomplished 3–4 days later than in the wild type. This delay was cRNA dose-dependent: at cRNA levels below 0.5 ng the M467T failed to achieve the wild type transport level. The M467K mutant displayed a normal K_m, but the V_{max} was between 5 and 35% of the wild type. The amount of rBAT protein was similar in normal and mutant-injected oocytes. In contrast to the wild type, the mutant proteins remained endoglycosidase H-sensitive, suggesting a longer residence time in the endoplasmic reticulum. We quantified the amount of rBAT protein in the plasma membrane by surface labeling with biotin 2 and 6 days after injection. Most of the M467T and M467K protein was located in an intracellular compartment. The converse situation was found in the wild type. Despite the low amount of M467T protein reaching the plasma membrane, the transport activity at 6 days was the same as in the wild type-injected oocytes. The increase in plasma membrane rBAT protein between 2 and 6 days was completely dissociated from the rise in transport activity. These data indicate impaired maturation and transport to the plasma membrane of the M467T and M467K mutant, and suggest that rBAT alone is unable to support the transport function.

The heterologous expression of rBAT in *Xenopus* oocytes elicits the sodium-independent, high affinity transport of cystine, dibasic amino acids, and some neutral amino acids ($b^{0,+}$-like activity) (1–3) by an obligatory exchange mechanism that accounts for the accumulation of such substrates in the oocytes (4, 5). The rBAT protein is located in the brush border of the epithelial cells of the proximal straight tubule and the small intestine (6, 7). Very recently, our group has demonstrated that rBAT is expressed in the "proximal tubule" cell line OK. An antisense sequence against rBAT abolishes *in vivo* the $b^{0,+}$-like activity expressed apically in these cells (8). All these data indicate a role of rBAT in cystinuria. This common inheritable disorder is due to the defective transport of cystine and dibasic amino acids through the epithelial cells of the renal tubule and the intestinal tract (9–11): the low solubility of cystine leads to the development of cystine calculi in the kidney. Three types of classic cystinuria have been described, but rBAT is responsible only for type I, as has been demonstrated by mutational and genetic analysis (12, 13).

Type I individuals who inherit one mutant rBAT gene are completely normal and do not hyperexcrete cystine or dibasic amino acids. Only the homozygotes for mutant rBAT genes display the above mentioned phenotype. Several mutations of rBAT have been described (12, 14–16). Among them, the most common point mutation is M467T, found in 26% of type I cystinuria chromosomes so far analyzed. This is also the only mutation that has been found in homozygosis in one Spanish family (12). Its relative, M467K, has been found as a compound heterozogous with the L678P mutant in one Italian patient (14). The methionine at position 467 is completely conserved among all the species in which rBAT sequences are known (1–3, 8). In the 4-transmembrane domain model proposed by Tate and co-workers (17) this residue lies in the third transmembrane domain, very close to the cytosol. There is little information regarding the dysfunctions provoked by rBAT mutations. Calonge and co-workers (12) reported preliminary experiments on the M467T mutant, showing a decrease in transport activity. Miyamoto and co-workers (18) obtained similar results with the E268K and T341A mutants. However, they do not provide any explanation for the reduced function.

The exact role of rBAT in the amino acid transport of system $b^{0,+}$-like is unknown. This is largely due to its unusual topology. Experimental evidence obtained using site-directed antibodies suggests a 4-transmembrane domain model for rBAT (17). This contrasts with common metabolite transporters, which appear to contain 8–12 transmembrane domains (19). Thus, it has been suggested that rBAT is only part of the

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functional transport unit (2, 3) rBAT is expressed in kidney and intestine as a 94-kDa protein in reducing conditions and as a 125-kDa complex in nonreducing conditions, suggesting a disulfide-linked heterodimer with another protein of 35–50 kDa (20, 21) This complex has also been detected in total membranes of oocytes injected with rBAT cRNA (20)

Here, we report the molecular basis for the defect seen in the M467T mutation and its relative M467K There is no significant difference in the functional properties of the M467T mutant compared with the wild type, but the M467K mutant shows a clear V_{max} defect The two mutants are expressed only as an endo H[1]-sensitive band that remains mostly inside the cell The lack of correlation between the rBAT protein in the plasma membrane and the expressed transport activity suggests that rBAT is necessary but not sufficient for the amino acid transport activity of system $b^{0,+}$-like

EXPERIMENTAL PROCEDURES

Uptake Experiments and Electrophysiological Studies—Oocyte management, injections, uptake measurements, and electrophysiological studies were as described elsewhere (1, 22) *Xenopus laevis* (H Kahler, Institut fur Entwicklungsbiologie, Hamburg, Germany) oocytes were defolliculated by collagenase (Boehringer Mannheim) treatment Only healthy looking stage VI oocytes were used The uptake of the labeled amino acids (DuPont NEN) was measured in 7–8 oocytes per individual data point at 25 °C for 5 min when initial rates were measured or for 3 h for accumulation experiments The data are expressed as the difference between the uptake in rBAT-injected oocytes and the uptake in uninjected oocytes (induced uptake) Uptake values in uninjected oocytes are the same as in water-injected oocytes For electrophysiological measurements the oocytes were each injected with 1 ng of cRNA, and two electrode voltage and current clamp recordings were performed 3–8 days later Recordings were performed at 22 °C using a Geneclamp amplifier (Axon Instruments, Foster City, CA) and MacLab D/A converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia) Amino acid induced voltage changes or currents were filtered at 10 Hz Experiments were repeated with two batches of oocytes, in all repetitions, qualitatively similar data were obtained The external control solution (ND96 medium) contained 96 mM NaCl, 2 mM KCl, 1 8 mM $CaCl_2$, and 5 mM HEPES at pH 7 5 The holding potential was −50 mV, but this was varied in one set of experiments to analyze the voltage dependence of amino acid induced currents The solution flow was adjusted to 20 ml/min, which guaranteed a complete solution exchange in the recording chamber within 10–15 s The maximal current amplitude induced during amino acid superfusion was measured Inward currents are shown with the prefix − All data are given as means ± S E where n is the number of oocyte measurements

Site directed Mutagenesis, cRNA Synthesis, and Injection—The construction of the M467T mutant in pSPORT 1 has been described elsewhere (12) For the construction of the M467K mutant we used the general procedure of Kunkel and co-workers (23) with additional steps described by Yan and Maloney (24) The mutagenic oligonucleotide was 5 -AAGC[T]TGTTCATCACGTT-3 (antisense strand, the mutated nucleotide (position 1400, Ref 1) is indicated between brackets) Mutants were identified by their acquisition of an *Alu*I restriction site (12), and a cassette between *Nco*I and *Bst*EII sites was completely sequenced Then, this cassette was ligated into pGEM4Z containing the cDNA from human rBAT that had been cut with the same enzymes Finally, the whole cassette was sequenced The three plasmids were isolated using a miniprep kit (Promega), linearized by *Xba*I restriction endonuclease digestion, and transcribed *in vitro* using T7 RNA polymerase (Promega) for the wild type and the M467T mutant and SP6 RNA polymerase (Pharmacia Biotech Inc) for the M467K in the presence of ^{7}mGpppG (NEB) as described elsewhere (1) Oocytes were injected (Inject+Matic-System, J A Gabay, Geneva, Switzerland) with 50 nl of cRNA at the concentrations given in the figures

Kidney Brush Border and Oocyte Membranes—Brush border membranes from rat kidney cortex were obtained as described (6) Total oocyte membranes were purified as described elsewhere (25) Briefly 30–50 oocytes were homogenized in 10 μl/oocyte buffer A (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7 5, plus 5 μg/ml leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) by 20 strokes of an Eppendorf Teflon-glass homogenizer The homogenate was centrifuged twice at 1,000 × g for 10 min at 4 °C to eliminate the yolk, and the supernatant was pelleted (100,000 × g, 90 min, 4 °C) and resuspended in 2 μl/oocyte buffer A Aliquots were used to quantify proteins by the method of Bradford (26) The membranes were stored at −20 °C until use

Endoglycosidase H Treatment—50–100 μg of total membranes or rat kidney brush borders was denatured by boiling for 5 min in the presence of 100 mM DTT and 0 54% SDS, and incubated in a NaP_i buffer (50 mM NaP_i, pH 5 5, 0 36% SDS, 0 5 mM phenylmethylsulfonyl fluoride) 10 milliunits of endo H (Boehringer Mannheim) was added The mixture was incubated for 18 h at 37 °C, and the reaction was stopped by the addition of Laemmli sample buffer (27) with DTT to 100 mM Samples were boiled for 5 min and stored at −20 °C until use

Surface Biotin Labeling and SDS PAGE—The procedure for biotin labeling of oocytes was adapted from that described in Ref 28 50–75 oocytes, which were injected with wild type or M467T rBAT cRNA, were washed five times in OR-2 medium (82 5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7 5 with NaOH) and then placed into an agarose coated Petri dish filled with 2 ml of OR-2 medium The fresh membrane-impermeant reagent NHS LC-biotin stock (2 mg/500 μl of OR 2) was carefully added to the dish After 10 min at room temperature the reaction was stopped by adding 1 ml of 500 mM glycine, pH 7 4 The oocytes were then washed three times in 500 mM glycine, pH 7 4, followed by two washes in OR-2 The oocytes were then transferred to a microcentrifuge tube, and 0 5 ml of lysis buffer (2% Nonidet P-40, 150 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, pH 7 4, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) was added The oocytes were passed 20 times through a 200-μl pipette, and the homogenate was centrifuged twice (1,000 × g, 10 min, 4 °C) to remove the yolk The supernatant was sonicated for 1 min and then centrifuged again at 1,000 × g (10 min, 4 °C) The supernatant was dialyzed overnight at 4 °C against streptavidin buffer (SAv buffer) (0 3% Nonidet P 40, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris, pH 8, plus 2 μM leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride) and then centrifuged for 30 min at 14,000 rpm at 4 °C to remove insoluble material The supernatant was incubated overnight with 75 μl of streptavidin-agarose bead suspension (previously washed three times in SAv buffer) Supernatant and pellet were separated by low speed (2,000 rpm) centrifugation The supernatant was precipitated with 5% trichloroacetic acid and resuspended in 150 μl of Laemmli sample buffer One-half was separated, and DTT was added to 100 mM Then the two halves (reduced and nonreduced) were boiled for 5 min and stored The pellets were eluted by adding 100 μl of Laemmli sample buffer without DTT and boiling for 5 min Then one-half was separated, and DTT was added to 100 mM Finally, the two halves were boiled once again and stored

SDS-PAGE was performed on total membrane proteins (endo H-treated or not) and the supernatants and pellets from the biotin-labeling experiments according to Laemmli (27) For rBAT Western blotting, reduced samples were transferred to Immobilon (Millipore) Following the transfer the filters were blocked with 5% non-fat dry milk and 0 02% sodium azide in phosphate-buffered saline for 1 h at 37 °C and incubated with the polyclonal antibody anti rBAT MANRX (6) at 1 100 dilution in 1% non-fat dry milk and 0 004% sodium azide in phosphate-buffered saline overnight at room temperature Detection of the immune complex was accomplished using ^{125}I-protein A (ICN) For β1-integrin Western blotting, nonreduced samples were transferred as above, blocked in 10% non-fat dry milk and 0 05% Tween-20 in phosphate-buffered saline for 30 min at 37 °C, and incubated with the monoclonal antibody 8C8 (29) at 1 10 dilution in the blocking solution overnight at room temperature Antibody binding was detected using the ECL Western blot detection system (Amersham Corp) Blots were quantified by scanning densitometry Immunoblots were performed under conditions in which autoradiography detection was in the linear response range All electrophoresis reagents were obtained from Bio-Rad NHS-LC-biotin was from Pierce, and streptavidin-agarose beads were from Sigma

RESULTS

We introduced the naturally occurring mutations M467T and M467K in the rBAT cDNA and, upon transcription *in vitro*, injected them into *Xenopus* oocytes As illustrated in Fig 1, L-Arg-induced transport was clearly reduced in both M467T

[1] The abbreviations used are endo H, endoglycosidase H, DTT, dithiothreitol, PAGE, polyacrylamide gel electrophoresis, ER, endoplasmic reticulum

and M467K compared with the wild type at 1.5 days after the injection. For 2.5 ng the uptake values were 21.4 ± 1.6 pmol/5 min/oocyte for the wild type group, 4.3 ± 1 for the M467T, and 2.3 ± 0.4 for the M467K; for 0.5 ng the values were 12.2 ± 0.9, 2.6 ± 0.8, and 0.7 ± 0.4; and for 0.05 ng the values were 4.4 ± 0.6, 0.1 ± 0.4, and 0 ± 0.4, respectively. Surprisingly, 7 days after the injection of 2.5 ng, the M467T achieved the same level of expression as the wild type (47.7 ± 4 pmol/5 min/wild type oocyte *versus* 42.2 ± 0.6 pmol/5 min/M467T oocyte). This recovery was dose-dependent since it was not observed at the lower doses (72% recovery at 0.5 ng and only 40% at 0.05 ng), suggesting that at physiological levels of rBAT mRNA there is no recovery (Fig. 1). The M467K-induced uptake was also time- and cRNA dose-dependent: at 0.05 ng the uptake was undetectable 1.5 days after the injection and was 13% of the wild type at 7 days; at 2.5 ng the uptake rose from 11 to 25% of wild type values. In other experiments the uptake induced in the M467K-injected oocytes was between 5 and 35% of control values. Similar results were obtained with current measurements (data not shown). Attempts to determine transport over a longer period failed because of the increased oocyte mortality, so we cannot preclude the possibility that the M467K (or the M467T at low cRNA doses) could reach the wild type uptake values. We also performed experiments in which 25 ng was injected and obtained similar results (see Fig. 3).

To assess whether the decrease in function was due at least in part to changes in the K_m for the different substrates we performed kinetic analysis of transport. The induced uptake was studied both by tracer measurements and by electrophysiological techniques: 3–4 days after injection by the former method and 8 days after injection by the latter (Table I). The wild type and the M467T were indistinguishable after 8 days of expression with regard to their similar K_m (in the 100 μM range, as reported in Refs. 1, 3, 22, and 30) and V_{max} for both L-Arg and L-Leu. Only after a shorter time of expression, *i.e.* 3–4 days, was a V_{max} decrease found for the three substrates used (65% for L-cystine, 54% for L-Leu, and 59% for L-Arg). The difference in the K_m of L-Arg and L-Leu observed only in the electric measurements could be due to the voltage clamp of the oocytes at −50 mV; more positive voltages led to a reduction of the L-Leu K_m without changing the L-Arg K_m (22). At day 6 and with 20 ng of cRNA injected, the M467K also showed a V_{max} decrease (65% for L-Arg) without any change in the K_m (27 ± 6 μM for the M467K and 40 ± 7 μM for the wild type). Kinetic analysis of the M467K mutant by electric measurements was also attempted, but the low induced currents precluded accurate estimations of the K_m and V_{max}.

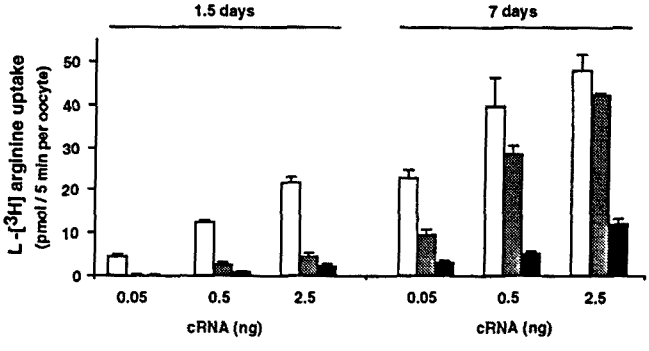

FIG. 1. **Time and cRNA dose dependence of the induced amino acid transport in wild type and mutant-injected oocytes.** Oocytes were injected with the indicated doses of cRNA of the wild type rBAT (*open bars*), M467T (*shaded bars*), or M467K (*closed bars*). The uptake of 50 mM L-Arg was determined for 5 min 1.5 and 7 days after the injection. Each bar is the mean ± S.E. of the induced uptake values measured in 6–8 oocytes. Another independent experiment gave similar results.

rBAT mediates an obligatory exchange of amino acids; no transport of amino acids occurs in the absence of amino acids on any side of the cell. This exchange accounts for accumulation of the substrates in the oocyte and it fully explains the rBAT amino acid-induced currents (4, 5). The L-Arg-induced currents measure the L-Arg inward-L-neutral amino acid outward mode of exchange, and the L-Leu-induced currents measure the reverse mode. Voltage modification leads to changes in these currents, reflecting, at least in part, changes in the modes of exchange; for instance, hyperpolarization reduces L-Leu-induced currents and increases L-Arg-induced currents. Therefore, we examined the possibility of a defect in the exchange by assaying the voltage dependence of L-Arg- and L-Leu-induced currents at day 8 after injection; as shown in Fig. 2 there was no difference between wild type and M467T-injected oocytes. The efflux of amino acids from the oocyte in the absence of amino acids in the extracellular medium was undetectable in either wild type or M467T-injected oocytes, as expected from an obligatory exchange (data not shown). Lastly, we examined the accumulation level of amino acids in both wild type and M467T-injected oocytes, and no differences were found (data not shown). In all, the data indicate no functional difference in the M467T mutant at high cRNA doses but a clear V_{max} decrease in the M467K.

Given that the M467T-injected oocytes displayed no functional defect despite the clear delay in the expression of transport, we next examined rBAT protein expression in the oocytes using the previously characterized polyclonal antibody MANRX in Western blot experiments (6). We injected a large dose of cRNA (25 ng) to ensure high levels of protein expression and measured uptake (Fig. 3*A*) and protein present in total membranes (Fig. 3*B*). The wild type protein appeared as a doublet, with one band of 94 kDa (band I) and one of higher mobility (85 kDa, band II). In contrast, only band II was present in the M467T mutant (Fig. 3*B*). This was also the case for

TABLE I

Kinetic parameters of the wild type (wt)- and M467T (MT)-induced amino acid transport activity

Kinetic parameters for the uninduced activity of transport of L-Arg, L-Leu, and L-cystine are given. Oocytes were prepared and injected with 2.5 ng of cRNA. K_m values are given in μM. V_{max} values are given in pmol/5 min/oocyte and in nA for tracer (3–4 days after injection) and electric (8 days after injection) measurements, respectively. Data (mean ± S.E.) correspond to representative experiments in which induced transport was measured varying the substrate concentration between 10 and 250 μM. 7–8 oocytes per triplicate were used for each data point. Different batches of oocytes from two different laboratories were used for the tracer and the electrophysiological studies. Therefore, the K_m values obtained are not comparable between the tracer and the electrophysiological studies.

J		3–4 days		8 days	
		wt	MT	wt	MT
L-Cystine	V_{max}	9.2 ± 0.6	3.2 ± 0.4	nd	nd
	K_m	43 ± 6	40 ± 10	nd	nd
L-Arginine	V_{max}	196 ± 16	81 ± 6	170 ± 4	181 ± 4
	K_m	93 ± 13	51 ± 8	30 ± 2	27 ± 2
L-Leucine	V_{max}	54 ± 4	24 ± 2	139 ± 3	133 ± 5
	K_m	78 ± 9	88 ± 14	213 ± 20	151 ± 24

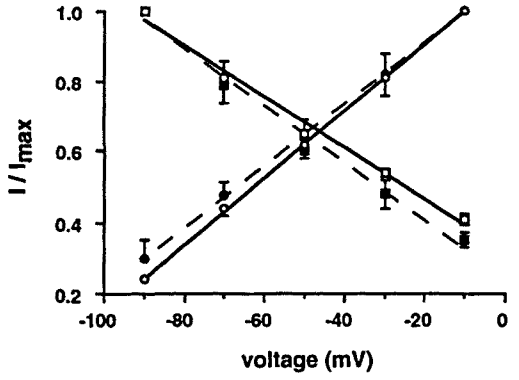

FIG. 2. **Voltage dependence of the wild type- and M467T rBAT-induced currents.** Currents were induced by superfusing wild type (*solid lines, open symbols*)- and M467T (*dashed lines, closed symbols*)-injected oocytes at the given holding potentials between −90 and −10 mV with L-Leu (1 mM, *circles*) or L-Arg (0.1 mM, *squares*) for 30 s. For the graph the currents were normalized against the maximal induced currents in each current-voltage experiment. The L-Leu- and L-Arg-induced currents were always the largest at −10 and −90 mV, respectively. When not visible, error bars are smaller than symbols.

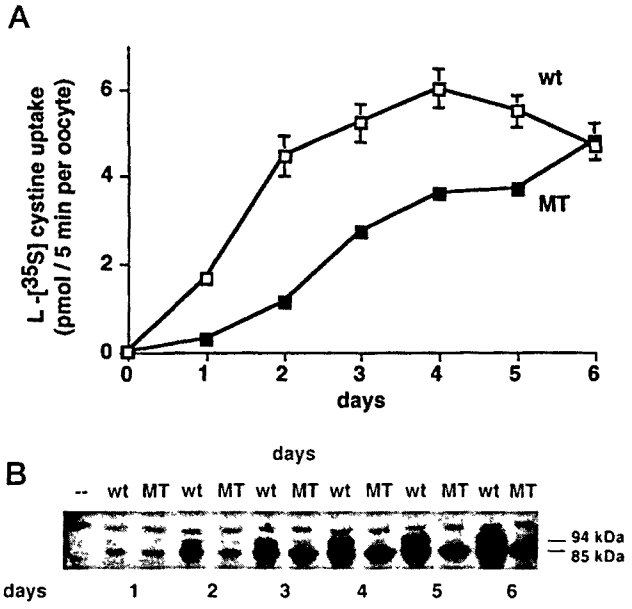

FIG. 3. **Time course of the induced amino acid transport and of the rBAT protein content in total membranes from wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. *A*, the induced uptake of 50 mM L-cystine was measured every day until day 6. Data are the mean ± S.E. of 6–8 oocytes. L-Cystine uptake in uninjected oocytes was 0.83 ± 0.08 pmol/5 min/oocyte at day 1 and 0.81 ± 0.05 pmol/5 min/oocyte at day 6. When not visible, error bars are smaller than symbols. *B*, total membrane proteins of oocytes were collected as described under "Experimental Procedures." 50 μg of reduced samples were run in a 7.5% PAGE system, transferred, incubated with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. Notice that the lower mobility band seen in the lanes also appeared in uninjected oocytes. Therefore, it is considered unspecific. --, uninjected oocytes.

the M467K (see Fig. 4). Quantification of the bands obtained in four experiments (data not shown) demonstrated that the total amount of wild type protein compared with the M467T was not significantly different at any day after injection and increased continuously until day 6 without any apparent saturation, despite uptake saturation. The amount of M467K protein was also similar to that of the wild type (data not shown). Electric measurements of the time course gave similar results. Thus, the different profiles of uptake observed for the wild type and M467T cannot be explained by the amount of rBAT protein present in total membranes.

From the Western blot in Fig. 3*B* it appeared that on the day after the injection only band II was detected in wild type-injected oocytes, suggesting that it could be a precursor of band I, most likely a non-fully glycosylated form of the protein. In fact, it is known from other membrane proteins, such as the cystic fibrosis transmembrane conductance regulator (31), low density lipoprotein receptor (32), sucrase-isomaltase (33), α_{IIb} integrin (34), and others, that some of their naturally occurring mutations produce only the endo H-sensitive form of the protein. This indicates intracellular accumulation of the mutant protein (probably in the ER) and failure to reach its normal location. The results of endo H digestions performed with total membranes of wild type-, M467T-, and M467K-injected oocytes are depicted in Fig. 4. Band II (85 kDa), the only one present in M467T and M467K, and the high mobility band of the wild type were endo H-sensitive, producing a new band of 71 kDa. This size agrees with the 72 kDa reported for the rBAT-expressing tunicamycin-treated oocytes (1). Band I shifted to 92 kDa when digested with endo H. rBAT from renal brush borders showed a similar shift, suggesting a similar glycosylation pattern for mature rBAT in oocytes and proximal tubule cells. The failure to detect band I in the M467T mutant suggests that the acquisition of the endo H-resistant condition is not necessary to its activity. This has also been demonstrated for other proteins expressed in oocytes, such as the *Torpedo* acetylcholine receptor γ and δ subunits (35) and glycophorin A (36).

The endo H sensitivity of band II suggested an intracellular location. To examine this hypothesis we performed surface labeling of intact oocytes with the membrane-impermeant reagent NHS-LC-biotin. We also attempted to determine whether the time course of the uptake correlated with the amount of wild type and M467T protein in the plasma membrane. Biotin labeling in oocytes has been successfully carried out in several studies (28, 37, 38). We opted for the method described by Müller and co-workers in Ref. 28 because it provided us with β1-integrin as an internal control for our biotinylation experiments. β1-integrin of *Xenopus* oocytes is expressed as two species of different molecular mass: one of 100 kDa (the pre-β1 form), endo H-sensitive, not biotin-labeled, and presumed to be localized to the cortical ER; and one of 115 kDa (the mature β1), endo H-resistant, biotin-labeled, and localized to the plasma membrane (28). Fig. 5 shows a representative biotin-labeling experiment of oocytes expressing wild type or M467T rBAT 2 or 6 days after the injection of 25 ng of cRNA. Labeled oocytes were lysed, and the biotinylated proteins were isolated by adsorption to streptavidin-agarose beads (see "Experimental Procedures") and probed for the presence of β1-integrin and rBAT. As expected, only the mature β1-integrin was biotinylated. In these conditions, most of the M467T protein remained in an intracellular compartment, whereas most of the wild type rBAT protein reached the oocyte surface. Densitometric measurements from three independent experiments showed that, at day 2 and day 6 in M467T-injected oocytes, 88% of the rBAT protein was in the supernatant. This value was only 35% in wild type-injected oocytes. As for the M467T, the M467K protein remained mostly intracellular, and only very small amounts reached the oocyte surface (data not shown). For the wild type, band II was detected in the intracellular fraction, and its presence in the plasma membrane was scarce; in some experiments it was not detectable, as shown in Fig. 5. In contrast, band I appeared at the plasma membrane, and its presence in the internal membranes was hardly detectable, suggesting an efficient biotin labeling. In agreement with the data obtained with total oocyte membranes (Figs. 3*B* and 4), band I was never observed in M467T-injected oocytes, even in the

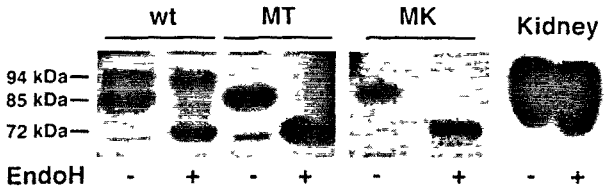

FIG. 4. **Sensitivity of wild type (*wt*), M467T (*MT*), and M467K (*MK*) rBAT proteins to the treatment with endo H.** Oocytes were injected with 25 ng of cRNA. Four days later, total membrane proteins were obtained. 50–100 μg of membrane proteins were digested or not with endo H and immunoblotted with MANRX. An equivalent amount of protein was loaded in each lane, except in kidney lanes, where rat kidney brush border membranes were loaded as a control. Another experiment was performed that gave the same results. The high mobility band seen below 72 kDa in the wild type and mutant lanes is not specific, since it also appeared in uninjected oocytes (not shown).

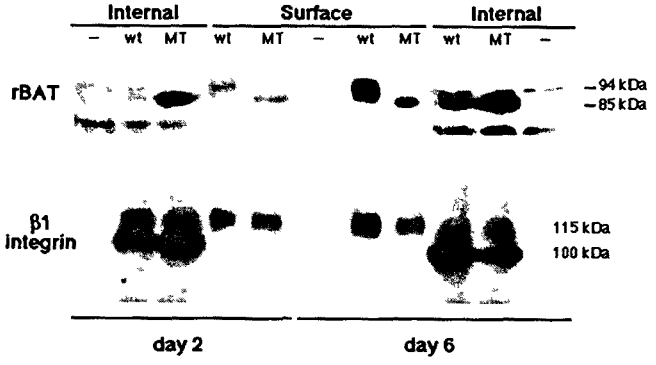

FIG. 5. **Biotin labeling of wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng of cRNA. Biotinylation was performed 2 and 6 days later as described under "Experimental Procedures." A representative experiment is shown. Times of exposure for 2 and 6 days are different (lower for 6 days). In the rBAT panel reduced samples were subjected to SDS-PAGE, probed with MANRX polyclonal antibody against rBAT, and revealed with ^{125}I-protein A. An equivalent of 16.5 oocytes was loaded in the precipitates (*Surface*), and 5 oocytes were loaded in the supernatants (*Internal*). The bands detected above and below the 85-kDa band in the internal fraction of the wild type and mutant lanes at days 2 and 6 are not specific, since they also appeared in uninjected oocytes (—). In the β1-integrin panel nonreduced samples were subjected to SDS-PAGE, probed with 8C8 monoclonal antibody against the oocyte β1-integrin, and revealed with the ECL system (Amersham Corp.) using an anti-mouse IgG coupled to peroxidase. An equivalent of 5 oocytes was loaded both in precipitates and supernatants. (—, uninjected oocytes).

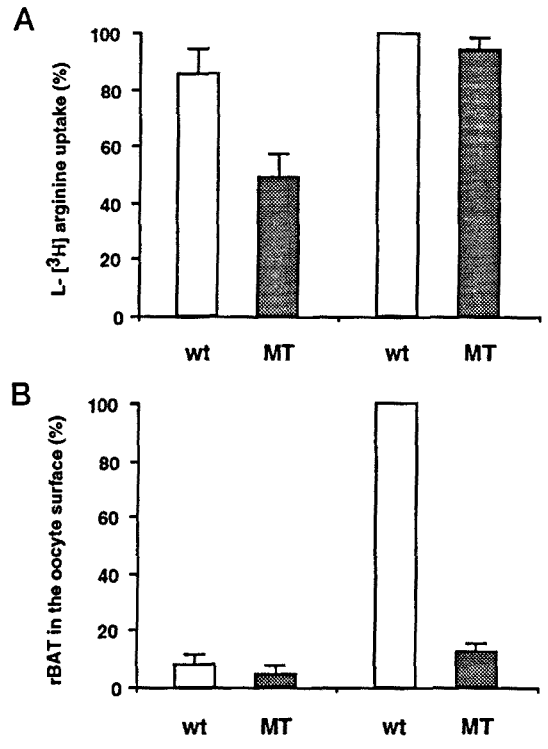

FIG. 6. **Induced amino acid transport and surface rBAT protein in wild type (*wt*)- and M467T (*MT*)-injected oocytes.** Oocytes were injected with 25 ng. *A*, 50 μM L-Arg-induced uptake was measured at 2 and 6 days. The wild type-induced uptake at day 6 was adjusted to 100. We present data (mean ± S.E.) of five experiments. *B*, in parallel, oocytes were treated as in Fig. 5. The densitometric quantification of the precipitates corrected for their β1-integrin control precipitates is depicted. The value for wild type at day 6 was adjusted to 100. Data (mean ± S.E.) of three experiments for 2 days and five experiments for 6 days are presented.

highly purified fraction corresponding to the biotin-labeled proteins (surface) (Fig. 5).

Despite the lower levels of M467T protein on the oocyte surface the recovery of the induced transport was total at 6 days. In Fig. 6*B* we present the densitometric quantification of the precipitates for three experiments (day 2) or five experiments (day 6). To obtain a better quantification, the signals in the rBAT precipitates were corrected for their β1-integrin content. The values of transport activity are also shown for comparison (Fig. 6*A*). Although there were no differences in the uptake, M467T surface rBAT protein was only 12% of the amount observed in wild type-injected oocytes at 6 days. Moreover, a 10-fold increase in plasma membrane rBAT protein was measured between 2 and 6 days in wild type-injected oocytes, despite an almost saturated transport at 2 days. Thus, there was a clear dissociation between protein in the plasma membrane and uptake: high increases in surface rBAT protein were not reflected in higher uptake rates. On the other hand, the difference between wild type and M467T surface rBAT protein at 2 days might correlate with the uptake values. The same is true of the increase in M467T rBAT plasma membrane protein from 2 to 6 days. In all, biotin-labeling experiments indicate that, in contrast to the wild type protein, M467T protein is mostly located in an intracellular compartment. A lack of correlation between surface rBAT protein and transport activity was also observed, suggesting the presence of rBAT molecules in the plasma membrane that are not functional. So, as we and others have proposed (1, 2, 20, 21), rBAT may be an essential but not a sufficient component of a complex of two or more proteins that mediates transport function.

DISCUSSION

In this paper we describe the molecular basis for the reduced transport observed in the rBAT point mutations M467T and M467K. Both mutants display an intracellular trafficking defect that impairs their transport to the oocyte surface.

Functional studies showed that transport was impaired in both mutants, the defect being more severe in the M467K. This may be caused by the more drastic amino acid change (from a nonpolar residue to a polar residue in the M467T and to a positively charged residue in the M467K). The magnitude of the defect, however, was dependent on the time after cRNA injection and on the amount of cRNA injected, especially for the M467T mutant. Thus, at high levels of cRNA the M467T mutant achieved the same levels as those induced by the wild type; this was not the case at lower levels. This "cRNA dose and time effect" may be related to the level of saturation of the quality-control machinery in the oocyte: only when the system is saturated (*i.e.* with high cRNA doses or after a longer time) does some M467T rBAT protein reach the surface. The dependence on the cRNA level might be important for the epithelial cell of the tubule *in vivo*; an rBAT mRNA level below the amount injected in the oocytes is conceivable.

The recovery of function is difficult to explain if the mutation affects only the amino acid translocation across the transporter, and it is better supported by an impairment of the intracellular transport of the molecule to its normal localization in the cell. An alteration of the transporter's turnover rate is also difficult to apply in this case because the total amount of wild type protein compared with the M467T is similar. Whole oocyte transport assays indicated no intrinsic functional defect in the M467T mutant: the K_m for the different substrates remained the same and the exchanger activity, measured as the voltage dependence of amino acid-induced currents and the efflux of amino acids in the absence of any amino acid in the extracellular medium, was not different from that of the wild type. Then, as expected from the above data, the amino acid accumulation level in the M467T-injected oocytes was similar to that in wild type-injected oocytes. The K_m of the M467K-induced transport was also the same as that of the wild type, but a V_{max} decrease was observed. This may reflect an additional intrinsic defect in the translocation pathway of amino acids. Nevertheless, the magnitude of this decrease is, as for the M467T but to a lesser extent, dependent on the time and on the cRNA injected, again suggesting a trafficking defect.

The normal rBAT protein accumulated in the oocyte in two forms: an endo H-sensitive form (band II) of 85 kDa and an endo H-resistant form (band I) of 94 kDa. Thus, rBAT followed the general pathway of membrane proteins, passing first through the endoplasmic reticulum and then through the Golgi complex, where it acquired the endo H-resistant condition, on its way to the plasma membrane. Band I was by far the major rBAT protein found in the plasma membrane in wild type-injected oocytes, as revealed by surface labeling with biotin. In contrast, both mutants remained in an endo H-sensitive form. We had no evidence that the two rBAT mutants were ever transported to the Golgi complex. However, we detected the presence of the endo H-sensitive form in the plasma membrane. This also occurred with the M467K mutant (data not shown). The mutants may traverse the Golgi complex without being processed; it has been shown that some naturally occurring mutants of the sucrase-isomaltase gene are endo H-sensitive and are missorted to the basolateral membrane of the epithelial cells of the jejunum. The authors suggested that conformational and/or structural alterations in the protein prevented the acquisition of the endo H-resistant condition (33).

It is worth mentioning the method we used to localize rBAT in the oocyte. Initial experiments with an oocyte membrane subfractionation protocol proposed by Thomas and co-workers (25), based on an earlier method (39), showed, surprisingly, the same localization for both the normal and the M467T mutant, in the "plasma membrane" fraction. Control Western blots with the oocyte β1-integrin showed the endo H-sensitive and the endo H-resistant forms of β1-integrin in the plasma membrane fraction, but neither form was detected in the intracellular fractions (data not shown). β1-integrin has been immunolocalized to the plasma membrane and to the cortical ER (28), which extends around the cortical granules and contacts the plasma membrane of the oocyte (40). The fractionation method may lead to the copurification of the plasma membrane and the cortical ER. Thus, the information so obtained should be interpreted with caution.

Therefore, surface labeling with biotin was used to quantify the amount of rBAT protein in the oocyte surface, using β1-integrin as a control in the biotinylation procedure. We observed a lower amount of M467T and M467K proteins at the plasma membrane and higher amounts located intracellularly. The converse situation was true for the wild type. Moreover, the overall rate of accumulation at the surface was higher for the normal protein. All these data strongly suggest a defect in the delivery of the mutants to the plasma membrane due to their retention in an intracellular compartment, probably the ER. The residence time in the ER is expected to be longer for both mutants. The intracellular retention could be due to an improper folding in the ER, as shown for the ΔF508 CFTR in cystic fibrosis (41) or α_1-antitrypsin deficiency (42). Met-467 lies on the third transmembrane domain according to the model of Tate and co-workers (17). The change to threonine would only slightly impair the folding of the protein, but the introduction of a lysine in the highly hydrophobic environment of the transmembrane domain would have a stronger effect. The complete carrier may be constituted only if rBAT is assembled with other proteins; this assembly might be impaired (see below). Assembly of proteins into native homo- or heterooligomers is essential for their transport out of the ER (43, 44). In eukaryotic cells, proteins that fail to fold or to assemble properly usually follow an ER-associated degradation pathway: ΔF508 CFTR (45) and the subunits of the T-cell receptor (44) are good examples. The slow rate of this pathway at low temperatures (46) makes its involvement in the defect displayed by these mutants unlikely, at least in oocytes, because they are continuously kept at 18 °C. Actually, the ΔF508 CFTR does not appear to be degraded in oocytes and cell lines cultured at low temperature (47–49) but is degraded in cells cultured at 37 °C (45). Whatever the cause of the retention in the ER, we propose that type I cystinuria should be added to the list of putative human protein-folding diseases given by Thomas and co-workers (50).

Here we have shown a clear lack of correlation between the amount of rBAT protein in the oocyte surface and the induced amino acid transport. The simplest explanation is that rBAT alone is unable to sustain its induced amino acid transport activity. So the question arises: what is the functional unit for the rBAT-induced $b^{0,+}$-like activity? Several findings support the hypothesis that it is a complex formed by rBAT and another protein. First, all the cloned metabolite and neurotransmitter transporters contain 8–12 transmembrane domains (19), which is in contrast to the 4-transmembrane domain model proposed for rBAT (17). This suggests that rBAT may not be the carrier but a modulator of it (*i.e.* delivering silent transporters to the cell surface, like the β subunit of the Na^+/K^+ ATPase (51)). Second, rBAT is expressed in kidney and intestine as a 94-kDa band in reducing conditions and as a 120–130-kDa band in nonreducing conditions (6, 20, 21), indicating a disulfide-linked heterodimer with a 30–40-kDa protein. Furthermore, this 120–130-kDa band is the only one detected upon cross-linking of kidney brush border membranes followed by reducing Western blot (20). Third, the rBAT homologous protein 4F2hc induces in oocytes a y^+L-like amino acid exchanger activity (2, 5, 52) and is expressed in different tissues as a disulfide-linked heterodimer of 120 kDa. Its 40-kDa component has been detected by ^{125}I surface labeling followed by immunoprecipitation and reduction of the complex. Unfortunately, only the 85-kDa component (4F2hc) has been cloned (53, 54). We have been unable to immunoprecipitate the rBAT 120–130-kDa band, most probably due to a steric masking of the epitope produced by the tight aposition of the other protein.[2] Fourth, so far the only heterologous system in which rBAT has been successfully expressed is the *Xenopus* oocyte. rBAT transiently expressed in COS cells is unable to reach the plasma membrane (21) or, if it arrives, no amino acid transport induction is detected (55). This correlates with the absence of the 120–130-kDa band in nonreducing Western blots from these cells (21).

[2] R. Estévez and M. Palacín, unpublished results.

Finally, Wang and Tate have recently shown that in nonreducing conditions, several high molecular weight rBAT-specific complexes (*i.e.* not present in uninjected oocytes) are detected in oocyte total membranes (20). In preliminary experiments, we detected such complexes in biotin-labeled oocytes (data not shown). Among them, only the 125-kDa complex was exclusively found at the surface, and its relative amount in wild type and M467T oocytes may correlate with transport function (data not shown). Its identical electrophoretic mobility in wild type and M467T oocytes, however, is surprising; perhaps a very small fraction of M467T protein matures (but so small as to be undetectable, even in overexposed films of the highly purified surface fraction) or, more likely, the endogenous subunit may display an anomalous mobility that renders the same size when it binds to either band I or II. Interestingly, the rBAT homologous protein 4F2hc, when it is bound to its very hydrophobic subunit (4F2 light chain), has an abnormal electrophoretic mobility (54).

In conclusion, the M467T and M467K mutants display a trafficking defect that maintains them in an intracellular location, probably the ER. A slower folding or assembly in the ER could be responsible for this defect. Thus, misfolding or a folding delay of the mutants may decrease the assembly rate with the endogenous subunit. The present data do not allow us to distinguish between these two possibilities. In any case, this phenotype can explain type I cystinuria. Direct confirmation of this requires analysis of the rBAT protein from tissue samples of patients carrying these mutations. A 125-kDa complex composed of rBAT and an endogenous oocyte protein may be the functional unit of transport. However, reconstitution experiments are needed to demonstrate that only a complex of rBAT with another protein is able to mediate transport. These studies await the cloning of this putative protein.

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