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Os transportadores ABC peroxissomais: estrutura e função da ALDP

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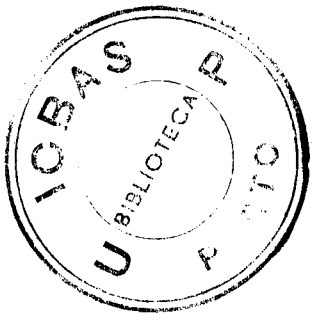

NOTAS PRÉVIAS.....

A autora declara que participou na concepção e execução da parte experimental, na interpretação dos resultados e na redacção dos manuscritos que constituem este trabalho:

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RESUMO

Os transportadores ABC (*ATP-binding cassette*) são proteínas com actividade ATPase que realizam o transporte de substratos através da membrana onde se encontram inseridas. O trabalho aqui apresentado focou-se no estudo dos transportadores ABC presentes na membrana do peroxissoma de mamíferos. Particular ênfase foi atribuída ao transportador ALDP, uma vez que defeitos no gene codificante para esta proteína (gene *ABCD*1) se encontram associados a uma doença genética humana conhecida como adrenoleucodistrofia ligada ao cromossoma X (X-ALD).

Numa primeira etapa, procedeu-se à caracterização molecular do gene *ABCD*1 em famílias Portuguesas afectadas com X-ALD. O protocolo preferencialmente adoptado consistiu na análise de toda a região codificante, recorrendo ao material biológico (RNA) de um doente do sexo masculino de cada família. Os resultados revelam uma elevada heterogeneidade molecular, à semelhança do que foi já descrito para outras populações.

Com o intuito de completar a caracterização das mutações encontradas, determinaram-se os níveis de RNA mensageiro e proteína ALDP presentes nos fibroblastos de pele, em cultura, dos doentes. Este tipo de análise complementar foi uma mais valia na caracterização de duas mutações de *splicing*. Os resultados sugerem que formas clínicas de severidade intermédia associadas a mutações de *splicing*, se podem dever (mesmo nos casos mais inesperados) à presença de baixos níveis de proteína ALDP normal. Nestes casos, a quantificação desta proteína poderá ser um dado fundamental na interpretação do fenótipo apresentado pelo doente.

Na membrana do peroxissoma de mamíferos são conhecidos, para além da ALDP, mais três transportadores *half*-ABC: ALDPR, PMP70 e PMP70R. Apesar de se assumir que estas proteínas dimerizam de forma a se tornarem funcionais, o certo é

que nunca foi claramente definido se, em condições fisiológicas normais, estas proteínas homodimerizam e/ou heterodimerizam. No sentido de esclarecer esta questão, procedeu-se à caracterização da estrutura quaternária de dois transportadores ABC peroxissomais, ALDP e PMP70, usando fígado de ratinho como material inicial. Os resultados obtidos indicam claramente que, pelo menos neste órgão, estas proteínas se encontram maioritariamente sob a forma homomérica, muito possivelmente homodimérica.

Apesar de algumas evidências indicarem que a ALDP está envolvida no metabolismo de ácidos gordos saturados de cadeia muito longa, a sua função exacta ainda permanece por determinar. Com o objectivo de identificar os substratos transportados por esta proteína foi desenvolvido um ensaio que consistiu na avaliação da susceptibilidade da ALDP a proteólise, na presença de determinados compostos. A ideia subjacente a este trabalho baseia-se na hipótese de que na presença do substrato correcto a conformação da ALDP se modifica, e que consequentemente se poderá observar alteração do padrão proteolítico. Os resultados sugerem que a ALDP transporta ácidos gordos saturados de cadeia longa e muito longa esterificados com Coenzima A.

ABSTRACT

The ABC (ATP-binding cassette) transporters are transmembrane proteins that couple ATP hydrolysis to the translocation of substrates across a biological membrane. The work described here focuses on the study of the ABC transporters from the mammalian peroxisomal membrane. Particular attention was given to the study of the ALDP transporter, because defects in its gene (*ABCD*1 gene) are associated with a human genetic disorder known as X-linked adrenoleukodystrophy (X-ALD).

This study was started by determining the mutational profile of the *ABCD*1 gene in Portuguese families affected with X-ALD. The protocol used consisted of analyzing the entire coding region of the gene, using the biological material (RNA) of a male patient from each family. The results show a great molecular heterogeneity, which is consistent with the data found in other populations.

In order to characterize further the mutations identified, the levels of *ABCD*1 mRNA and ALDP protein in patient's skin cultured fibroblasts were determined. This kind of analysis proved crucial in characterizing two splicing mutations. The data suggest that less severe or late onset phenotypes, when associated with splicing mutations, may be due (even in the most unexpected cases) to the presence of low levels of normal protein. In these cases, the quantification of ALDP is essential in understanding the phenotype displayed by the X-ALD patients.

Besides ALDP, there are three more half-ABC transporters in the mammalian peroxisomal membrane: ALDPR, PMP70 and PMP70R. It has long been assumed that these proteins dimerize in order to become functional. However, it was never determined whether these proteins engage in homodimers and/or heterodimers under normal physiological conditions. In order to clarify this question, the quaternary structures of ALDP and PMP70 from mouse liver peroxisomes were characterized. The

results clearly show that, at least in this organ, the majority of these two peroxisomal ABC-transporters are homomeric assemblies, most probably homodimers.

Although some evidence indicates that ALDP is somehow implicated in the metabolism of saturated very long-chain fatty acids, its exact role was never established. Aiming at the identification of the substrates transported by this protein, a protease-based assay to search for substrate-induced conformational alterations on ALDP was developed. The idea underlying this work is that in the presence of the correct substrate, the conformation of ALDP would change and consequently a different susceptibility to proteolysis could be observed. The results suggest that ALDP is directly involved in the transport of long- and very long-chain acyl-CoA esters across the peroxisomal membrane.

RÉSUMÉ.....

Les transporteurs ABC (*ATP-binding cassette*) sont des protéines avec de l'activité ATPasique qui participent au transport de substrats à travers les membranes dans lesquelles ils sont insérés. Le travail ici présenté, est centré sur l'étude des transporteurs ABC de la membrane peroxysomale chez les mammifères. Une attention particulière a été apportée à l'étude du transporteur ALDP, protéine codée par le gène *ABCD*1, dont la déficience est associée à une maladie génétique humaine appelée adrénoleucodystrophie liée au chromosome X (X-ALD).

Tout d'abord, on a procédé à la caractérisation moléculaire du gène *ABCD*1 chez des familles Portugaises affectées par l'X-ALD. Le protocole suivit a consisté à analyser la totalité de la région codante en utilisant le matériel biologique (RNA) d'un patient de sexe masculin de chaque famille. Les résultats montrent une forte hétérogénéité moléculaire en accord avec les données décrites pour d'autres populations.

Dans le but de compléter la caractérisation des mutations identifiées, les niveaux en ARN messager d'*ABCD*1 et en protéine ALDP ont été déterminés dans des fibroblastes cutanés cultivés de patients. Cette analyse complémentaire s'est avérée déterminante dans la caractérisation de deux mutations d'épissage. Les résultats obtenus suggèrent que les formes cliniques les moins sévères, lorsqu'elles sont associées à des mutations d'épissage, peuvent être dues (même dans les cas les plus inattendus) à la présence de concentrations faibles de protéine ALDP normale. Dans ces cas, la quantification de la protéine ALDP se révèle fondamental pour l'interprétation du phénotype clinique du malade.

Au delà de l'ALDP, dans la membrane peroxysomale des mammifères trois autres hémi-transporteurs ABC: ALDPR, PMP70 et PMP70R on été retrouvés. Il est

convenu que ces protéines se dimérisent pour former un transporteur fonctionnel. Il n'a cependant jamais été clairement établi si dans des conditions physiologiques normales, ces protéines s'homodimérisent et/ou s'hétérodimérisent. Dans le but de clarifier ce point, la caractérisation de la structure quaternaire des protéines ALDP et PMP70 a été effectuée à partir de foie de souris. Les résultats obtenus indiquent nettement que, au moins dans ce tissu, ces protéines se retrouvent majoritairement sous forme homomérique, plus probablement homodimérique.

Bien que plusieurs évidences indiquent que ALDP est impliqué dans le métabolisme des acides gras saturés à très longue chaîne, sa fonction exacte reste encore à déterminer. Afin d'identifier les substrats transportés par cette protéine, un essai a été développé de façon à évaluer la susceptibilité de l'ALDP à être clivée par protéolyse en présence de certains composés. L'idée à l'origine de ce travail repose sur l'hypothèse qu'en présence du substrat adéquat, la conformation de l'ALDP est modifiée, et par conséquent son profil protéolytique en sera également altéré. Les résultats obtenus suggèrent que l'ALDP serait impliquée dans le transport d'esters d'acyl-CoA composés d'acide gras saturés à chaînes longues et très longues.

ABREVIATURAS

ABC	*ATP-binding cassette*
AGCML	Ácidos gordos saturados de cadeia linear muito longa
ALDP	*Adrenoleukodystrophy protein*
ALDPR	*ALDP-related protein*
AMN	Adrenomieloneuropatia
CCALD	*Cerebral childhood adrenoleukodystrophy*
CFTR	*Cystic fibrosis transmembrane conductance regulator*
CoA	Coenzima A
CSGE	*Conformational sensitive gel electrophoresis*
GTE	Gliceril trierucato
GTO	Gliceril trioleato
mRNA	Ácido ribonucleico mensageiro
NBD	*Nucleotide binding domain*
PCR	*Polymerase chain reaction*
PMP70	*Peroxisomal membrane protein of 70kDa*
PMP70R	*PMP70-related protein*
RT-PCR	*Reverse transcription polymerase chain reaction*
TAP	*Transporter associated with antigen processing*
TMD	*Transmembrane domain*
VLCS	*Very long-chain acyl-CoA synthetase*
X-ALD	Adrenoleucodistrofia ligada ao cromossoma X

ÍNDICE

INTRODUÇÃO

"LORENZO"

Once upon a time I made a lion roar -
he was sleeping in the sunbeams on the old zoo floor.
I had gone to see the park where my papa used to play,
it's called Villa Borghese and it's on the way
to East Africa.

Down on Grand Comoro Island, where I grew past four,
I could swim and fish and snorkel on the ocean floor,
and the wind laughed, and the wind laughed through the trees as if to say,
here's a child who'll want the world to go his way
in East Africa, in East Africa.

Suddenly for me the world turned upside down -
far from my friends the lions and the dolphins came this awful sound.

Dark shadows, sounds of thunder raging over me,
came this monster called 'A-dre-no-leu-ko-dys-tro-phy'
Where's my East Africa?

Well they said, they said, they said (the ones who know it all)
they said from now on for you there will be no more standing tall,
so I took my parents' hands, I lifted my head to say
I'll just have to be a hero, there's no other way!
Back to East Africa
Back to East Africa
Back to East Africa
Come with me I'm going back, going back to East Africa.

Michaela and Lorenzo Odone

1. A família dos transportadores ABC (*ATP-binding cassette*)

Em 1982, Higgins e colaboradores (Higgins et al., 1982) clonaram e sequenciaram pela primeira vez, na íntegra, a sequência nucleotídica de um transportador ABC: o transportador de histidina da bactéria *Salmonella typhimurium*. Desde então, muitos outros membros desta família de proteínas têm sido identificados em vários organismos (revisão em (Higgins, 1992)). Refira-se que, por exemplo, 5% do genoma de *Escherichia coli* codifica transportadores ABC (Linton and Higgins, 1998).

1.1. Aspectos estruturais

Um transportador ABC funcional típico é constituído por quatro domínios: dois domínios que compreendem, no seu conjunto, cerca de 12 segmentos hidrofóbicos que atravessam a membrana formando um canal (vulgo TMDs, *transmembrane domains*) e dois domínios onde ocorre ligação e hidrólise de ATP (vulgo NBDs, *nucleotide binding domains*) (Higgins, 1992).

Apesar do elevado número de transportadores ABC conhecidos, a região dos NBDs apresenta-se bastante conservada entre os diferentes membros desta família de proteínas[1]. Para além das sequências comuns a qualquer proteína com actividade ATPase (i.e., Walker A (GX_4GKS/T; X corresponde a qualquer aminoácido) e Walker B ($R/KX_{(6-8)}\Phi_4D$; Φ corresponde a qualquer aminoácido hidrofóbico) (Walker et al., 1982)) salientam-se três motivos específicos dos transportadores ABC: (1) a sequência LSGGQ denominada por *Signature Motif* (Bianchet et al., 1997), (2) um

[1] Refira-se que certas proteínas, apesar de apresentarem conservação dos motivos exclusivos da família de transportadores ABC, não se encontram associadas à função de transporte. Estas proteínas são, genericamente, denominadas por ABC-ATPases, não possuem regiões membranares e desempenham papéis noutros processos celulares, tais como: reparação de DNA (exs. UvrA (Thiagalingam and Grossman, 1993) e Rad50 (Hopfner et al., 2000)), tradução proteica (ex. EF-3 (Belfield et al., 1995)), etc. O estudo desta subclasse de proteínas ABC está fora do âmbito deste trabalho.

resíduo de histidina (*His-Loop*) no contexto $\Phi_4H\Psi$ (Ψ corresponde a qualquer aminoácido com carga) situado a 30-40 aminoácidos a jusante do Walker B e (3) um *Glutamine-loop* localizado entre o Walker A e a sequência *Signature Motif* (Kerr, 2002).

Atendendo à estrutura primária, os transportadores ABC podem ser classificados como *full*-ABC, se os quatro domínios (2TMDs + 2NBDs) estiverem contidos na mesma cadeia polipéptidica (Fig. 1A; ex. CFTR; *cystic fibrosis transmembrane conductance regulator* (Riordan et al., 1989)) ou *half*-ABC, caso o respectivo gene codifique somente um domínio TMD e um domínio NBD (Fig. 1B; ex. Tap1/Tap2; *transporter associated with antigen processing* (Hyde et al., 1990)). Por analogia com a estrutura dos transportadores *full*-ABC, pressupõe-se que as proteínas *half*-ABC dimerizam entre si para se tornarem biologicamente activas. Assim, alguns transportadores *half*-ABC associam-se em complexos homodiméricos (exs. *ABCG2* (Kage et al., 2002), *ABCB10* (Graf et al., 2004)) e outros em complexos heterodiméricos (exs. Tap1/Tap2 (Kelly et al., 1992), *ABCG5/ABCG8* (Graf et al., 2003)).

Nos organismos procarióticos, para além dos transportadores *full*- e *half*-ABC, é ainda frequente encontrar as quatro unidades estruturais em quatro polipéptidos independentes, os quais interactuam entre si formando um complexo proteico activo (Holland and Blight, 1999). Certos transportadores ABC, nomeadamente os que estão envolvidos na importação de compostos para o interior da célula, possuem ainda uma quinta subunidade: uma proteína auxiliar, localizada no periplasma, que reconhece especificamente o composto a ser transportado e que o entrega ao complexo membranar que realizará o transporte (Shuman and Panagiotidis, 1993) (Fig. 1C).

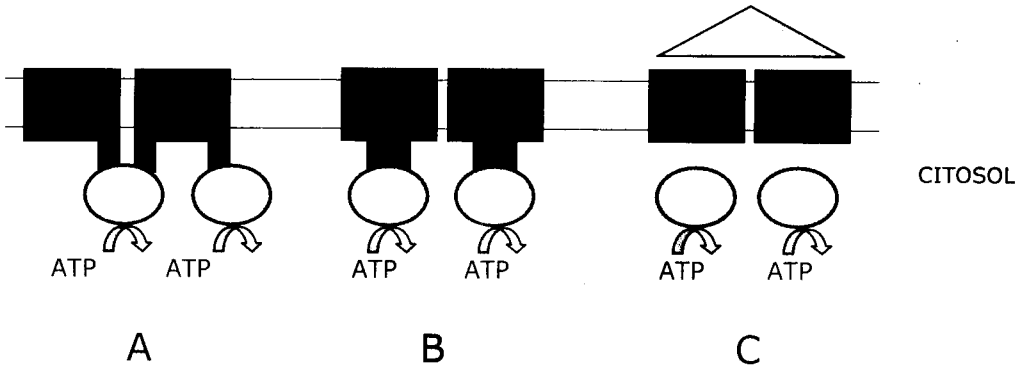

Fig. 1 Organização dos quatro domínios estruturais dos transportadores ABC. A) transportador *full*-ABC, B) transportadores *half*-ABC e C) transportador ABC exclusivo de um sistema bacteriano, onde o triângulo representa a proteína extracelular que se liga ao substrato. Os rectângulos e os círculos representam os TMDs e NBDs, respectivamente. (Adaptado de (Schneider and Hunke, 1998))

1.2. Aspectos funcionais

Os transportadores ABC realizam o transporte activo de vários substratos[2] (Higgins, 1992) cuja natureza química é, no seu conjunto, muito variada: lípidos (por ex. colesterol, ácidos biliares), proteínas, iões, antibióticos, etc. (Holland and Blight, 1999). O sentido do transporte é único para cada proteína, pelo que certos transportadores importam e outros exportam substratos (Saurin et al., 1999).

Certos transportadores *half*-ABC podem estabelecer interacções com mais do que uma proteína da mesma classe (i.e., podem originar diferentes tipos de heterodímeros). Esta capacidade confere versatilidade funcional, uma vez que diferentes complexos transportam, geralmente, substratos diferentes. Esta afirmação pode ser ilustrada recorrendo ao caso dos transportadores *half*-ABC *white, brown* e *scarlet* de *Drosophila melanogaster* (Ewart and Howells, 1998). Neste organismo, a proteína *white* dimeriza com as outras duas, possibilitando o transporte de diferentes metabolitos percursores dos pigmentos dos olhos. O complexo *white/brown* transporta guanidina (percursor do pigmento vermelho) e o complexo *white/scarlet* transporta

[2] Uma vez que o ATP é o único composto modificado (hidrolisado) durante o mecanismo de transporte, só este, atendendo à definição clássica, deveria ser denominado por substrato. No entanto, a palavra substrato será usada ao longo do texto referindo-se à substância que é transportada.

triptofano (percursor do pigmento castanho). Assim, devido às diferenças de especificidade de substrato, mutações nos genes codificantes para *brown*, *scarlet* ou *white* traduzem-se em moscas com olhos castanhos, vermelhos ou brancos, respectivamente (Ewart and Howells, 1998).

1.3. Mecanismo de transporte: modelos actuais

Os transportadores ABC podem, em certa medida, ser comparados a enzimas convencionais que na presença de um substrato e ATP modificam, de alguma forma, a sua conformação para realizar o transporte. Vários esforços têm sido efectuados no sentido de obter estruturas de alta resolução, que permitam compreender de que forma os quatro domínios estruturais se articulam entre si (Jones and George, 2004). Atendendo às dificuldades experimentais inerentes ao estudo de qualquer proteína intrínseca de membrana, vários grupos de investigação decidiram recorrer à análise das subunidades "solúveis" (domínios NBDs) de transportadores ABC bacterianos (por exemplo, a subunidade HisP do complexo permease de histidina (Hung et al., 1998)), ao estudo de proteínas ABC-ATPases (por exemplo, Rad50 (Hopfner et al., 2000)) ou ainda à sobre-expressão e análise dos domínios catalíticos dos transportadores ABC (por exemplo, TAP1 (Gaudet and Wiley, 2001)). Actualmente, encontram-se também já disponíveis as estruturas completas de três transportadores ABC: MsbA de *Escherichia coli* (Chang and Roth, 2001), MsbA de *Vibrio cholera* (Chang, 2003) e BtuCD de *Escherichia coli* (Locher et al., 2002).

O transportador MsbA pertence à subclasse *half*-ABC, e é responsável pelo transporte de lípido A do folheto interno para o folheto externo da membrana plasmática. A estrutura deste transportador de *E. coli* (Fig. 2A, resolução de 4.5Å) foi obtida na ausência de substrato e nucleótidos, apresentando uma forma aberta, com o formato de um " V " invertido, na qual os NBDs se encontram separados cerca de 50Å (Chang and Roth, 2001). De acordo com os dados obtidos, Chang e Roth (Chang and

Roth, 2001) propuseram um mecanismo de acção em "tesoura", ou seja: (1º) ocorre hidrólise de ATP que é acompanhada pela entrada do lípido A no canal transmembranar formado pelos dois monómeros de MsbA; (2º) os dois NBDs até então afastados dimerizam e o substrato, dada a presença de vários resíduos polares no interior do canal, inverte a sua orientação; (3º) com a ligação de ATP, os NBDs separam-se e a estrutura membranar abre-se libertando o lípido. Algumas críticas têm sido tecidas a esta estrutura, nomeadamente, ao facto dos dois NBDs apresentarem uma distância tão grande entre si (revisão em (Jones and George, 2004)). Esta característica não se verifica na estrutura da proteína MsbA de *Vibrio cholerae* (obtida com uma resolução de 3.8Å). Neste caso, os autores sugerem que a conformação deste transportador é muito mais compacta e que os NBDs se associam após ligação ao ATP (Chang, 2003), sendo consensual com os dados obtidos por outras metodologias (nomeadamente experiências de *cross-linking* e estudos de mutagénese). Assim, permanece por esclarecer se a estrutura obtida para a proteína MsbA de *E. coli* representa um artefacto de cristalização ou uma verdadeira conformação de transição (Jones and George, 2004).

O transportador BtuCD é uma proteína *full*-ABC que transporta vitamina B_{12}, do meio extracelular para o citosol. Neste caso, o substrato é entregue ao complexo membranar por uma proteína (denominada por subunidade BtuF) que se localiza no periplasma. A estrutura desta proteína (Fig. 2B, resolução de 3.2Å) foi também obtida na ausência de substrato e nucleótidos sendo semelhante à estrutura anteriormente prevista por métodos bioquímicos e moleculares (Locher et al., 2002). Surpreendentemente, a região transmembranar é constituída por 20 α-hélices e não 12 como é usual. No mecanismo de transporte proposto é sugerido que a ligação de ATP provoca a dimerização dos NBDs, o que desencadeia o afastamento dos domínios transmembranares e consequentemente a entrada de vitamina B_{12} no canal.

As diferenças encontradas nos mecanismos de acção propostos para os transportador MsbA e BtuCD poderão ser explicadas, em parte, pela existência de artefactos nas estruturas e/ou pelo facto da organização estrutural, natureza do substrato e orientação do transporte ser diferente nos dois casos (Jones and George, 2004). Na proteína MsbA, a entrada de substrato no canal é efectuada pelo lado para o qual se encontram orientados os NBDs, enquanto que na proteína BtuCD o substrato é entregue, por uma proteína acessória, pelo lado oposto ao dos NBDs.

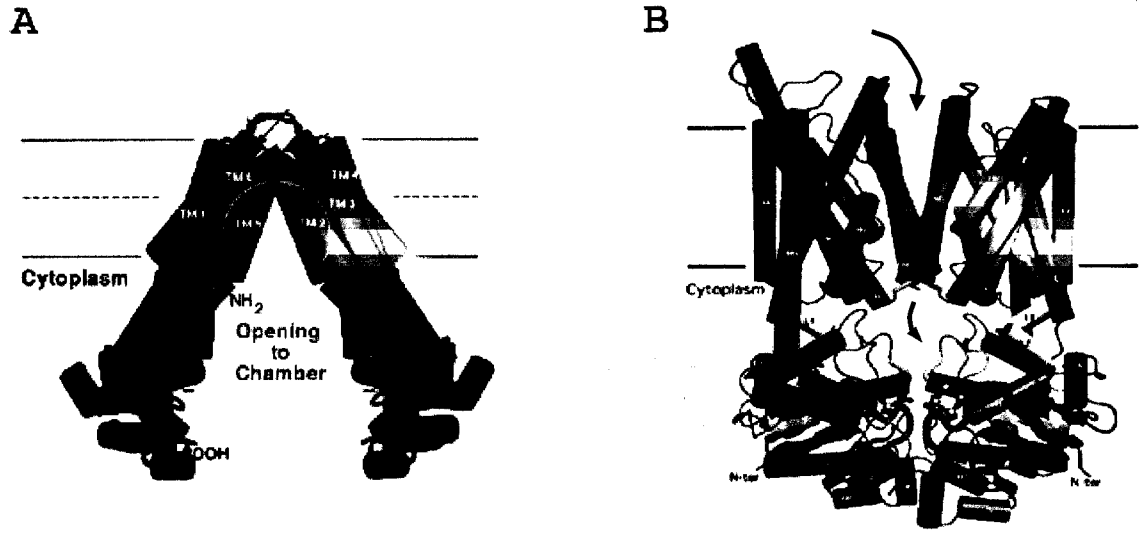

Fig. 2 Estruturas de dois transportadores ABC de *E.coli*. A) Representação da estrutura do dímero MsbA. Os domínios TMDs e NBDs encontram-se representados a vermelho e a verde, respectivamente. B) Representação da estrutura do transportador BtuCD. Os dois domínios transmembranares (denominados por BtuC) encontram-se representados a roxo e a vermelho, e os dois domínios NBD (denominados por BtuD) encontram-se representados a verde e a azul. As setas indicam o trajecto do transporte. (Adaptado de (Chang and Roth, 2001 e Locher et al., 2002))

A aquisição de novas estruturas, destes e de outros transportadores ABC, obtidas em diferentes etapas do mecanismo de transporte permitirá, no futuro, dar resposta a várias perguntas. Por exemplo: o que é que determina a orientação de transporte? Como é que os transportadores ABC reconhecem especificamente o substrato a transportar? Como é que estas proteínas efectuam o transporte dos substratos?

2. Relevância dos transportadores ABC na saúde humana

O elevado interesse pelo estudo dos transportadores ABC é em grande parte justificado pelo facto de vários membros desta família de proteínas se encontrarem associados à função de desintoxicação celular, tendo consequentemente implicações na área do tratamento do cancro. Refira-se que a sobre-expressão natural de alguns transportadores ABC em células neoplásicas (por exemplo, a glicoproteína P) está na base dos mecanismos moleculares que conferem resistência a estas células contra certas drogas citotóxicas usadas em tratamentos quimioterapêuticos (Ambudkar et al., 2003). Por outro lado, várias doenças genéticas têm vindo a ser associadas a defeitos nestas proteínas (Borst and Elferink, 2002). Actualmente, são conhecidas 15 patologias distintas das quais se salientam a fibrose quística, a doença de Tangier e a adrenoleucodistrofia ligada ao cromossoma X (X-ALD). Esta última é, até ao momento, a única patologia resultante de defeitos num transportador ABC localizado na membrana do peroxissoma.

2.1. X-ALD: aspectos clínicos

A X-ALD é uma doença neurodegenerativa que afecta cerca de 1:21000 indivíduos do sexo masculino (Dubois-Dalcq et al., 1999; Bezman et al., 2001). Vários quadros clínicos da doença podem ser destacados, atendendo à idade dos primeiros sintomas, órgãos afectados e à progressão dos sintomas neurológicos (revisão em (Moser et al., 2001). Esta variabilidade fenotípica é encontrada entre indivíduos da mesma família (Moser et al., 2001) e até mesmo entre gémeos monozigóticos (Sobue et al., 1994; Korenke et al., 1996). Os três fenótipos mais comuns são: a forma infantil (CCALD; *Childhood Cerebral ALD*), a adrenomieloneuropatia (AMN) e a forma Addison. Os dois primeiros representam, no seu conjunto, cerca de 80% dos casos (van Geel et al., 1997).

A forma infantil é a forma mais severa, sendo caracterizada pela desmielinização da substância branca do cérebro que, por sua vez, desencadeia uma resposta inflamatória cerebral (McGuinness and Smith, 1999). A progressão dos sintomas neurológicos é muito rápida (2-3 anos) levando à morte dos doentes na primeira década de vida.

A adrenomieloneuropatia manifesta-se em indivíduos com idade superior a 20 anos de idade. É caracterizada pela progressão lenta (na ordem de décadas) dos sintomas clínicos, que se encontram inicialmente limitados à espinal-medula e ao sistema nervoso periférico (forma pura de AMN). Em cerca de metade dos casos AMN existe uma inflamação progressiva rápida no cérebro (forma cerebral de AMN), à semelhança da forma infantil (Moser et al., 2001).

Doentes X-ALD com a forma Addison apresentam insuficiência das glândulas supra-renais, sem qualquer tipo de envolvimento neurológico. Na maioria dos casos (95%) esta forma evolui ou coexiste com qualquer outro quadro clínico da doença (Moser et al., 2001). Refira-se ainda que cerca de 35% dos casos idiopáticos de Addison poderão estar associados à X-ALD (Laureti et al., 1996; Aubourg and Chaussain, 2003).

Relativamente às portadoras, cerca de 50% desenvolvem sintomas idênticos a um indivíduo afectado com a forma pura de AMN (Moser, 1997). Raramente apresentam insuficiência das glândulas supra-renais (<1%) (el-Deiry et al., 1997) ou formas da doença ditas cerebrais (<3%) (Moser et al., 2001; Fatemi et al., 2003).

2.2. Caracterização bioquímica da X-ALD

O único parâmetro bioquímico reconhecidamente alterado, nos tecidos e plasma de doentes afectados com X-ALD, corresponde ao aumento (cerca de 2-10 vezes (Moser et al., 1999)) dos níveis de ácidos gordos saturados de cadeia linear muito longa (AGCML; >C22) (Igarashi et al., 1976). Esta acumulação é mais acentuada nas glândulas supra-renais e substância branca do cérebro, onde se chega mesmo a observar a presença de inclusões lipídicas (Schaumburg et al., 1972; Powers and Schaumburg, 1973; Igarashi et al., 1976). A contribuição da acumulação deste tipo de ácidos gordos para o aparecimento da doença ainda permanece por esclarecer. Inclusivamente, coloca-se a questão se este excesso de ácidos gordos é efectivamente a causa primária da doença. Por exemplo, os três ratinhos modelo para a X-ALD (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997) apesar de, à semelhança dos doentes, apresentarem níveis aumentados de AGCML nunca desenvolvem a forma mais severa da doença (CCALD). No entanto, Pujol e colaboradores (Pujol et al., 2002) verificaram que estes animais, numa fase já tardia da sua vida (> 15 meses de idade) começam a exibir algumas características de um fenótipo AMN puro, verificando-se alterações neurológicas confinadas ao sistema nervoso periférico mas sem apresentação de desmielinização ou de qualquer tipo de resposta inflamatória cerebral.

A acumulação de AGCML verificada nos doentes X-ALD é frequentemente explicada por uma deficiência na degradação destes ácidos gordos (Singh et al., 1984a), processo este que ocorre exclusivamente no peroxissoma (Lazarow and De Duve, 1976; Singh et al., 1984b). O sistema de β-oxidação peroxissomal de AGCML encontra-se esquematizado na Fig. 3.

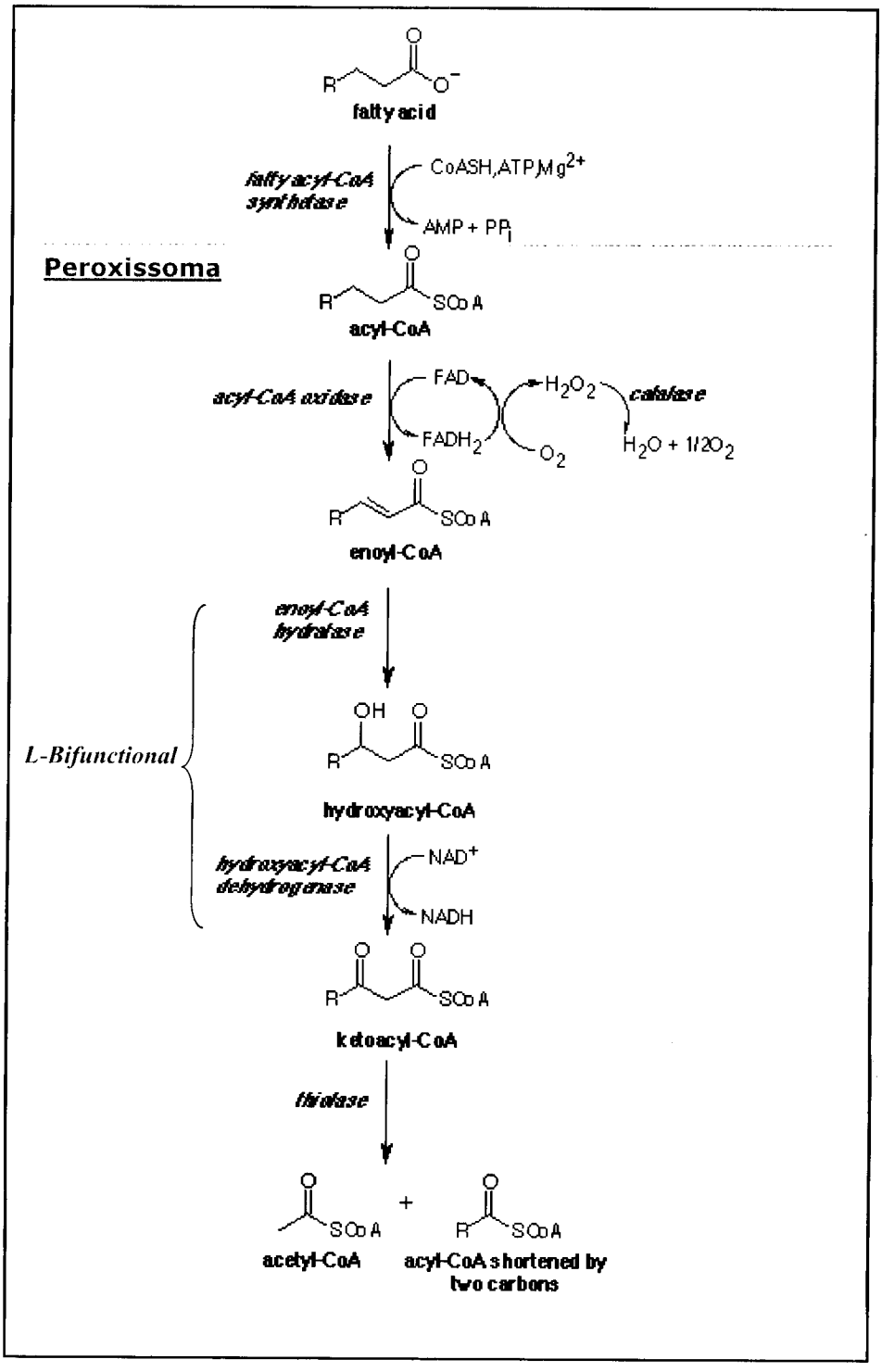

Fig. 3 Via de β-oxidação peroxissomal de AGCML. Todas as enzimas foram identificadas na matriz do organelo, com excepção da enzima acil-CoA sintetase cuja localização subcelular ainda se desconhece. O terceiro e quarto passos desta via metabólica são catalisados pela mesma enzima *L-bifunctional* (Furuta et al., 1980) que, tal como indicado no esquema, apresenta dupla actividade. (Adaptado http://www.peroxisome.org)

2.2.1. Diagnóstico laboratorial

O diagnóstico bioquímico de doentes X-ALD do sexo masculino baseia-se na determinação dos níveis de AGCML (nomeadamente ácido tetracosanóico (C24:0) e ácido hexacosanóico (C26:0)) em amostras biológicas como plasma e/ou células em cultura (por exemplo, fibroblastos de pele, amniócitos, etc.). A acumulação de AGCML é independente da idade dos doentes, da presença/ausência de sintomas e da amostra usada para análise (Moser et al., 1999). No entanto, nos indivíduos do sexo feminino isto nem sempre se verifica. Assim, mesmo que a análise seja efectuada simultaneamente em plasma e fibroblastos de pele em cultura, pelo menos 5% das portadoras apresentam níveis de AGCML normais pelo que não podem ser identificadas recorrendo unicamente à análise bioquímica (Moser et al., 1999). Nestes casos, o estudo molecular é indispensável para o estabelecimento do diagnóstico de portadora X-ALD.

2.2.2. Terapia com óleo de Lorenzo

O desenvolvimento de uma terapia eficaz para a X-ALD torna-se complicado dado que os mecanismos responsáveis pelo aparecimento desta doença ainda não são completamente conhecidos. Nos doentes que apresentam insuficiência das glândulas supra-renais a terapia de substituição hormonal compensará o defeito, mas a sua eficácia é diminuta no panorama global da X-ALD. Vários têm sido os esforços dirigidos para o desenvolvimento de uma terapia que impeça o aparecimento/evolução dos sintomas neurológicos, aumentando de alguma forma a qualidade e esperança de vida dos doentes. Apesar de ainda não existir tratamento eficaz para a X-ALD, o óleo de Lorenzo merece aqui uma atenção especial.

O óleo de Lorenzo é uma mistura de gliceril trioleato (GTO; C18:1) e gliceril trierucato (GTE, C22:1) na proporção de 4:1 (Rizzo et al., 1989). A ingestão deste

óleo, conjuntamente com a restrição alimentar de AGCML, permite a normalização dos níveis deste tipo de ácidos gordos num curto espaço de tempo, nomeadamente no plasma dos doentes (cerca de 4 semanas (Moser et al., 2001)). Este efeito deve-se a dois factores: (1) os AGCML que se acumulam nos doentes provêm da dieta e da síntese endógena (Kishimoto et al., 1980; Van Duyn et al., 1984) e (2) a elongação dos ácidos gordos saturados e monoinsaturados é efectuada pela mesma maquinaria enzimática (Bourre et al., 1976), pelo que a introdução de GTO/GTE na dieta inibe a síntese endógena de ácidos gordos saturados de cadeia muito longa. Este regime dietético foi aplicado em doentes X-ALD de todo o mundo (revisão em (Moser et al., 2003)), mas os resultados revelam que apesar da normalização dos níveis de AGCML no plasma e em vários tecidos, não se observa regressão ou diminuição na progressão dos diferentes parâmetros clínicos nos indivíduos que já apresentem envolvimento neurológico (van Geel et al., 1999; Moser et al., 2003). Muito possivelmente, tal insucesso deve-se ao facto dos ácidos monoinsaturados não ultrapassarem a barreira hematoencefálica, pelo que a acumulação de AGCML no cérebro não é impedida usando esta estratégia (Poulos et al., 1994; Rasmussen et al., 1994). Actualmente, a utilização do óleo de Lorenzo é recomendada, como forma de prevenção, em doentes neurologicamente assintomáticos e com uma ressonância magnética de imagem normal (Moser et al., 2003). Assim, salienta-se a importância da identificação de doentes e portadoras X-ALD vários anos antes do aparecimento dos primeiros sintomas.

2.3. Caracterização molecular da X-ALD

Na década de oitenta, vários estudos realizados com células de doentes X-ALD indicaram que toda a via de β-oxidação peroxissomal estava funcional com excepção da activação dos AGCML por esterificação com coenzima A (ver Fig. 3) (Singh et al., 1984a; Hashmi et al., 1986; Wanders et al., 1987; Lazo et al., 1988). Esta observação

levou os autores a sugerir que o gene envolvido nesta doença codificava uma enzima da família das VLCS (*very long-chain acyl-CoA synthetase*). No entanto, vários anos mais tarde, Mosser e colaboradores (Mosser et al., 1993) recorrendo a técnicas de clonagem posicional, identificaram o gene envolvido na X-ALD (actualmente conhecido por *ABCD*1) o qual, surpreendentemente, codifica um transportador ABC. Os dados bioquímicos que originaram a hipótese anterior são, desta forma, difíceis de compreender (revisão em (Moser et al., 2001)), permanecendo por esclarecer se algum membro da família das enzimas VLCS (Steinberg et al., 2000; Lewin et al., 2001) está realmente relacionado com os mecanismos patogénicos que estão na base da X-ALD (Jia et al., 2004).

O gene *ABCD*1 compreende 21kb de DNA genómico e está localizado no cromossoma X (Migeon et al., 1981). Vários *pseudogenes* contendo a região dos exões 7-10 daquele gene foram identificados em diferentes *locus* - 2p11, 10p11, 16p11, 20ptel e 2q11 (Braun et al., 1996; Eichler et al., 1997). Mutações no gene *ABCD*1 têm sido identificadas em todos os doentes X-ALD (Moser et al., 2001), encontrando-se actualmente descritas mais de 600 alterações genéticas que se distribuem ao longo de toda a sequência (base de dados em http://www.x-ald.nl (Kemp et al., 2001)). Todos os tipos de mutação foram encontrados, sendo as mutações do tipo *missense* as mais frequentes. É importante realçar que a maioria das mutações não são recorrentes, e que a mutação mais comum, c.1415-1416delAG, ocorre somente em 8% dos casos. De acordo com os dados actuais, não é possível estabelecer uma correlação genótipo/fenótipo na X-ALD, uma vez que o mesmo quadro clínico pode estar associado a diferentes tipos de mutação e, de forma semelhante, a mesma mutação pode estar associada a qualquer um dos fenótipos (inclusivamente entre indivíduos da mesma família) (Moser et al., 2001).

A expressão da proteína codificada pelo gene *ABCD*1 (denominada por ALDP - *adrenoleukodystrophy protein*) é ubíqua, sendo maioritariamente encontrada no cérebro, nas glândulas supra-renais, pulmão, coração e intestino (Fouquet et al.,

1997; Berger et al., 1999). A ALDP é um transportador *half*-ABC que se localiza na membrana do peroxissoma (Contreras et al., 1994; Mosser et al., 1994), com o NBD voltado para o citosol (Contreras et al., 1996) (Fig. 4). Para além da ALDP, foram identificados mais três transportadores *half*-ABC na membrana do peroxissoma de mamíferos: a ALDPR (*ALDP-related protein*; (Lombard-Platet et al., 1996)), a PMP70 (*peroxisomal membrane protein of 70kDa*; (Kamijo et al., 1990)) e a PMP70R (*PMP70-related protein*; (Holzinger et al., 1997a; Shani et al., 1997)).

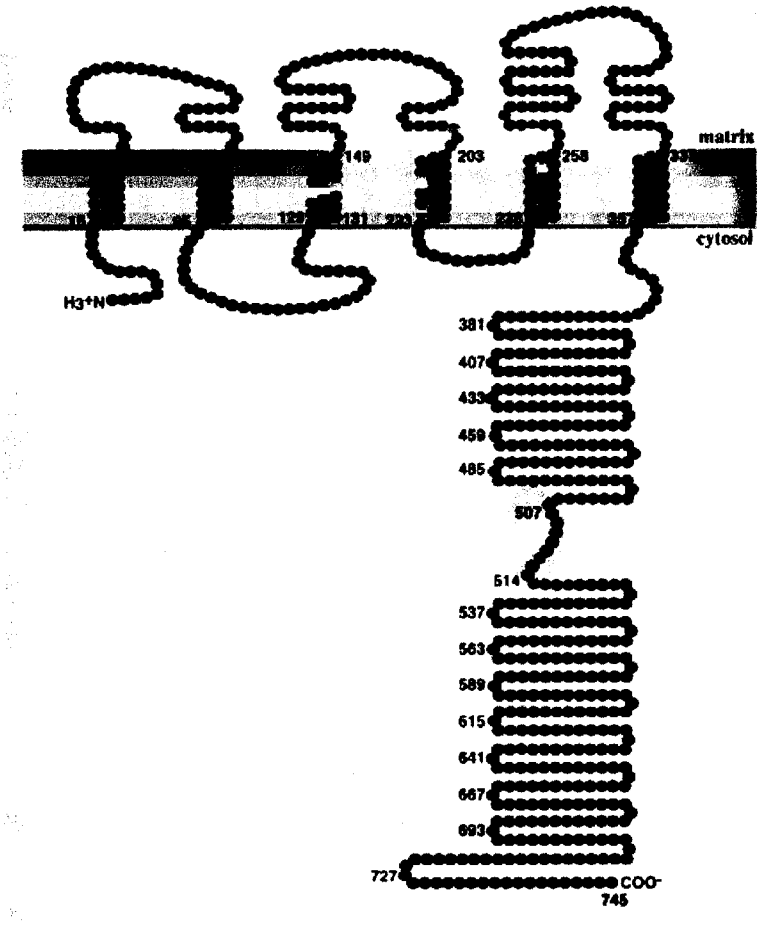

Fig. 4 Modelo hipotético para a topologia da ALDP na membrana do peroxissoma. Os círculos a vermelho indicam algumas das mutações encontradas em doentes X-ALD. (Adaptado http://nave.em.mpg.de)

3. Linhas de investigação na área dos transportadores ABC peroxissomais

Actualmente são conhecidos 49 transportadores ABC no Homem. Atendendo ao grau de conservação das suas sequências, estas proteínas foram agrupadas em sete subfamílias distintas (Dean et al., 2001), sendo a subfamília D a que compreende os quatro transportadores ABC peroxissomais (tabela 1). Seguindo as recomendações do *Human Gene Nomenclature Committee*, cada transportador passou a ser designado pela abreviatura da família de proteínas (i.e., ABC) seguida da letra da subfamília à qual pertence, e de um número (http://www.gene.ucl.ac.uk). No entanto, neste trabalho recorre-se à nomenclatura antiga de forma a tornar o texto mais claro.

Tabela 1. Localização dos genes codificantes para os quatro transportadores ABC peroxissomais conhecidos no Homem.

Proteína	nº aa	Localização do gene	Referências
ALDP (*ABCD1*)	745	Xq28	(Mosser et al., 1993)
ALDPR (*ABCD2*)	740	12q11	(Lombard-Platet et al., 1996)
PMP70 (*ABCD3*)	659	1p21	(Kamijo et al., 1990)
PMP70R (*ABCD4*)	606	14q24	(Holzinger et al., 1997a; Shani et al., 1997)

3.1. Determinação da estrutura quaternária

Tal como foi referido anteriormente, os transportadores *half*-ABC dimerizam para se tornarem funcionais, adoptando uma estrutura semelhante à dos transportadores *full*-ABC. Assim, a existência de quatro transportadores *half*-ABC na membrana do peroxissoma levanta a hipótese destas proteínas existirem como homodímeros e/ou heterodímeros. Como é óbvio, a determinação do tipo de interacções estabelecidas, sobretudo pela ALDP, assume especial importância na caracterização bioquímica da X-ALD. Considerando que cada transportador apresenta

especificidade para um determinado substrato (ou classe de substratos) e assumindo-se que a ALDP é capaz de estabelecer interacções com mais do que um transportador ABC peroxissomal então, na ocorrência de uma mutação no gene *ABCD1*, seria de se esperar a acumulação dos substratos específicos de cada complexo que envolvesse a ALDP. Pelo contrário, se a ALDP originar somente homodímeros então a variabilidade de substratos acumulados deveria ser menor. Saliente-se que, até ao momento, não se conseguiu detectar alteração em qualquer outro parâmetro bioquímico para além dos níveis de AGCML.

A abordagem da problemática da homo e heterodimerização, entre os transportadores ABC peroxissomais, esteve já na base de três estudos:

- no primeiro, Smith e colaboradores (Smith et al., 1999) recorreram a experiências de co-imunoprecipitação de proteínas sintetizadas *in vitro* (ALDP, ALDPR e PMP70) que haviam sido previamente misturadas com ALDP marcada radioactivamente. O anticorpo usado reconhecia um epítopo que foi adicionado ao C-terminal das proteínas sintetizadas *in vitro*, tendo o resultado sido analisado por autorradiografia. Os autores verificaram que a ALDP estabelece interacções com ela mesma e com as outras proteínas. No entanto, o método usado levanta sérias dúvidas relativamente à relevância das observações realizadas dado que sendo estas proteínas tão hidrofóbicas, na ausência de uma membrana não se encontram, muito possivelmente, na conformação correcta;

- no segundo trabalho, Liu e colaboradores (Liu et al., 1999) utilizaram duas abordagens: *yeast two-hybrid*, utilizando unicamente a região C-terminal (que contém o domínio NBD) dos transportadores ABC peroxissomais e co-imunoprecipitação recorrendo a células manipuladas geneticamente para expressar estas proteínas. Os resultados obtidos levaram estes autores a sugerir que os transportadores ABC peroxissomais têm capacidade de homo e heterodimerizar. No entanto, a metodologia utilizada suscita algumas dúvidas: (1) dado que os NBDs podem ser permutáveis entre certos transportadores ABC (Teem et al., 1993), não seria surpreendente

também observar interacções entre os NBDs dos transportadores ABC peroxissomais com os NBDs de um transportador ABC de outra subfamília. Infelizmente, um controlo deste tipo não foi incluído no ensaio de *yeast two-hybrid*; (2) nas experiências de imunoprecipitação, os homogeneizados celulares usados não sofreram qualquer tipo de tratamento para remoção de proteínas insolúveis, questionando-se se as interacções observadas são de facto específicas.

- o terceiro estudo foi realizado por Tanaka e colaboradores (Tanaka et al., 2002) que, usando um anticorpo contra a PMP70, foram capazes de imunoprecipitar conjuntamente a ALDP e PMP70, a partir de fígado de rato. No entanto, como muitas outras proteínas foram também encontradas no imunoprecipitado, fica por determinar se a interacção ALDP/PMP70 é real.

Por tudo isto, é legítimo afirmar-se que permanece completamente por esclarecer que tipo de complexos envolvendo os transportadores ABC peroxissomais podem ser encontrados em condições fisiológicas normais.

3.2. Determinação da função

À semelhança da estrutura quaternária, também a função dos transportadores ABC peroxissomais de mamíferos permanece por definir. No entanto, atendendo aos papéis que os transportadores ABC peroxissomais desempenham em leveduras e plantas considera-se que, de alguma forma, também os transportadores ABC peroxissomais de mamífero estejam envolvidos no metabolismo lipídico. Em *Saccharomyces cerevisiae* os únicos transportadores ABC peroxissomais conhecidos (Pxa1p e Pxa2p) associam-se um com o outro (Hettema et al., 1996; Shani and Valle, 1996) de forma a transportar, para o interior do peroxissoma, acil-CoAs de cadeia longa (Hettema et al., 1996). Em *Arabidopsis thaliana*, o único transportador ABC peroxissomal conhecido (COMATOSE ou Ped3p (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002) transporta acil-CoAs de cadeia longa e muito longa e

ácido 2,4-diclorofenoxibutírico para a matriz do peroxissoma (Zolman et al., 2001; Footitt et al., 2002) onde são degradados via β-oxidação.

Na expectativa de determinar o papel fisiológico de cada um dos transportadores ABC peroxissomais, alguns grupos de investigação têm recorrido ao desenvolvimento de ratinhos *knock-out*. Actualmente, existem modelos animais para a ALDP (cujas principais características foram já descritas na secção 2.2.) (Forss-Petter et al., 1997; Kobayashi et al., 1997; Lu et al., 1997), ALDPR (Berger et al., 2003) e PMP70 (Jimenez-Sanchez et al., 1997), bem como duplos *knock-out* que foram produzidos com a intenção de averiguar a existência de uma interacção funcional e/ou estrutural entre ALDP/ALDPR (Berger et al., 2003) e ALDP/PMP70 (Jimenez-Sanchez, 1998). Todos estes animais permanecem em estudo, pelo que ainda se desconhece quais os substratos transportados por cada uma destas proteínas. Relativamente à ALDP, tem sido especulado que esta proteína se encontra directamente envolvida no metabolismo de AGCML (Braiterman et al., 1998; Dubois-Dalcq et al., 1999; Aubourg and Dubois-Dalcq, 2000; Kemp et al., 2001); isto porque uma deficiência em ALDP (quer nos doentes X-ALD quer nos ratinhos *knock-out*) resulta na acumulação de AGCML, que são degradados no mesmo organelo onde a ALDP se localiza. Três hipóteses para a função desta proteína têm sido apontadas: transporte de AGCML livres, transporte de AGCML esterificados com Coenzima A, ou transporte de algum co-factor essencial à via de β-oxidação peroxissomal. Tais hipóteses não são, no entanto, unanimemente aceites. De facto, recentemente, McGuinness e colaboradores (McGuinness et al., 2003) propuseram uma nova hipótese para a função da ALDP. Estes autores assumem que a acumulação de AGCML nos ratinhos deficientes em ALDP se deve a anomalias na mitocôndria, tendo proposto que esta proteína transporta um composto (de natureza ainda desconhecida) de uma via metabólica comum aos dois organelos.

Uma das questões que tem vindo a ser debatida neste contexto relaciona-se com a hipótese dos quatro transportadores ABC peroxissomais poderem apresentar

funções redundantes. De uma forma geral, estas proteínas coexistem na mesma célula pelo que se assume que desempenham papéis diferentes. No entanto, em determinados tipos celulares os padrões de expressão são assimétricos e específicos (por exemplo, a ALDP e a ALDPR são expressas no córtex e na medula das glândulas supra-renais, respectivamente) (Fouquet et al., 1997; Holzinger et al., 1997b; Berger et al., 1999). Esta observação pode significar que os transportadores ABC peroxissomais participam em vias metabólicas diferentes e que a sua expressão dependa do grau de especialização da célula onde se encontram, ou então que os transportadores ABC desempenham funções idênticas, mas em diferentes tipos celulares.

A existência de uma interacção funcional entre os diferentes transportadores ABC peroxissomais poderia, eventualmente, justificar a falta de correlação genótipo/fenótipo verificada na X-ALD. Assim, mutações ou polimorfismos num dos outros transportadores ABC peroxissomais poderiam contribuir para uma actividade de β-oxidação de AGCML residual, o que possivelmente influenciaria o curso clínico da doença. No entanto, a existência de mutações nos genes codificantes da ALDPR, PMP70 e PMP70R nunca foi demonstrada em doentes X-ALD. É contudo importante salientar que a sobre-expressão da ALDPR ou PMP70 em fibroblastos de pele em cultura de doentes X-ALD permite reverter a acumulação de ácidos gordos de cadeia muito longa (Braiterman et al., 1998; Flavigny et al., 1999; Netik et al., 1999) tal como acontece quando as células são transfectadas com o cDNA *ABCD1* (Cartier et al., 1995; Shinnoh et al., 1995; Braiterman et al., 1998). Apesar de ainda não se saber se esta acção é específica ou inespecífica (note-se que os ratinhos *knock-out* para a ALDPR e PMP70 não apresentam AGCML aumentados (Jimenez-Sanchez, 1998; Berger et al., 2003)) estas proteínas, nomeadamente a ALDPR por apresentar um maior grau de conservação com a ALDP, têm sido alvo de vários estudos com o sentido de avaliar o potencial da estimulação da síntese endógena destes transportadores como futuro alvo terapêutico para a X-ALD (revisão em (Moser et al., 2004)).

OBJECTIVOS

Este trabalho foi orientado de forma a contribuir para a compreensão dos mecanismos patogénicos envolvidos na X-ALD, bem como para a caracterização bioquímica e estrutural do peroxissoma. O estudo desenvolvido compreendeu três linhas de investigação que se complementam entre si: determinação da epidemiologia genética da X-ALD em Portugal, caracterização da estrutura e da função da proteína ALDP.

Sucintamente, pretendeu-se:

1. Identificar e caracterizar mutações no gene *ABCD*1 de doentes X-ALD.

2. Evidenciar a importância da caracterização de mutações, nomeadamente as que se encontram associadas a defeitos de *splicing*, recorrendo ao estudo do RNA mensageiro e à quantificação dos níveis de ALDP.

3. Determinar que tipo de complexos envolvendo os transportadores ABC peroxissomais, designadamente a ALDP, existe em condições fisiológicas normais.

4. Identificar os substratos transportados pela ALDP.

TRABALHO EXPERIMENTAL E RESULTADOS

Este capítulo contém os resultados experimentais que permitiram a elaboração de quatro manuscritos, que serão referenciados ao longo do texto pelos números romanos a eles associados:

I. *Molecular characterization of 21 Portuguese families: identification of eight novel mutations in the ABCD1 gene*

II. *Characterisation of two mutations in the ABCD1 gene leading to low levels of normal ALDP*

III. *Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo*

IV. *Probing substrate-induced conformational alterations in ALDP by proteolysis*

TRABALHO I

Molecular characterization of 21 Portuguese families: identification of eight novel mutations in the ABCD1 gene

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Molecular characterization of 21 X-ALD Portuguese families: identification of eight novel mutations in the *ABCD1* gene

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is the most common inherited peroxisomal disorder. The gene associated with X-ALD, *ABCD1*, encodes a peroxisomal ATP-binding cassette half-transporter. In this study, we describe the molecular characterization of 21 affected Portuguese families. The complete coding region of the *ABCD1* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) or genomic PCR. After conformation-sensitive gel electrophoresis analysis, fragments with a conformational heteroduplex pattern were sequenced. Using this strategy, we have identified 14 missense mutations, two nonsense mutations, two splicing site defects, and three small deletions, two of them resulting in frameshifts. Eight of the genetic alterations characterized in this study are novel. The levels of the *ABCD1* transcript as well as the levels of ALDP in cultured skin fibroblasts of male probands were also determined in most cases. The levels of the *ABCD1* transcript in one patient (corresponding to a nonsense mutation) were below the detection limit of Northern-blotting analysis. ALDP was found at normal levels in only three patients, absent in five (corresponding to a double missense, two nonsense, and two frameshift mutations), and decreased in all the others.

Keywords: X-ALD; ABCD1 gene; Mutations; Peroxisome disorders

1. Introduction

The adrenoleukodystrophy (X-ALD, OMIM #300100) is an inborn X chromosome linked disease. This neurodegenerative disorder is characterized by great clinical expression variability. According to the age of onset, the organs affected and the rate of progression of neurologic symptoms seven phenotypes can be considered in males: childhood cerebral form (CCER), adolescent cerebral ALD, adrenomyeloneuropathy (AMN), adult cerebral ALD, Addison disease only (AO), olivo-ponto-cerebellar, and asymptomatic. CCER is the most severe phenotype with an onset at 3–10 years of age; after the first symptoms appear the course of the disease is rapidly progressive leading to death. The adrenomyeloneuropathy phenotype has an onset in young adulthood. AMN patients who have a normal brain MRI with neurological involvement confined to the spinal cord and peripheral nerves are referred to as 'pure AMN.' However, almost 40% of the AMN patients have or develop cerebral disability being referred to as AMN-cerebral. The Addison-only X-ALD form is characterized by adrenocortical insufficiency without nervous system involvement. However, these patients are at high risk to develop neurologic symptoms (for review see [1]).

The main biochemical abnormality associated with X-ALD is the accumulation of unbranched saturated very long chain fatty acids (VLCFA) in plasma and tissues [2]. This is due to an impaired capacity of the peroxisome to metabolize these fatty acids via β-oxidation [3]. The increase in the VLCFA levels provides a reliable diagnostic tool for prenatal and postnatal identification of affected males [1]. Most carrier women can also be identified using this criterion [4].

The ALD gene (presently known as *ABCD1* gene) was identified by positional cloning in 1993 [5]. It

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comprises 10 exons spanning approximately 21 kb of genomic DNA [6] and it codes for ALDP (adrenoleukodystrophy protein)—a 75 kDa peroxisomal membrane protein which belongs to the ATP-binding cassette half-transporters superfamily [7].

A variety of disease-causing mutations in the *ABCD1* gene have been described [8]. Most of them are private to one kindred. The majority of them are missense mutations resulting in the absence of immunodetectable ALDP [9].

So far, no correlation between clinical phenotype, levels of VLCFAs, and genotype was found [1]. In spite of this limitation, identifying the mutation affecting a new family is still the best approach to characterize the members of the family under study. Besides allowing the confirmation of a clinical suspicion, in many cases this is the only way of defining the carrier status (for a discussion see [4]).

In this work, we report the results of the molecular characterization (ALD protein analysis included) performed in Portuguese families affected with X-ALD.

2. Patients, material, and methods

2.1. Patients

We included in this study affected males and obligatory carrier women of 21 unrelated families (see Table 1). All the index cases analysed had increased VLCFA levels, in plasma and/or fibroblasts. The classification of X-ALD phenotypes was performed according to the current criteria [1].

2.2. Screening of mutations in the ABCD1 gene

The screening of mutations by RT-PCR was exclusively done in male patients following the strategy described recently [10]. When no biological material from male patients was available, the screening of mutations in carrier women was performed using genomic DNA according to the method of Boehm et al. [11]. In this case, only amplified DNA fragments displaying an altered migration pattern upon conformation-sensitive gel electrophoresis (CSGE) analysis [10,12] were subjected to sequencing. The alterations identified were confirmed by sequencing a second independent PCR product. The sequencing reactions were performed on both strands using the Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All the alterations identified at cDNA level were confirmed in the genomic DNA through restriction fragment length polymorphism (RFLP) analysis, whenever possible, or by sequencing.

Table 1
Phenotype classification according to clinical data

Family[a]	Phenotype[b]	Age of onset of clinical phenotype	Adrenal insufficiency
1	cAMN	33y	+
	CCER	5y	–
	Asy (2y)	–	–
2	Carrier[c]	–	–
3	AO	8y	+
4	CCER	5y	+
5	CCER	13y	+
	cAMN	35y	+
6	AO	11y	+
	AO	Unknown	+
7	CCER	6y	–
8	CCER	8y	Unknown
9	pAMN	41y	+
	AO	30y	+
10	pAMN	29y	+
	AO	33y	+
	Asy(11y)	–	–
11	pAMN	21y	+
12	aCER	14y	+
13	cAMN	29y	+
14	cAMN	23y	+
	pAMN	27y	
15	pAMN	23y	+
16	CCER	7y	+
17	CCER	8y	–
	Asy (11y)	–	–
18	Carrier[c]	–	–
19	CCER	9y	+
20	pAMN	32y	+
21	Carrier[c]	–	–

[a] The mutation which affects each X-ALD family was found by screening the entire *ABCD1* coding region. This analysis was only performed for each index case (the first patient listed in the families with more than one individual affected). In the relatives, only the region containing the mutation identified in the index case was analysed.

[b] CCER—childhood cerebral form, cAMN AMN-cerebral, pAMN—pure AMN, aCER—adolescent cerebral ALD, AO—Addison disease only.

[c] In these families only carrier women are known.

To verify if the novel alterations found in this work were mere polymorphisms, a screening in a sample of 100 control alleles was performed using RFLP analysis after genomic DNA PCR (see Table 2 for details). Since the mutation c.2000A > T has no RFLP associated, a restriction site-generating PCR strategy was employed using ALDe10-F [11] as the forward primer and the primer 5′-CCCCATCGAACTGTAGCAAGTGTCTG-3′ which generates a mutation specific restriction site for the enzyme *Dde*I.

A similar strategy was used to detect mutation c.1964T > C. In this case, genomic DNA was subjected to PCR amplification using the primer 5′-GGAGGGCCGGTGGGTGATGAAG-3′ (located in exon 9) which eliminates a mutation specific restriction site for the enzyme *Sap*I and ALDe8/9-F as forward primer [11].

Table 2
Summarized data of the molecular diagnosis performed

Family no.	Type of genetic alteration	Exon	RT-PCR fragment[a]	Nucleotide change	Amplicon used[b]/RFLP associated[c]	ALDP (WB)	*ABCD1* mRNA (NB)	References
Missense								
1	S103R	1	F2	c.309C > G	ALDe1A/+*Cfo*I	Diminished	Detectable	[19]
2	S108W	1	–	c.323C > G	ALDe1B/+*Rle*AI	Not done	Not done	[18]
3	S108L	1	–	c.323C > T	–	Normal	Not done	[20]
4	L114P	1	F2	c.341T > C	ALDe1B/–*Eco*RII	Diminished	Detectable	Novel mutation
5	[R236H; G512S]	1	F3	[c.707G > A;	ALDe1C/+*Nco*I			Novel mutation
		6	F6	c.1534G > A]	ALDe6/+*Pst*I	Not detectable	Not done	[16,17]
6	G266R	1	F3	c.796G > A	–	Normal	Detectable	[21]
7	R518W	6	F6	c.1552C > T	ALDe6/–*Hpa*II	Diminished	Detectable	[22]
8	R518Q	6	F6	c.1153G > A	ALDe6/–*Bam*HI	Diminished	Not done	[23]
9	R545W	6	–	c.1633A > T	ALDe6/+*Tsp*RI	Not done	Not done	Novel mutation
10	R591W	7	F7	c.1171C > T	ALDe7/–*Aci*I	Normal	Not done	[24]
11	L655P	9	F8	c.1964T > C	ALDe8/9/–*Sap*I[d]	Diminished	Detectable	Novel mutation
12	R660W	9	F7/F8	c.1978C > T	ALDe8/9/+*Bsr*I	Diminished	Detectable	[16,17,25]
13	H667L	10	F8	c.2000A > T	ALDe10/+*Dde*I[d]	Diminished	Detectable	Novel mutation
Nonsense								
14	Q574X	7	F6	c.1720C > T	ALDe7/–*Alw*NI	Not detectable	Detectable	Novel mutation
15	W601X	8	F7	c.1802G > A	ALDe8/9/–*Bsr*I	Not detectable	Not detectable	[9]
Frameshift								
16	fs G298	1	F3	[c.893delG; c.894C > T]	ALDe1C/–*Nla*IV	Not detectable	Detectable	Novel mutation
17, 18	fs E472	5	F5	c.1415–1416delAG	–	Not detectable	Detectable	[21,26,27]
Microdeletion								
19	F175del	1	F2	c.522–524delCTT	[d]	Diminished	Detectable	Novel mutation
Splicing defect								
20	Splicing	IVS1	–	c.900G > A	–	Not done	Not done	[10]
21	Splicing	IVS7	–	c.1760+1G > A	–	Not done	Not done	[18]
Polymorphism								
1, 5	F673F	8	F8	c.2019C > T	–*Taq*I	–	–	[28]
1, 2, 5, 11, 13	3′UTR		F8	–	–*Drd*I	–	–	[27]

[a] RT-PCR fragment (defined according to [10]) which shows heteroduplex molecules in a CSGE analysis. The mutations, for which no fragment is indicated, were detected in the genomic DNA due to the impossibility to perform a RT-PCR-based strategy.
[b] Nomenclature according to Boehm et al. [11].
[c] New (+) or deleted (–) restriction sequence site in the presence of the genetic alteration.
[d] For a detailed description see materials and methods.

For the screening of the c.522-524delCTT mutation, a fragment of 64 bp was amplified using the forward primer 5′-CCCACGCCTACCGCCTCTAC-3′ and the reverse primer 5′-CGCCCGTCCATGTTGCTGAC-3′. To resolve the 64 bp fragment (non-mutated fragment) from the 61 bp fragment (arising from the c.522-524delCTT mutation) a 12% non-denaturing polyacrylamide gel was used.

Gene mutation nomenclature used follows the recommendations of Dunen and Antonarakis [13], and gene symbols follow the recommendation of the Hugo Gene Nomenclature Committee [14].

2.3. Northern-blotting analysis

Electrophoresis and blotting onto nylon membranes were performed exactly as recommended (Northern-Max-Gly-Ambion, Austin, TX). Hybridization and chemiluminescent detection were performed using the Dig Northern-Starter Kit and the Dig wash and block

buffer set (Roche Diagnostics, Mannheim, Germany) according to the manufacturer instructions.

For the synthesis of a non-radioactive probe, a fragment of 1273 bp, obtained with primers F3U and F6L [10] was cloned in the pGemT-easy vector (Promega, Madison, WI). Transcription labelling of RNA was done according to the manufacturer's procedure (Dig Northern-Starter Kit, Roche Diagnostics) using Sp6 RNA polymerase. The amount and quality of the RNA blotted was monitored using the supplied β-actin probe (Northern-Starter Kit, Roche Diagnostics), after membrane stripping.

2.4. Immunoblotting analysis

Total cell lysates were prepared from cultured skin fibroblasts of probands and controls (from a 75 cm^2 confluent culture flask). After trypsinization, the cells were washed three times with 20 mM Tris–HCl, pH 7.5, and 0.25 M sucrose. The pellet, obtained by centrifugation at 2000*g* for 10 min at 4 °C, was resuspended in 5 mM Tris–HCl, pH 7.5, and disrupted by sonication exactly as described [10]. An aliquot was removed for protein quantification and EDTA/EGTA, pH 8.2 to final concentration of 1 mM each, and 10 μL of a protease inhibitor cocktail (Sigma–Aldrich # P8340, Saint Louis, MO) (for a cell suspension of 10^6 cells), were immediately added to the remaining lysate. Protein was quantified by the Lowry method [15], using BSA as standard.

Total lysate protein (80 μg per lane) was resolved on 10% (w/v) polyacrylamide SDS-gels. Western-blotting onto nitrocellulose membranes was performed as described by the manufacturer (Schleicher and Schuell, Dassel, Germany).

The mouse anti-human ALDP monoclonal antibody 1D6 (Euromedex, France) was used in immunoblotting at 1:1000 (v/v) dilution. An alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma–Aldrich) was used as second antibody, at 1:5000 (v/v) dilution, for a chromogenic detection (Biorad, Munchen, Germany). The detection limit of normal ALDP using this protocol is 4–8% of the control values, as determined by loading decreasing amounts of total protein lysates from control individuals.

3. Results and discussion

To minimize the expensive process of sequencing the complete set of amplified fragments (8 and 10 fragments in the RT-PCR [10] and genomic PCR [11] based strategies, respectively) a conformation-sensitive gel electrophoresis (CSGE) analysis was performed to narrow the region to be sequenced. In all the cases analysed heteroduplex molecules were detected in at least one fragment.

Using this strategy, we have identified the mutations affecting all the X-ALD Portuguese families, from which biological material was available. A total of 23 genetic alterations were found in the 21 families studied (see Table 2). The majority of the alterations characterized are missense mutations (14 out of 23). Additionally, two nonsense mutations, two splicing site mutations, and three small deletions were found. Two of these small deletions result in frameshifts. Finally, two polymorphisms were detected.

The levels of the *ABCD*1 transcript as well as the levels of ALDP in cultured skin fibroblasts were determined by northern and Western-blotting analysis, respectively (see Table 2).

Eight out of the 23 genetic alterations described in this study are novel. None of these eight mutations were detected in 100 control X-chromosomes. A detailed description for each one is presented below.

L114P. CSGE analysis of RT-PCR amplified fragments from the proband of family 4 (see Table 2) indicated the presence of an alteration in fragment F2. Sequencing of this fragment revealed a transition c.343T > C deleting an *Eco*RII recognition site. No biological material was available from the patient's mother and so the origin of the mutation remains unknown. The effect of this missense mutation L114P in ALDP levels was determined by immunoblotting. Normally migrating ALDP was detected upon Western-blotting analysis of cultured fibroblasts total protein. However, the levels of ALDP in this patient were decreased when compared to control values. Normal levels of the *ABCD*1 transcript were detected by Northern-blotting analysis.

R236H. CSGE analysis of the cDNA fragments from the proband of family 5, showed the presence of heteroduplex molecules in products F3, F6, and F8. After sequencing two missense mutations—R236H and G512S—were found. The first one (detected in fragment F3) arises from a transition c.709G > A and originates a novel restriction site for *Nco*I. The other, corresponding to the transition c.1536G > A (detected in fragment F6) was already described by other authors as being associated with X-ALD in at least three other families [16–18]. Thus, it cannot be ascertained at this moment whether the R236H alteration (a conservative amino acid substitution) is a disease-causing mutation per se.

Using the RFLP analysis (see Table 2), we found both missense alterations in affected individuals of the same family. Sequencing of fragment F8 demonstrated the presence of two polymorphisms. One is located downstream of the STOP codon (c.2246G > C) and the other (c.2019C > T) in exon 8. This last neutral alteration was also detected in two out of 100 control alleles. In the proband from family 5 no ALDP could be detected by immunoblotting.

R545W. Since no fibroblasts from any of the two affected males from family 9 were available, a PCR/CSGE analysis [11] was performed using genomic DNA isolated from peripheral leukocytes. An alteration was found in exon 6. The transversion c.1633A > T identified originates a recognition site for the *Tsp*RI restriction enzyme. Mutational analysis of this family revealed the presence of this missense mutation in all affected members.

L655P. RT-PCR/CSGE analysis of the proband from family 11 revealed the existence of heteroduplex molecules in fragment F8. Sequencing of this cDNA fragment revealed a novel mutation: a transition c.1966T > C resulting in the change of leucine by a proline at codon 655. The polymorphism downstream of the STOP codon (c.2246G > C) was also found. The patient's mother was heterozygous for both alterations. Normal levels of the *ABCD*1 mRNA were found in this patient by Northern-blotting. ALDP levels, however, were decreased as assessed by immunoblotting.

H667L. Heteroduplex molecules were detected in cDNA fragment F2 of the proband from family 13. DNA sequencing revealed the existence of a transversion c.2002A > T that leads to the missense mutation H667L. Besides his daughter, shown to be heterozygous for this mutation, no other relatives could be analysed since no biological material was available. Decreased levels of ALDP were found in this patient by immunoblotting. Normal levels of the *ABCD*1 mRNA were detected by Northern-blotting.

Q574X. The transition c.1720C > T, resulting in a nonsense mutation at codon 574, was found in a patient from family 14 after RT-PCR/CSGE analysis and sequencing of cDNA fragment F6. Although another X-ALD patient is known in this family, no biological material from this or other relatives is available. As expected, no ALD protein could be detected in this patient by immunoblotting. However, normal levels of the *ABCD*1 mRNA were found after Northern-blotting analysis.

fs G298. RT-PCR/CSGE analysis of the proband from family 16, revealed the existence of heteroduplex molecules in the cDNA fragment F3. The sequencing data revealed a single G nucleotide deletion at position 895 (G895del) and a C to T transition in the adjacent base pair c.896C > T. This alteration eliminates a *Nla*IV restriction site. Due to the deletion, a frameshift mutation at amino acid G298 is originated. The patient's mother is heterozygous for both alterations. As expected, no ALD protein could be detected by immunoblotting, despite the fact that normal amounts of the *ABCD*1 mRNA were found by Northern-blotting.

F175del. Heteroduplex molecules displaying large electrophoretic shifts were detected in the CSGE analysis of cDNA fragment F2 of the proband from family 19. After sequencing, a three base pair deletion c.524–526delCTT was identified. Although this alteration affects two codons in the open reading frame of the *ABCD*1 transcript, the predicted consequence in the primary structure of the resulting protein is just a phenylalanine deletion at position 175—all the other amino acids in this one amino acid truncated version of ALDP remain unchanged. Normal levels of the *ABCD*1 transcript were detected by Northern-blotting analysis. Decreased levels of ALDP were found by immunoblotting.

In this work, the molecular characterization of the *ABCD*1 gene in 21 X-ALD families is presented. All but one of the mutations found (the AG dinucleotide deletion in exon 5 detected in families 17 and 18) were private to a family, illustrating the enormous genetic heterogeneity of X-ALD [1]. As already noticed in other X-ALD populations, the majority of the genetic alterations described here are missense mutations (14 out of 21), most of which result in lower steady-state levels of ALDP.

In the absence of data concerning the structure and function of ALDP, the effects of single amino acid alterations on the biochemical role performed by this protein are difficult to infer. In principle, any missense mutation leading to decreased levels of ALDP (but with no effect on the levels of the *ABCD*1 transcript) could interfere with the peroxisomal targeting mechanisms of the newly synthesized ALDP molecules, their correct membrane insertion or their correct folding. While most of the missense mutations presented here may interfere with any of these processes, it is highly unlikely that this is the case for the S108L, G266R, and R591W missense mutations. In these mutated cell lines normal levels of ALDP were found. Thus, it is possible that these amino acids play some important roles on the function of ALDP itself. Additional data will be necessary to prove this hypothesis.

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TRABALHO II

Characterisation of two mutations in the ABCD1 gene leading to low levels of normal ALDP

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ORIGINAL INVESTIGATION

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Characterisation of two mutations in the *ABCD1* gene leading to low levels of normal ALDP

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Abstract A variety of mutations have been identified in the X-linked adrenoleukodystrophy (X-ALD) gene, none of which is prevalent. In this work we describe a reverse transcription polymerase chain reaction (RT-PCR)-based strategy specially suited to the molecular characterisation of mutations in index cases. After RT-PCR amplification of the X-ALD transcript a conformation-sensitive gel electrophoresis analysis is performed followed by sequencing of the fragments with altered mobility. Two X-ALD patients were studied using this strategy. In both cases, splice site mutations were found. The first patient studied has a single base substitution at the first position of the invariant GT dinucleotide donor splice site of intron 8. In spite of this alteration, small quantities of correctly spliced mRNA molecules were easily detected. In agreement with these data, a small amount of ALDP was found by western blotting analysis. An alteration at the ˜1 position of the donor splice site of exon 1 was detected in the second patient. This mutation results in the utilisation of a cryptic 5 splice site within intron 1. Nevertheless, this transition also allows for some correct splicing. Western blotting analysis revealed the existence of normal-migrating ALDP. However, as expected, the levels of this protein were greatly decreased. Taken together, our data suggest that some less severe or late-onset forms of X-ALD associated with splice mutations result from the production of small amounts of normal ALDP. It is proposed that the quantification of ALDP levels in these patients could provide important insights concerning the correlation between clinical phenotype and amount of normal ALDP.

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Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100) is a neurodegenerative disorder characterised by progressive demyelination of the nervous white matter system and adrenal insufficiency (Moser et al. 1995). Great phenotypic variability can be found in this disease, even within the same kindred. The three most common phenotypes are childhood cerebral ALD (CCALD), adrenomyeloneuropathy (AMN) and Addison-only (AO). CCALD, the most devastating phenotype, is associated with a rapidly progressive cerebral inflammatory demyelinating reaction, with an onset before 10 years of age. Adrenomyeloneuropathy, which manifests itself after 20 years of age, is characterised by being slowly progressive with initial symptoms limited to the spinal cord and peripheral nerves (AMN pure). Eventually, cerebral involvement can develop (AMN cerebral). The AO form is associated with adrenal insufficiency without nervous system involvement (reviewed by Moser 1997).

Biochemically, the accumulation of very long chain fatty acids (VLCFAs) is the hallmark of all X-ALD forms. The pathogenic mechanisms linking this excess of VLCFAs to the clinical features remain unknown (Dubois-Dalcq et al. 1999).

In 1993, the X-ALD gene was identified using positional cloning strategies (Mosser et al. 1993). The gene (presently known as *ABCD1*) is 21 kb long and contains 10 exons. It encodes a peroxisomal transmembrane protein of 745 amino acids, ALDP, which belongs to subfamily D of the ATP-binding cassette (ABC) transporters superfamily (Mosser et al. 1994).

Mutations in the *ABCD1* gene have been identified in the majority of the X-ALD patients examined to date (listed at http://www.x-ald.nl). The data indicate that al-

most 60% of the X-ALD mutations are non-recurrent. Thus the identification of the mutation affecting a new family is always a demanding task. Several strategies have been developed in order to solve this problem. However, the major breakthrough in this field came from the work of Boehm et al. (1999). By optimising primer sequences and PCR conditions these authors were able to develop a method leading to the amplification of all exonic sequences of the *ABCD1* gene. However, by using genomic DNA as the starting material, this strategy is of limited value in the characterisation of splicing site mutations. These include not only the classical splice and branch site mutations but also, as described recently (Liu et al. 2001), many exonic mutations leading to exonic-splice enhancer inactivation.

In this work, we describe a method for determining the genetic alterations present in a new index case. The strategy consists in amplifying the *ABCD1* transcript by RT-PCR, followed by sequencing of the fragment(s) displaying abnormal migration upon conformation-sensitive gel electrophoresis (CSGE). Using this method we have identified the mutations present in all the X-ALD Portuguese families studied to date (in preparation). Here, we demonstrate the advantages of this technique over genomic DNA-based approaches by describing two splice site mutations associated with AMN phenotypes. Our findings indicate that expression of low levels of normal ALDP may account for some of the less severe or late-onset phenotypes observed in patients affected with splice site mutations.

Materials and methods

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from skin fibroblasts of male patients, using a total RNA isolation kit (Roche) according to the manufacturer s instructions. RT and PCR reactions were performed in a single tube using the Titan system (Roche), according to the protocol supplied by the manufacturer. In a typical reaction, 300 ng of total RNA template and 10 pmol of each primer were used. Eight partially overlapping fragments, containing the entire coding region of the *ABCD1* gene were amplified, using the primers listed in Table 1. The same RT-PCR conditions were used for all the amplifications: reverse transcription at 50 °C for 30 min, initial denaturation at 94 °C for 2 min; 10 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 45 s followed by 27 cycles with time extension increments of 5 s over each preceding cycle and a post-elongation step at 68 °C for 7 min. All reactions were carried out in a Unoll thermocycler (Biometra).

CSGE analysis

CSGE analysis was carried out in MDE gels (28×16.5×0.75 cm) prepared with 1× MDE standard solution (FMC BioProducts), 15% urea (w/v), 53.4 mM Tris (Merck), 9 mM taurine and 0.3 mM EDTA, pH 8.5. For heteroduplex formation, amplified products derived from affected and control individuals were mixed together in equal amounts (approximately 50 ng, as estimated by agarose gel electrophoresis in the presence of ethidium bromide), denatured at 95 °C for 3 min and gradually cooled down for 30 min to 37 °C. Aliquots of 2–3 µl of this mixture were added to 1 µl of loading buffer [40% (w/v) sucrose, 0.01% (w/v) each of bromophenol blue and xylene cyanol] and subjected to electrophoresis at a constant voltage of 600 V for 8 h at room temperature. The gels were stained with silver by a standard procedure (Budowle et al. 1991).

Immunoblotting analysis

Fibroblasts were cultured from skin biopsies in a standard Dulbecco s modified medium. Cells at 90% confluence were harvested with a cell scraper and centrifuged at 2000 *g* for 10 min at 4 °C. The cell pellet was resuspended in lysis buffer containing 0.3 M NaCl, 20 mM Tris-HCl pH 7.0, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0 and a protease inhibitor cocktail (Sigma #P8340) at a 1:450 (v/v) final dilution. The samples were sonicated three times for 15 s (with 30 s intervals on ice) using a Vibra Cell/Sonics & Materials (model VC 130) equipped with a microtip and set to a power of 40 W. After a clarifying spin at 2000 *g* for 10 min at 4 °C, the supernatants were centrifuged at 137000 *g* for 30 min at 4 °C in the 65.13 angular rotor (Sorvall). The pellets were then resuspended in 0.1% (w/v) Triton X-100 and the protein quantified by the Lowry method, using bovine albumin as standard (Lowry et al. 1951). Different amounts of protein from control individuals and patients were analysed by SDS-PAGE using 1.0-mm-thick, 10% polyacrylamide gels. Western-blotting onto nitrocellulose membranes was done as described by the manufacturer (Schleicher & Schuell).

The anti-ALDP monoclonal antibody 1D6 (Euromedex) and the anti-α subunit of the mitochondrial ATPase complex (F1α) (Molecular Probes) were used in western blots at 1:1000 (v/v) and 1:5000 (v/v) dilutions, respectively. The primary antibodies were detected either with a peroxidase-conjugated sheep anti-mouse antibody when the ECL system (Apbiotech) was used or with an alkaline-phosphatase-conjugated goat anti-mouse antibody when chromogenic substrates (Biorad) were used.

Table 1 Primers used for RT-PCR amplification and sequencing of the ABCD1 transcript (according to GenBank entry Z21876)

Fragment	Length (bp)	Forward primer sequences (5 →3)	Reverse primer sequences (5 →3)
F1	424	GGTCAGAGCAACAATCCTTCCA	GCCACATACACCGACAGGAAG
F2	469	AAGCTGGCATGAACCGGGTAT	AGGAGTGGCTTGGTCAGGTTG
F3	446	TACTACCGGGTCAGCAACATGG	ACATACCACAGGCGTTCCAGAA
F4	446	GTGGCCAACTCGGAGGAGATC	TTGAAGTGACAGCGCTGAACATC
F5	438	CACTGGCTACTCAGAGTCAGATGCA	CCTTCCTCCACCCTGATGTTGA
F6	415	CAGGTGGTGGATGTGGAACA	CGTCCTTCCAGTCACACATAGC
F7	387	TGATCTACCCGGACTCAGTGGAG	AGTCCAGCTTCTCGAACTTCCAG
F8	421	AAGTACGCCCTCCTGGATGAAT	TCCTTCATGTGATCCGAGCTTG

Miscellaneous

Genomic DNA was extracted from fibroblasts or peripheral leukocytes following a standard method (Miller et al. 1988). PCR reactions were performed as described previously by Boehm et al. (1999).

All fragments showing a conformational heteroduplex pattern in CSGE analysis were sequenced in both directions. The sequencing reactions were performed with the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer s instructions.

Northern blotting analysis was performed essentially as described in the Dig-Northern Starter kit technical bulletin of the manufacturer (Roche).

Immunofluorescence studies on cultured fibroblasts were performed as described previously (Watkins et al. 1995).

Gene mutation nomenclature used in this article follows the recommendations of Dunnen and Antonarakis (2001), and gene symbols follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001).

Results

Mutation screening strategy

The mutation screening strategy presented in this work may be divided into four steps: (1) cell culture of fibroblasts from index (male) patients; (2) RNA isolation and RT-PCR amplification of the *ABCD1* transcript; (3) DNA heteroduplex formation (achieved by mixing amplified cDNA fragments from the patient with the corresponding cDNA fragments from control individuals) followed by CSGE analysis; and (4) sequencing of the DNA fragment(s) displaying a mobility shift upon CSGE.

A diagram of the RT-PCR strategy, the critical step in the protocol described here, is shown in Fig. 1A. The complete coding region of the ALDP transcript, plus 89 bp from the 5 and 51 bp from the 3 untranslated regions, are amplified in eight RT-PCR reactions. The primers used in these reactions were selected considering several parameters. First, from the several pairs of primers tested in trial experiments only those providing high yields were selected. Second, primers were chosen so that fragments of 300˜500 bp were obtained. This length, besides allowing the sequence analysis of the entire fragment in only one sequencing reaction, represents the optimal size for a CSGE analysis (Ganguly et al. 1993), the next step in our mutation detection protocol. Third, since some single base mismatches within 50 bp of the ends of a DNA fragment may not result in a mobility shift upon CSGE analysis (Ganguly et al. 1993), primers were designed so that there was a minimum overlapping region of approximately 100 bp between all the amplified fragments. Obviously, this choice also ensures the detection of mutations located in primer annealing sequences, which otherwise would be missed by the introduction of the correct sequence by the PCR primers. Finally, all the primers had similar annealing

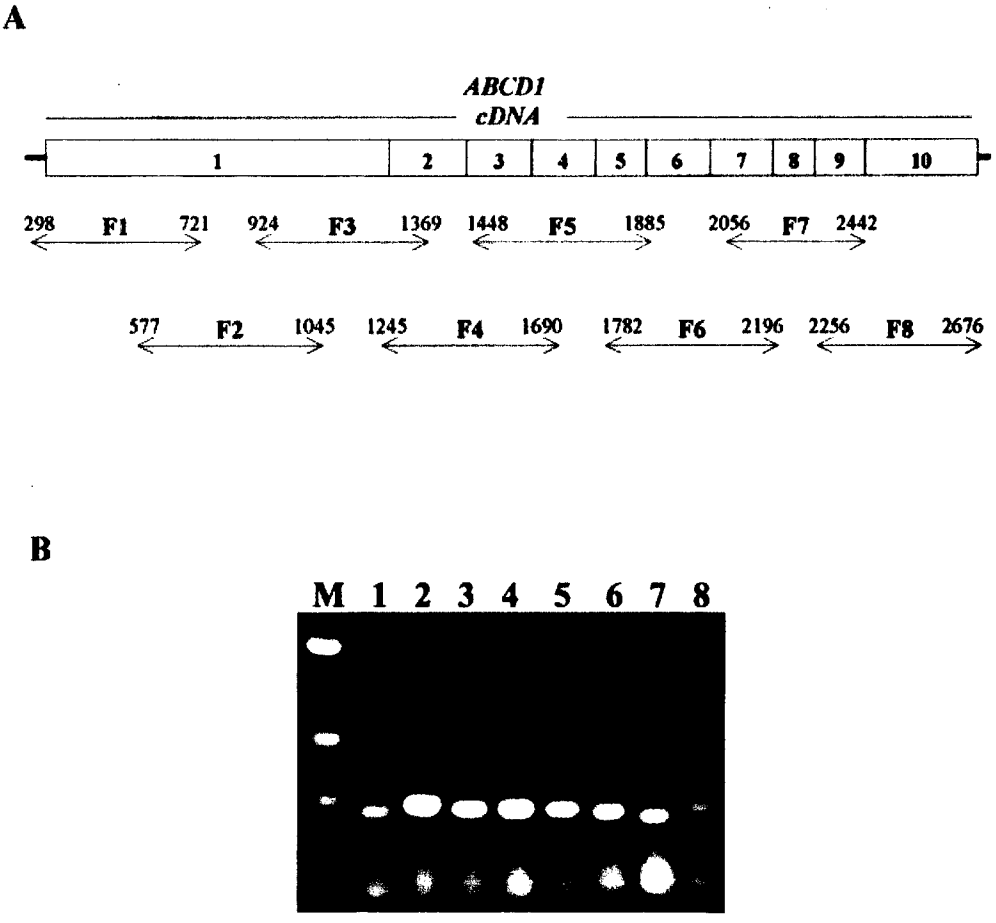

Fig. 1 **A** Schematic representation of the *ABCD1* cDNA. The *boxes* represent the 10 exons and the *arrows* indicate the eight amplified fragments (F1 to F8). The 5 position of the primers, in the cDNA sequence, is also indicated. **B** Agarose gel electrophoresis of RT-PCR amplified fragments F1 to F8 from a control individual (lanes *1* to *8*, respectively). Each lane contains 1/25 of the RT-PCR reaction. *M* indicates 0.1 μg of the DNA molecular weight marker 100 bp ladder (Roche)

temperatures, allowing amplification of the eight DNA fragments using a single thermal program.

The results of a typical RT-PCR amplification using total RNA isolated from a control fibroblast cell line are shown in Fig. 1B. The identity of each DNA fragment was confirmed by DNA sequencing.

Characterisation of the *ABCD1* gene mutation in patient A

Patient A is a 23-year-old man who developed normally until the age of 9 years, when he was diagnosed with Addison disease. At that time no neurological involvement could be observed. Five years later, he was biochemically diagnosed as an adrenoleukodystrophy patient (VLCFA levels were found to be increased in both plasma and skin fibroblasts). At the age of 21 years, muscular weakness and difficulty in walking led him to a new clinical evaluation: spastic paraparesis with neurophysiological abnormalities with an altered spinal cord MRI and a normal cerebral MRI were found. One and a half years later, the patient displayed a cerebral AMN sub-phenotype associated with an affected cerebellum. Currently the patient is in a vegetative state.

As a first step to characterising the mutation present in patient A, the levels of *ABCD1* mRNA in total RNA isolated from skin fibroblasts were analysed by northern blotting. In contrast with the results obtained with control cell lines, no hybridising band was detected in the RNA sample of patient A (data not shown), suggesting a problem in the synthesis and/or stability of the *ABCD1* transcript in this patient.

The *ABCD1* mRNA was then analysed by the RT-PCR strategy described above, which is a far more sensitive technique. All the RT-PCR amplifications resulted in normal-sized products, with exception of fragment F7. In this reaction several DNA fragments were consistently amplified (Fig. 2A), suggesting a splicing defect. When these DNA fragments were analysed by CSGE, multiple heteroduplexes were observed (data not shown), reinforcing this idea. In order to test this hypothesis and also as an attempt to define the mutation causing these events, all the DNA fragments obtained were purified and sequenced on both strands. Fragment A (Fig. 2A) corresponds to the normal 387 bp cDNA fragment ˇ no sequence alteration could be found in this molecule. Thus, at least a small fraction of the *ABCD1* transcript is correctly spliced in this patient. Fragment B is 300 bp long and derives from an exon-skipping event: exon 7 was spliced to exon 9. Clearly, the mutation present in patient A interferes with the efficiency of intron 8 removal. Sequence analysis of fragment C provided the explanation of this observation. In this 537 bp DNA fragment intron 8 was retained. A single base substitution was detected in this DNA molecule: a transition of guanosine to adenosine in the invariant GT dinucleotide within the 5 splice site of intron 8 (c.1867+1G>A).

The mutation found in patient A was also confirmed at the genomic DNA level using two different strategies. After amplification of the genomic region containing exon 8, intron 8 and exon 9 (ALDe8/9) from both patient A and his mother, using the protocol developed by Boehm et al. (1999), a RFLP analysis was performed with the *Hph*I restriction enzyme (the G to A transition eliminates a *Hph*I cleavage site). As expected, no restriction fragments were detected with patient s DNA sample; his mother was found to be heterozygous for the mutation using this criterion (data not shown). Finally, sequencing analysis of the genomic amplicon from patient A corroborated the mutation data described above.

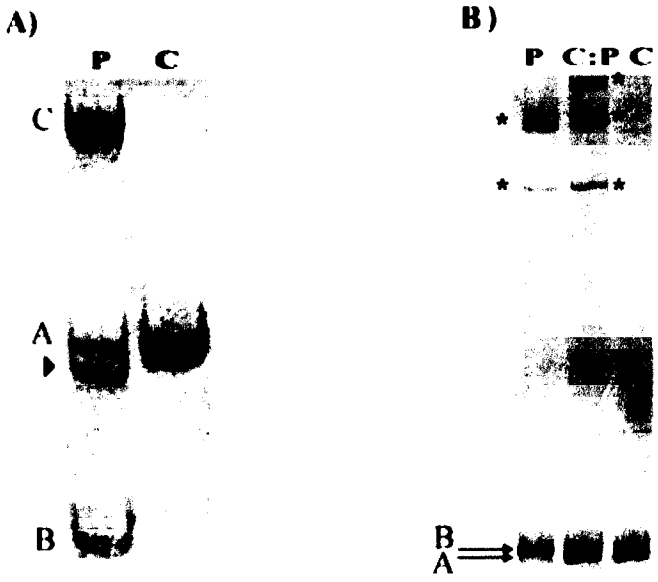

Fig. 2 **A** Silver-stained non-denaturing 5% polyacrylamide gel showing the amplified product F7 derived from patient A (lane *P*) and from a control individual (lane *C*). Bands *A*, *B* and *C* represent the normal, the exon 8 skipped and the intron 8 retained amplified molecules, respectively. The *black triangle* indicates a non-specific amplification product corresponding to a fragment of desmoplakin cDNA (GenBank entry NM_004415). The amplification of this DNA fragment, which has never been detected in other X-ALD patients studied in our laboratory (unpublished results), is probably the result of the extremely low levels of *ABCD1* transcript present in this patient. **B** CSGE analysis of fragment F3 from patient B. *C* control, *C:P* mixture control:patient, *P* patient (for details see Materials and methods). Heteroduplex bands are indicated with *asterisks*. Bands *A* and *B* represent the two *ABCD1* transcripts detected in patient B

Although the mutation detected in this patient breaks the so-called GT-AG rule of nuclear RNA splicing (reviewed by Madhani and Guthrie 1994), our data strongly suggest that some correctly spliced *ABCD1* transcripts are present in patient A s cells. In order to prove this hypothesis, the presence of ALDP in an enriched membrane protein fraction obtained from the patient s cultured skin fibroblasts (see Materials and methods) was assessed by semi-quantitative western blotting analysis. As shown in Fig. 3 (lane 10), a weak signal corresponding to ALDP is visible when 70 µg of this protein fraction are analysed. Densitometric analysis of the blot shown in (Fig. 3) indicates that the levels of ALDP in patient A correspond to 0.4% of control values.

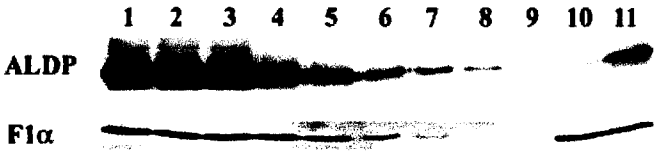

Fig. 3 An enriched protein membrane fraction obtained from cultured skin fibroblasts was analysed by immunoblotting using the antibodies directed to ALDP and to F1α subunit of the mitochondrial ATPase complex. In order to quantify the ALDP present in the patients studied, a calibration curve was done by mixing equal amounts of protein derived from three different control cell lines (25 μg of each in lanes *1*, *2* and *3*). Lanes *4* to *9* correspond to decreasing amounts of the mixture (10, 5, 2, 1, 0.5 and 0.25 μg, respectively). Lanes *10* and *11* correspond to 70 μg of protein derived from the cultured cells of patients A and B, respectively

Characterisation of the *ABCD1* gene mutation in patient B

Patient B is a 44-year-old man displaying an AMN pure sub-phenotype of X-ALD. He was diagnosed with Addison disease at the age of 22 years. Ten years later a spastic paraparesis was manifested and the biochemical diagnosis (increased VLCFA levels in plasma and fibroblasts) established him as an X-ALD patient.

In order to characterise the mutation affecting this patient, we analysed the *ABCD1* mRNA using the RT-PCR strategy described above. Agarose gel electrophoresis of the amplification products revealed the presence of all eight cDNA fragments displaying the expected size. Thus, a CSGE analysis was performed. Heteroduplex DNA molecules were detected only in fragment 3 (Fig. 2B) and fragment 4 (not shown), suggesting an alteration in the overlapping region of these two fragments (Fig. 1A).

Interestingly, several heteroduplex DNA molecules were detected when a mixture of patient and control DNA was analysed (Fig. 2B, lane C:P*f*). However, two of these molecules were already detectable when the DNA fragments F3 and F4, of patient B alone (i.e. without mixing with the corresponding fragments obtained from control individuals) were analysed (Fig. 2B, lane P*f*). Furthermore, in addition to these two bands, representing heteroduplex DNA molecules, two homoduplex DNA bands (see below) were also detected in this lane. These results are compatible with the existence in patient B of two different RNA populations, neither of which has the normal sequence.

Sequencing analysis of the two homoduplex DNA fragments observed in patient B (obtained after excising the bands from a non-denaturing polyacrylamide gel) confirmed this interpretation. The faster-migrating DNA fragment (band A in Fig. 2B) has the normal sequence with only one exception ˇ a transition of a guanosine to adenosine at the ˇ1 position of the donor splice site of exon 1 (c.900G>A). This single base mutation does not result in an amino acid exchange (codon 300 GAG is changed to GAA, both coding for glutamic acid). Thus, this population of *ABCD1* transcripts encodes the normal ALDP protein.

The c.900G>A transition was also found in the second DNA fragment sequenced (band B in Fig. 2B). However, in this case an additional alteration was found: the first nine nucleotides of intron 1 (GTGGGGCAG) were retained in this molecule. This incorrect splicing event derives from the activation of a non-consensus donor splice site at position +10 of intron 1. The result of this alteration is the insertion of three codons (encoding Val-Gly-Gln) at position 300 in the translation reading frame of this incorrectly spliced *ABCD1* transcript. It remains to be determined whether this extended version of ALDP is functional and/or stable in vivo (see also Discussion).

The mutation found in this patient was analysed using the genomic DNA-based approach of Boehm et al. (1999) (data not shown). Only the c.900G>A transition was found, validating all the RT-PCR-based mutation data described above.

The existence of ALDP in cultured fibroblasts from patient B was assessed using semi-quantitative western blotting analysis. The results presented (Fig. 3, lane 11; see legend for details) indicate the existence of normal-migrating ALDP in these cells. However, the levels of ALDP in this patient are clearly reduced, being only 7% of the control values.

Discussion

In this work we describe a RT-PCR-based approach for the characterisation of mutations in the *ABCD1* gene. The strategy is intended to speed up the identification and characterisation of mutations in new index (male) patients clinically and biochemically diagnosed as X-ALD. In this statement we do not include just splice site mutations ˇ it is obvious that only a transcript analysis can shed some light on the consequences of this kind of alteration. As discussed by Liu et al. (2001) many exonic single point mutations (missense mutations included) also lead to splicing defects by inactivating exonic splicing enhancers. Furthermore, the prevalence of this kind of splicing mutation in human genetic diseases may be greater than that associated with mutations disrupting the splice and branch sites (Liu et al. 2001). Thus, using a RT-PCR-based approach in the characterisation of mutations seems to be the right strategy. However, it should be emphasised that RT-PCR-based approaches are not suitable for the determination of the carrier status. For this purpose only genomic DNA-based approaches should be used, as discussed by Boehm et al. (1999).

The effects of the splice site mutation found in patient A are quite striking (Fig. 4A). Although the mutation identified in the 5 splice site of intron 8 (c.1867+1G>A) breaks the so-called GT-AG rule, some correctly spliced transcripts were detected in our analysis. It should be said that this result was obtained with two independent skin biopsy samples (one collected at the age of 14 years and the other collected 9 years later) and that particular care was taken to avoid cross-contamination during RNA isolation and RT-PCR. The fact that a small amount of normal ALDP could be detected by western blotting analysis strongly suggests that, indeed, some correctly spliced

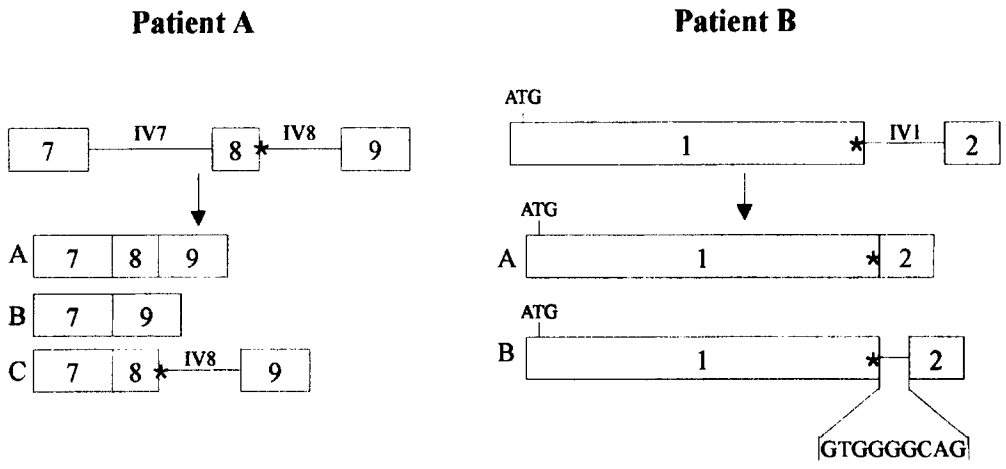

Fig. 4 Schematic representation of the mRNA populations present in the two patients studied. The *boxes* and *lines* represent the exons and introns, respectively. The *asterisks* indicate the mutations found. Letters *A*, *B* and *C* correspond to the cDNA fragments detected by RT-PCR (see also Fig. 2)

ABCD1 transcripts are produced in patient A's cells. In this respect, patient A may not be the only example of this phenomenon. Indeed, two out of three known X-ALD patients affected with similar mutations [G to A transitions at the +1 position of the donor splice sites of intron 5 (Lira et al. 2000), intron 6 (Fanen et al. 1994) and intron 7 (Feigenbaum et al. 1996)] presented late-onset forms of the disease. Although no data concerning the *ABCD1* transcript or ALDP levels were provided in those studies, it is possible that some correct splicing does occur when this type of mutation is present in introns 5 and 6.

The splice site mutation found in patient B (a G to A transition at the ˇ1 position of the donor splice site of exon 1) also leads to a severe alteration in the splicing process of the *ABCD1* transcript (Fig. 4B). Indeed, the most abundant transcript detected in this patient derives from a splicing event in which the first 9 bases of intron 1 are retained. However, in spite of this alteration correctly spliced RNA molecules were also detected. Thus, some fully functional ALDP protein must be produced in the cells of this patient. Western blot analysis revealed the existence of normally migrating ALDP protein corresponding to approximately 7% of the amount found in controls. However, since the abnormally spliced *ABCD1* transcript has the potential to encode a protein of similar size to ALDP (an extended version of ALDP with an insertion of Val-Gly-Gln at position 300), we cannot exclude the possibility that the signal observed upon western-blotting analysis represents a mixture of two polypeptides. Thus, the value of 7% concerning the levels of immunoreactive ALDP in patient B fibroblasts should be regarded as a maximum value of normal ALDP.

Taken together, our results suggest that some less severe or late-onset forms of X-ALD associated with splice mutations may result from the production of small amounts of fully functional ALDP. Our data also point to the necessity of expressing the ALDP levels found in these patients as percentages of control values. The dichotomous classification presently used by most authors to characterise the presence of ALDP in X-ALD patients is too simple to be informative. The fact that both patients characterised in this study were ALDP-negative when their cultured skin fibroblasts were analysed by immunofluorescence using a commercially available anti-ALDP antibody (data not shown; see Materials and methods) illustrates this point.

In the absence of structural/functional data concerning ALDP, the effects of missense mutations on the clinical phenotype are always difficult to predict. On the other hand, some splicing mutations do not abolish completely the production of normal ALDP. As shown here, this may be true even for the less expected cases such as the GT to AT donor site splicing mutation found in patient A. A complete characterisation of these mutations, and in particular the determination of ALDP levels in these cells, should provide data concerning the correlation of levels of ALDP versus clinical phenotype. Obviously, such information is of great importance in any future attempt to treat this disease by gene therapy.

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TRABALHO III

Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo

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Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo

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Abstract

ALDP, ALDPR, PMP70 and PMP70R are half ATP-binding cassette (ABC) transporters of the mammalian peroxisomal membrane. By analogy with other members of this family, it is assumed that peroxisomal ABC transporters must dimerize to become functional units. However, not much is known regarding the type of dimers (i.e., homodimers and/or heterodimers) that are formed in vivo under normal expression conditions. In this work, we have characterized the quaternary structure of mouse liver PMP70 and ALDP. The PMP70 protein complex was purified to apparent homogeneity using a two-step purification protocol. The ALDP-containing protein complex was characterized by preparative immunoprecipitation experiments. In both cases, no evidence for the existence of heteromeric interactions or for the presence of accessory proteins in these ABC transporter protein complexes could be obtained. Our data indicate that the majority (if not all) of mouse liver PMP70 and ALDP are homomeric proteins.

Keywords: Half ABC transporter; Peroxisome; Adrenoleukodystrophy; X-ALD; Membrane protein purification

1. Introduction

The ATP-binding cassette transporters (ABC transporters) represent one of the largest families of proteins, with 48 ABC transporters already identified in human. These proteins are molecular pumps that couple ATP hydrolysis to the transport of substrates (e.g., cholesterol, bile salts, polypeptides, iron) across biological membranes (reviewed in Ref. [1]). Structurally, the members of this family have two transmembrane domains, each comprising several α-helices, and two conserved nucleotide binding domains (reviewed in Ref. [2]). In eukaryotes, the ABC proteins are classified either as full transporters, if this core of four domains is encoded by only one gene (e.g., CFTR), or as half-transporters, if the corresponding genes encode only one transmembrane domain and one nucleotide binding domain (e.g., Tap1 and Tap2). The latter class of proteins becomes functional only after dimerization (reviewed in Ref. [3]).

In mammals, four half ABC transporters are present in the peroxisomal membrane: the adrenoleukodystrophy protein (ALDP or *ABCD1* [4]), the ALDP-related protein (ALDPR or *ABCD2* [5]), the peroxisomal membrane protein of 70 kDa (PMP70 or *ABCD3* [6]) and the PMP70-related protein (PMP70R or *ABCD4* [7,8]). By analogy with other members of the half ABC transporter family (e.g., Tap1 and Tap2), it has been assumed for long that these peroxisomal transporters must dimerize to become functional units.

Abbreviations: ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; ALDPR, ALDP-related protein; PMP70, peroxisomal membrane protein of 70 kDa; PMP70R, PMP70-related protein; X-ALD, X-linked adrenoleukodystrophy; ESI-MS, electrospray ionization mass spectrometry

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Whether dimerization results in the formation of homodimers only or if heterodimers can also be formed is an issue that did not receive much attention up to now. In fact, although all these peroxisomal proteins were identified several years ago, only three studies have addressed this problem. In one of these studies, yeast two-hybrid experiments together with Western blotting analysis of immunoprecipitates obtained from cultured cells overexpressing peroxisomal transporters led to the idea that ALDP, ALDPR and PMP70 are quite promiscuous entities capable of engaging in both homo- and heterodimerization processes [9]. However, due to the nature of the experimental techniques used in that study, it is not evident what kind of interactions prevails in vivo (i.e., under normal expression conditions of the peroxisomal transporters). Exactly the same remark can be made regarding the in vitro experiments described by Smith et al. [10]. In that work, a co-immunoprecipitation assay employing in vitro synthesized proteins was used to identify protein–protein interactions involving ALDP, PMP70 and ALDPR. Finally, in the third study addressing this issue, purified peroxisomes from rat liver were used in immunoprecipitation experiments. The authors were able to demonstrate co-immunoprecipitation of ALDP with PMP70 [11]. However, several other peroxisomal proteins were also found in the immunoprecipitates. Thus, it is not clear whether the PMP70–ALDP interaction is direct or indirect.

Defining the kind of protein interactions in which peroxisomal transporters are involved in vivo is an important task having major implications on our knowledge on the biochemistry of the mammalian peroxisome. If it is assumed that peroxisomal transporters exist in the peroxisomal membrane solely as homodimers, then only four different substrates (or families of substrates) are expected to be transported by these proteins. If, however, peroxisomal transporters are promiscuous entities capable of interacting with each other, then 10 different combinations of transporters can be formed raising the possibility that 10 different substrates (or families of substrates) use these transporters to cross the peroxisomal membrane. In the particular case of ALDP, it is likely that such information is of fundamental importance to understand the pathogenesis mechanism in X-linked adrenoleukodystrophy (X-ALD), a human genetic disease caused by mutations in the ALDP gene (reviewed in Ref. [12]) and that affects 1:21,000–1:100,000 males [13,14].

Although the exact function of ALDP is not presently known, it has been proposed that it transports very long chain fatty acids (VLCFAs) into the peroxisomal compartment or some factor necessary for the peroxisomal β-oxidation of these compounds [4,15]. This conclusion derives from the fact that X-ALD patients accumulate VLCFAs in many organs (liver included [16]) and body fluids (reviewed in Ref. [12]), a biochemical alteration that was also observed in several ALD knockout mice [16–18]. However, the correlation between accumulation of VLCFAs and clinical phenotype in X-ALD is far from being clear [19], raising the possibility that other (still unknown) biochemical factors contribute to the pathology. To define the biochemical role of ALDP, it is of the utmost importance to know the kind of protein–protein interactions in which ALDP is involved in vivo. If ALDP is a homodimeric protein, then only one primary biochemical defect should occur in the absence of a functional protein. If, however, ALDP is capable of forming homo- and heterodimers, then it is plausible to assume that the metabolism of more than one type of substrates will be affected by the absence of functional ALDP.

In this work, we have characterized the quaternary structure of mouse liver PMP70 and ALDP. After solubilization of peroxisomal proteins using the mild detergent digitonin, a simple two-step procedure was used to purify PMP70. The ALDP protein complex was characterized by preparative immunoprecipitation experiments. Our results indicate that both half ABC transporters exist in the peroxisomal membrane predominantly (if not exclusively) as homomeric proteins, probably dimers.

2. Materials and methods

2.1. Purification of mouse liver peroxisomes

Peroxisomes were isolated from the liver of C57/BL6 male mice (1–3 months of age) by differential centrifugation and purified through a Nycodenz gradient essentially as described [20,21]. The Nycodenz gradients (6 ml of 30% and 2 ml of 25% (w/v) Nycodenz in 5 mM imidazole–HCl, pH 7.4 and 1 mM EDTA–NaOH, pH 7.4) were centrifuged in the fixed-angle rotor T1270 (Sorvall, Ultra Pro80 centrifuge) at 60 000 × *g* for 30 min. Peroxisomes obtained in this way were estimated to be 90% pure presenting a minor contamination with mitochondria and endoplasmic reticulum.

2.2. Solubilization of peroxisomal proteins

In a typical experiment, 1 mg of purified peroxisomes were incubated in 500 μl of solubilization buffer (see bellow) for 30 min at 4 °C with gentle agitation. Protein samples were centrifuged at 105 000 × *g* for 1 h at 4 °C using the rotor T1270. To determine yields of solubilization, equivalent portions of supernatants and pellets were subjected to Western blotting analysis using antibodies directed to mouse peroxisomal ABC transporters. Good solubilization yields (approximately 80%) were obtained when using a buffer (hereafter referred to as buffer A) containing 1% (w/v) digitonin [added from a 5% (w/v) stock solution prepared as suggested by the manufacturer (Calbiochem)], 50 mM Tris–acetic acid, pH 7.5, 0.1 M potassium acetate-acetic acid, pH 7.4, 0.25 M 6-amino-caproic acid, 1 mM EDTA–NaOH, pH 7.4, 1:500 (v/v)

protease inhibitor cocktail (Sigma P8340) and 0.1 mg/ml phenylmethylsulfonyl fluoride.

2.3. Sucrose density gradient analysis

Detergent-solubilized proteins (2 mg of protein in 1 ml of buffer A) were loaded onto the top of a discontinuous sucrose gradient [2 ml of 10%, 1.8 ml of 15.5%, 1.7 ml of 21%, 1.5 ml of 25%, 1.2 ml of 30%, 1.0 ml of 35% and 0.3 ml of 40% (w/v) sucrose in a buffer containing 50 mM Tris–acetic acid, pH 7.5, 10 mM potassium acetate–acetic acid, pH 7.4, 1 mM EDTA–NaOH pH 7.4 and 0.1% (w/v) digitonin]. In the experiment shown in Fig. 1, centrifugation of the sucrose gradients was performed in the swinging bucket rotor TST 41.14 rotor (Sorvall, OTD75B centrifuge) at 160000 × *g* for 16 h at 4 °C. In Fig. 2, the sucrose gradient was centrifuged at 180000 × *g* for 16 h at 4 °C using the swinging bucket rotor TH-641 (Sorvall, Ultra Pro80 centrifuge). Twelve fractions of 875 μl were collected from the bottom of the tube and subjected to precipitation with 10% (w/v) trichloroacetic acid.

Treatment with urea of digitonin-solubilized peroxisomal proteins was done as follows: 200 μg of purified peroxisomes were incubated with 50 μl of buffer A for 15 min at 4 °C with gentle agitation. After adding 25 μl of 9 M urea (final concentration of 3 M), protein samples were incubated 15 min at room temperature, diluted with 925 μl of buffer A and loaded onto the top of a sucrose gradient.

2.4. Purification of mouse liver PMP70 complex

The purification protocol was started with a pellet of 3.3 mg of purified peroxisomes. The organelles were solubilized for 30 min at 4 °C, in 550 μl of buffer B (buffer A without EDTA, plus 2 mM $MgCl_2$ and 0.5 mM $MnCl_2$). The sample was subjected to centrifugation at 105000 × *g* for 1 h at 4 °C to remove insoluble material. The supernatant was added to 150 μl (bed volume) of ATP-agarose (C-8 attachment; Sigma) previously equilibrated in buffer C (buffer B containing 0.1% (w/v) digitonin). After incubation for 1 h at 4 °C in an end-over-end shaker, the ATP-agarose slurry was washed twice with 1.5 ml of buffer C supplemented with 5 mM $MgCl_2$ (final concentration). The proteins were then eluted with 600 μl of buffer C containing 10 mM $MgCl_2$ and 10 mM ATP for 1 h at 4 °C with occasional shaking. Eluted proteins were loaded onto the top of a sucrose gradient (rotor TH-641), centrifuged and fractionated, as described above.

2.5. Immunoprecipitation of mouse liver ALDP

Two milligrams of purified peroxisomes were solubilized in 2 ml of buffer A containing 5 mM iodoacetamide for 30 min at 4 °C and subjected to centrifugation (105000 × *g* for 1 h at 4 °C) to remove insoluble material. One aliquot (220 μl) of the supernatant (sample "T") was kept on ice. The remaining supernatant was halved. Each half was added to

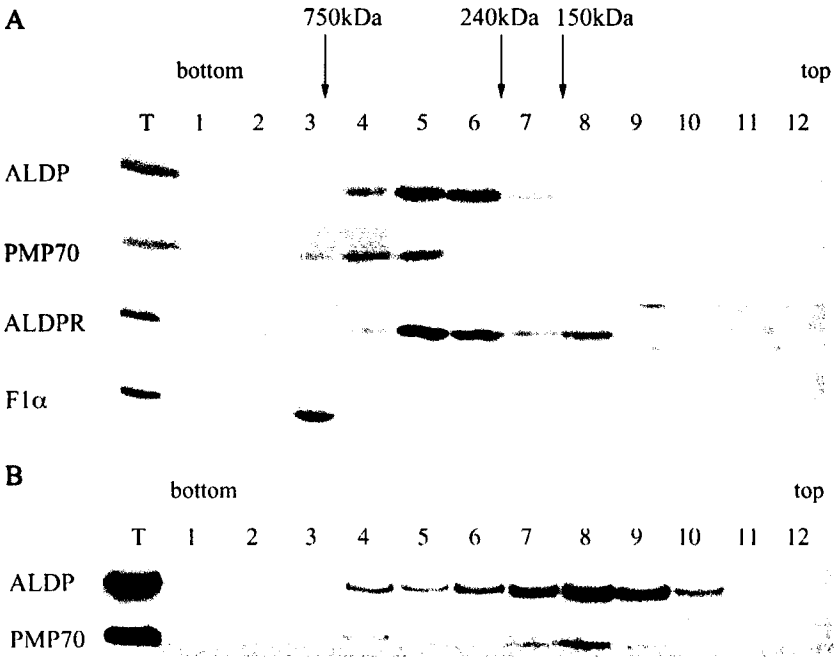
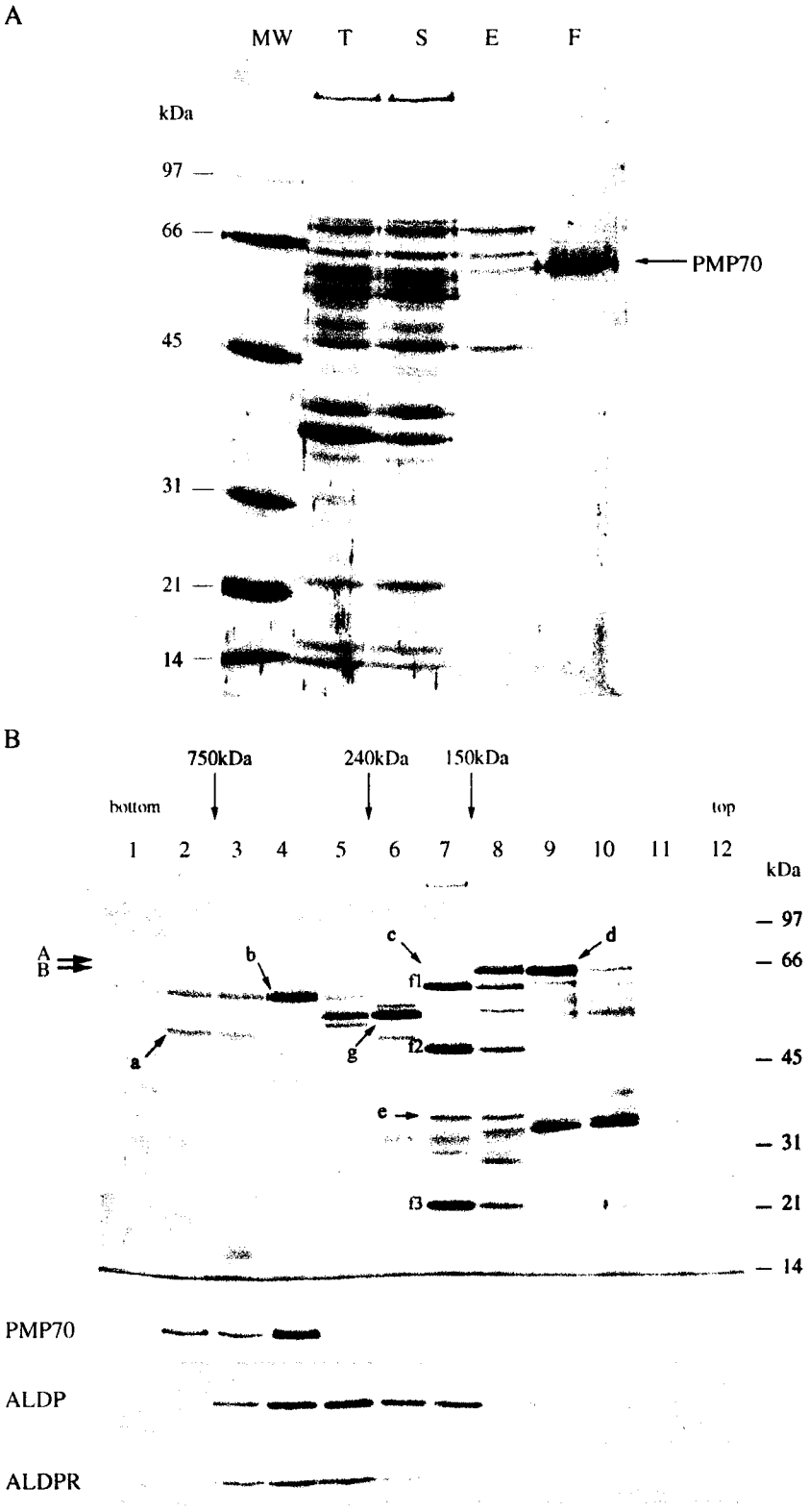


Fig. 1. Sedimentation analysis of digitonin-solubilized mouse liver ALDP, PMP70 and ALDPR. (A) Digitonin-solubilized peroxisomal proteins were loaded onto the top of a sucrose gradient. After centrifugation, the gradient was fractionated starting from the bottom (lane 1) to the top (lane 12). Equivalent portions of each fraction (corresponding to 210 μg of peroxisomal protein) were precipitated with trichloroacetic acid and analyzed by immunoblotting using antibodies directed to ALDP, PMP70, ALDPR and the α subunit (F1α) of the mitochondrial ATPase complex (molecular mass of 750 kDa). The positions of catalase (240 kDa) and palmitoyl-CoA oxidase (150 kDa) in the gradient are also indicated. Lane T, 70 μg of peroxisomal protein. (B) Digitonin-solubilized peroxisomal proteins (200 μg) were incubated in the presence of 3 M urea, subjected to sucrose gradient centrifugation and analyzed as described above.

12 mg of CNBr-activated Sepharose 4B beads that had been previously coupled with anti-ALDP or pre-immune immunoglobulins G (IgGs; see below). The samples were incubated for 3 h at 4 °C, in an end-over-end shaker. The beads were collected by centrifugation (10000 × *g* for 10 s) and the supernatants (samples "S") were removed and kept on ice. After washing the beads four times with 1 ml of buffer A containing 0.1% (w/v) digitonin, immunoprecipitated proteins were eluted with 150 μl of Laemmli sample buffer [22] at 85 °C for 10 min. Fifteen microliters of 1 M dithiothreitol were subsequently added to the eluate. Samples "T" and "S" were subjected to



precipitation with trichloroacetic acid before SDS-PAGE analysis.

2.6. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS of tryptic digests from protein-containing polyacrylamide gel slices was carried out using a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). Samples were digested as described [23] and analyzed by HPLC-MS using a Discovery Bio Wide Pore C18, 5 mm (15 cm × 0.32 mm) HPLC column (Supelco, Bellefonte, USA). Proteins were identified by search with peptide-mass fingerprinting data from mass spectrometry on Protein Prospector [24].

2.7. Miscellaneous

Proteins were quantified by the Lowry method, using albumin as standard [25]. SDS-PAGE analysis was performed in 0.75-mm-thick, 11% polyacrylamide gels using the Laemmli discontinuous buffer system [22]. Silver staining of polyacrylamide gels was performed as described [26]. Western blotting onto nitrocellulose membranes was done as described by the manufacturer (Schleicher & Schuell). The polyclonal antibodies directed to mouse ALDP (serum 1664 [27]) and ALDPR (serum 7373 [28]) were used in immunoblotting analysis at 1:1000 (v/v) dilution. The rabbit antibody directed to rat PMP70 (Zymed Laboratories) and the mouse anti-α subunit of the mitochondrial ATPase complex (Molecular Probes) were used in immunoblotting analysis at 1:2000 (v/v) dilution. The primary antibodies were detected with a horseradish peroxidase conjugated donkey antirabbit or sheep antimouse antibodies (Amersham Biosciences) using ECL super signal West-dura substrates (Pierce).

Control and antimouse ALDP IgGs were isolated using protein A-Sepharose according to the manufacturer's instructions (Amersham Biosciences). The isolated IgGs were coupled to CNBr-activated Sepharose 4B beads at a ratio of 200 μg of protein per 12 mg of resin following the manufacturer's recommendations (Amersham Biosciences).

Densitometric analysis of Western blots was performed using the UN-SCAN-IT automated digitizing system.

3. Results

3.1. Mouse liver PMP70, ALDP and ALDPR behave as large protein complexes upon solubilization with digitonin

As a first step to characterize the quaternary structure of peroxisomal half ABC transporters, a preliminary set of experiments was performed to find the best solubilization conditions. These experiments were carried out taking into consideration two variables: (1) high yield in the solubilization procedure, so that the extracted protein population is representative of the population that exists in vivo and (2) mildness of the solubilization step to avoid disrupting protein–protein interactions. The first variable was quantified by densitometric analysis of Western blots containing known amounts of total peroxisomal proteins and the corresponding solubilized protein fractions; yields of 80% were routinely obtained (compare lanes Per and T in Fig. 3). The second variable was empirically assessed by subjecting solubilized proteins to centrifugation sedimentation analysis (see below).

For practical reasons, related to antibody availability and facility in obtaining highly purified peroxisomal fractions, these studies were performed with mouse liver proteins. The results of one of these experiments are shown in Fig. 1A. After solubilization of purified mouse liver peroxisomes using the mild detergent digitonin, PMP70, ALDP and ALDPR were found as large protein complexes (apparent molecular mass of 300–400 kDa) upon sucrose gradient centrifugation. Considering that all these proteins have a predicted molecular mass around 70 kDa, the estimated molecular masses may appear too large even if we assume that all these proteins are indeed in a dimeric state. However, it should be noted that the sedimentation properties of a solubilized membrane protein also depend on its partial specific volume, shape and amount and type of protein-bound detergents/membrane lipids. Thus, no conclusions regarding the true size of the observed protein complexes can be inferred from these experiments. In fact, by the same line of reasoning, it could be argued that the solubilized proteins are in the monomeric state. However, this is clearly not the case as shown by the following experiment. Digitonin-solubilized peroxisomal proteins were incubated in the

Fig. 2. Isolation of the PMP70-containing protein complex. (A) Digitonin-solubilized peroxisomal proteins (lane S, derived from 2 μg of starting material) were subjected to an ATP-agarose affinity chromatography. Eluted proteins (lane E, derived from 8 μg of peroxisomal protein) were loaded onto a sucrose gradient and centrifuged. The PMP70-enriched fraction (fraction 4 of the sucrose gradient; see below) was selected (lane F; derived from 80 μg of peroxisomal protein). Lane T, 2 μg of peroxisomal protein. Lane MW, molecular mass markers. A silver-stained polyacrylamide gel is shown. (B) ATP-agarose affinity chromatography eluted proteins (derived from 250 μg of peroxisomal proteins) were subjected to sedimentation analysis. The upper panel shows a silver-stained gel and the lower panel a Western blotting using the PMP70, ALDP and ALDPR antibodies. The identity of several proteins was determined by ESI-MS (see also Table 1): a—mitochondrial ATP-synthase α-chain, a subunit of the 750 kDa complex V [34]; b—PMP70; c—peroxisomal D-bifunctional protein, a homodimer of 154 kDa [35]; d—peroxisomal L-bifunctional protein, a 79-kDa protein monomeric in its native state [36]; e—urate oxidase (probably the trimeric form [37]). Some other proteins can be easily identified considering their abundance, molecular mass and subunit composition: f1, f2 and f3—72, 52 and 21 kDa subunits of palmitoyl-CoA oxidase, respectively, a dimeric protein with a native molecular mass of 150 kDa [38]; g—catalase, a tetrameric complex of 240 kDa [39]. Arrows A and B on the left indicate the expected migration positions of ALDP and ALDPR, respectively. The positions of the molecular mass standards (kDa) are indicated.

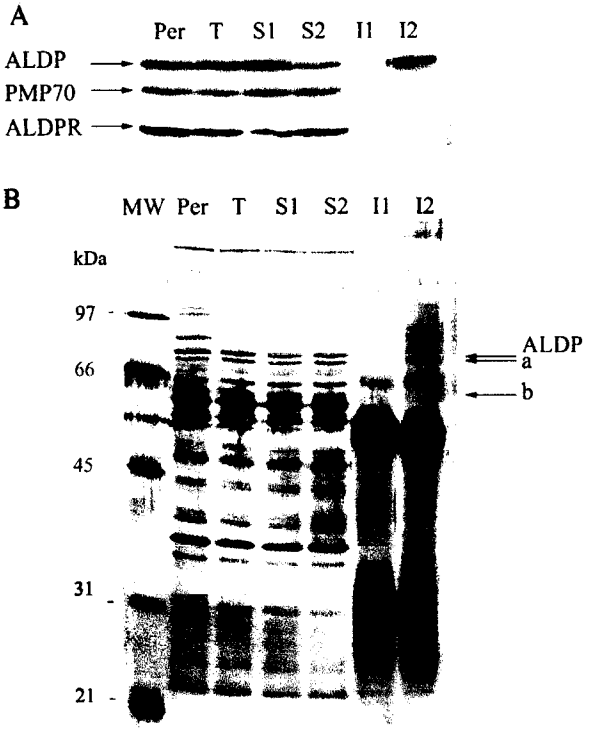


Fig. 3. Characterization of the ALDP-containing protein complex. Digitonin-solubilized peroxisomal proteins (lane T) were incubated with CNBr-activated Sepharose 4B beads previously coupled to control or anti-ALDP IgGs (see Materials and methods). After centrifugation, the supernatants were recovered, the beads were washed and the immunoprecipitated proteins were eluted with Laemmli sample buffer. Lanes S1 and I1: supernatant and immunoprecipitated proteins, respectively, obtained with control IgGs. Lanes S2 and I2: supernatant and immunoprecipitated proteins obtained with anti-ALDP IgGs, respectively. The protein samples were subjected to Western blotting using antibodies directed to ALDP, ALDPR and PMP70 (panel A) or analyzed by SDS-PAGE followed by silver staining (panel B). In panel A, protein loading was the following: lanes T, S1 and S2—proteins derived from 70 μg of peroxisomal protein; lanes I1 and I2—immunoprecipitated proteins derived from 140 μg of peroxisomal protein; lane Per: 70 μg of peroxisomal protein. In panel B, aliquots derived from 2 μg of peroxisomal protein were loaded in lanes T, S1 and S2. Lanes I1 and I2 contain immunoprecipitated proteins from 140 μg of peroxisomal protein. Lane Per—2 μg of peroxisomal proteins. Arrows a and b indicate the expected migration positions of ALDPR and PMP70, respectively. Molecular mass markers are shown on the left-hand lane (lane MW).

presence of urea, a chaotropic agent frequently used to disrupt protein–protein interactions. After diluting the solution with digitonin-containing buffer (see Materials and methods), the protein sample was subjected to sedimentation analysis. Although a fraction of several peroxisomal proteins precipitated after this treatment and were found at the bottom of the centrifuge tube (data not shown), a small amount of PMP70 and ALDP remained soluble. Two different populations of PMP70 were detected in this experiment (see Fig. 1B): a minor fraction of PMP70 resisted to the urea treatment in an apparently intact conformation (fraction 4 of the gradient); the majority of soluble PMP70, however, was found in fractions 7–8 of the sucrose gradient (apparent molecular mass of 100–150 kDa). Similar results were obtained for ALDP, although in this case the vast majority of the protein was detected in fraction 7–8 of the sucrose gradient (see Fig. 1B). These data taken together indicate that under the solubilization conditions employed, peroxisomal transporters are indeed components of protein complexes.

3.2. Purification of mouse liver PMP70

As shown above, digitonin-solubilized PMP70, ALDP and ALDPR behave as 300–400 kDa protein complexes upon centrifugation sedimentation analysis. Such apparent molecular mass could be explained by assuming that these proteins are dimers that bind large amounts of detergent (see Discussion). Alternatively, this behavior could reflect the existence of larger protein complexes comprising, in addition to the transporters, other proteins. Examples of ABC transporter protein complexes containing accessory proteins are abundant (e.g., tapasin and the TAP transporter of mammals [29]) or the maltose binding protein and the ABC maltose transporter of *Escherichia coli*; reviewed in Ref. [30]).

To clarify this issue, the PMP70-containing protein complex was purified. For this purpose, a simple two-step procedure was developed. In the first step, digitonin-solubilized peroxisomal proteins were subjected to an ATP-agarose affinity chromatography (see Materials and methods for details). Western blotting analysis revealed that more than 90% of the amount of PMP70 applied to the affinity matrix was recovered after this step (data not shown). Interestingly, several other proteins were specifically eluted from the ATP-agarose matrix (see Figs. 2A and B and Table 1). Although some of these proteins may indeed bind ATP (this is surely the case for mitochondrial complex V; reviewed in Ref. [31]), the majority of the proteins identified bind adenine-containing molecules (e.g., NADP, FAD, NAD), but not ATP itself (see legend to Fig. 2). In the second step of the purification procedure, the high sedimen-

Table 1
Identification of ATP-agarose affinity chromatography purified proteins using ESI-MS

Protein band	Protein name	MW (Da)	Mean error (ppm)	Sequence coverage (%)	MOWSE score[a]
A	ATP synthase alpha chain	59,753	8.9	32.0	1.216e + 008
B	PMP70	75,483	− 15.1	19.0	4.619e + 007
C	Peroxisomal D-bifunctional enzyme	79,525	− 3.25	18.0	1.928e + 007
D	Peroxisomal L-bifunctional enzyme	78,244	0.534	22.0	1.896e + 007
E	Urate oxidase	35,039	14.0	32.0	5.289e + 006

[a] The MOWSE score reported by MS-Fit is based on the scoring system described in Ref. [40].

tation coefficient of digitonin-solubilized PMP70 was explored. As shown in Fig. 2A, a homogeneous PMP70 preparation was obtained: no stoichiometric amounts of other proteins co-purifying with PMP70 could be detected upon SDS-PAGE analysis. Furthermore, ESI-MS analysis of the protein band containing PMP70 failed to reveal the presence of any protein other than PMP70 itself. We conclude that the majority of mouse liver PMP70 is a homomeric protein.

It may be interesting to note that high amounts of PMP70 (5 μg/mg of total peroxisomal protein) can be easily obtained using the method described here. Biochemical experiments to further characterize the structure/function of this protein are under progress.

3.3. Characterization of the mouse liver ALDP protein complex

In rat liver, ALDP and ALDPR are much less abundant proteins than PMP70 [11]. This is probably also the case for mouse liver ALDP and ALDPR. Indeed, although these two mouse proteins are also retained in an ATP-agarose matrix, as revealed by Western blot analysis (see Fig. 2B), no protein band corresponding to either of these proteins can be detected in the polyacrylamide gels shown in Fig. 2. Thus, to characterize the quaternary structure of ALDP, a different strategy was used. Digitonin-solubilized mouse liver peroxisomes were subjected to an immunoprecipitation experiment using the anti-ALDP antibody. Immunoprecipitated proteins were then analyzed by Western blotting using antibodies directed to ALDP, PMP70 and ALDPR. As shown in Fig. 3A, about 80% of the ALDP protein was immunoprecipitated by the anti-ALDP antibody. Most importantly, neither PMP70 nor ALDPR could be detected in the immunoprecipitate.

To corroborate these observations, the immunoprecipitated proteins were also subjected to SDS-PAGE and stained with silver. As shown in Fig. 3B, besides antibody-derived protein bands, only one protein band displaying exactly the electrophoretic behavior of ALDP is visible in the immunoprecipitate obtained with the anti-ALDP antibody. Taken together, these data strongly suggest that the majority of mouse liver ALDP (if not all) is a homomeric protein assembly.

4. Discussion

The most critical step in any protocol aiming at the purification of a membrane protein resides in the solubilization procedure. Solubilization conditions have to ensure high yields in the extraction of the protein(s) under study, and, if the aim is to isolate protein complexes, they have to be gentle enough so that protein–protein interactions are preserved. When determining the best solubilization conditions for a given protein it is important to define the variable "solubility". In general terms, a soluble protein should remain in the supernatant after centrifugation for 1 h at $105\,000 \times g$ or should elute in the included volume from a gel filtration medium with very large pores such as Sepharose 4B [32]. In this work, we used the former criterion. Unfortunately, these considerations are sometimes overlooked and it is not uncommon to find in the literature experiments involving membrane proteins in which the detergent extract was just subjected to a low speed centrifugation (e.g., 5 min at $15\,000 \times g$) before immunoprecipitation. The risk associated with this type of procedure is high: If the protein under study is not completely in solution, then it is likely that other insoluble proteins will be co-immunoprecipitated not because they are truly associated with the protein of interest but because they reside in the same membrane fragments. This is probably the reason why the data reported here are so different from recent results suggesting that rat liver PMP70 and ALDP are components of the same protein assembly [11].

The aim of the work presented here was to determine the type of protein–protein interactions in which peroxisomal half ABC transporters are involved in vivo. In particular, we were interested in assessing whether these transporter proteins can engage in heteromeric interactions with each other and/or with other unrelated proteins under normal expression conditions. It should be noted that data suggesting that all these peroxisomal ABC transporters interact with each other have already been described. Indeed, pairwise interactions involving PMP70, ALDP and ALPR were observed when using a combination of yeast two-hybrid assays and Western blotting analysis of immunoprecipitates obtained from cultured cells overexpressing epitope-tagged peroxisomal transporters [9]. While these results suggest that these proteins have the potential to interact with each other forming both homo- and heterodimers, the nonquantitative nature of these techniques does not allow us to infer which kind of interactions (if any) prevail in vivo.

Considering the unexpectedly high apparent molecular mass of digitonin-solubilized peroxisomal ABC transporters (300–400 kDa), we started our studies by addressing the possibility that these transporters interact with other nonrelated proteins. For this purpose, we isolated chemical amounts of the PMP70-containing complex. Our results clearly show that no other protein in stoichiometric amounts was co-purified with PMP70. Thus, the strikingly high sedimentation coefficient of PMP70 (and of the other two ABC transporters characterized in this work) is an intrinsic property of these digitonin-solubilized proteins. What is the reason for such high sedimentation coefficients? The most plausible explanation is to assume that all these proteins are dimers (approximate molecular masses of 140 kDa) that bind an amount of detergent that equals 100–150% of their masses (140–210 kDa). Detergent/protein ratios of 1:1.5 (w/w) are quite common for solubilized hydrophobic proteins (e.g., Ref. [33]). The fact that the majority of urea-treated PMP70 and ALDP (presumably representing mono-

meric protein) display an apparent molecular mass of 100–150 kDa supports this interpretation. However, it has to be emphasized that further experiments are necessary to confirm this hypothesis. Thus, at this moment, other possibilities (e.g., a homotetrameric structure), although unlikely, cannot be formally excluded.

To assess whether peroxisomal ABC transporters are capable of forming heterodimers, we focused our attention on ALDP. To characterize the quaternary structure of this protein, we performed immunoprecipitation experiments using mouse liver peroxisomes. Western blotting analysis clearly show that neither PMP70 nor ALDPR could be co-immunoprecipitated with ALDP. Silver staining of a polyacrylamide gel containing the immunoprecipitated proteins confirmed this observation: No protein band other than the one corresponding to ALDP could be observed. Thus, the vast majority (if not all) of mouse liver ALDP is a homomeric protein.

Due to the fact that antibodies directed to mouse PMP70R are not yet available, the properties of this protein regarding its native molecular mass and interactions with the other three peroxisomal half ABC transporters could not be assessed in this work. Although there are no data whatsoever to support the existence of heteromeric interactions involving PMP70R, our data do not allow us to exclude this possibility. Indeed, PMP70R is much less abundant than PMP70 and ALDP (less than 1/70 and 1/10, respectively [11]) and thus, the presence of PMP70R in our silver-stained gels would not be noted. Further work is necessary to clarify this issue.

In conclusion, our results indicate that mouse liver PMP70 and ALDP are mostly (if not exclusively) homomeric protein assemblies. Determining whether this observation can be extended to other mouse organs and to other organisms, humans in particular, is of main importance to understand the pathophysiology of X-ALD.

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TRABALHO IV

Probing substrate-induced conformational alterations in ALDP by proteolysis

Probing Substrate-Induced Conformational Alterations in ALDP by Proteolysis

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Abstract

The adrenoleukodystrophy protein (ALDP) is a half-ABC (ATP-binding cassette) transporter localized in the peroxisomal membrane. Dysfunction of this protein is the cause of the human genetic disorder X-linked adrenoleukodystrophy (X-ALD), which is characterized by the accumulation of saturated very long-chain fatty acids (VLCFAs). This observation suggests that ALDP is involved in the metabolism of these compounds. Whether ALDP transports VLCFA or their derivatives across the peroxisomal membrane or some cofactor essential for the efficient peroxisomal β-oxidation of these fatty acids is still unknown. In this work, we used a protease-based approach to search for substrate-induced conformational alterations on ALDP. Our results suggest that ALDP is directly involved in the transport of long- and very long-chain acyl-CoAs across the peroxisomal membrane.

Introduction

X-linked adrenoleukodystrophy (X-ALD) is the most common inherited peroxisomal disorder with a minimum incidence of 1:21000 in the male population [1]. It is a progressive neurodegenerative disease affecting mainly the nervous system white matter and the adrenal cortex. The clinical presentation is extremely variable and, frequently, different phenotypes occur in the same family. Common to all the X-

ALD phenotypes, however, is the accumulation of saturated unbranched very long-chain fatty acids (VLCFA) in plasma and tissues of the patients [2].

The gene responsible for X-ALD (*ABCD*1 gene) was identified in 1993 [3]. It encodes a half ATP-binding cassette (ABC) transporter, the so-called ALDP (or *ABCD*1). Half ABC-transporters are homo or heterodimeric protein assemblies that couple ATP hydrolysis to the transport of substrates across a biological membrane [4]. Data suggesting that ALDP from mouse liver is a homodimeric protein were recently described (see discussion in [5]).

Several hypotheses have been forwarded to explain how dysfunction of ALDP leads to accumulation of VLCFA in X-ALD (e.g., [3, 6]). However, the possibility that ALDP may play a direct role in the metabolism of these fatty acids is still the most plausible (e.g., [7-10]). The observations supporting this hypothesis are not limited to the fact that β-oxidation of VLCFAs is a peroxisomal event [11] and that ALDP is a peroxisomal membrane protein [12]. Indeed, a variety of genetic and biochemical studies on ALDP-related peroxisomal ABC-transporters from other organisms have led to the same conclusion. For example, disruption of Pxa1p and/or Pxa2p, the only two peroxisomal ABC-transporters known in *Saccharomyces cerevisiae*, results in an impaired growth of these mutants on long-chain fatty acids as the sole carbon source [13-15]. Biochemical characterization of these proteins demonstrated their involvement in the transport of acyl-CoAs across the peroxisomal membrane [14, 16]. More recently, a similar role was proposed for the Comatose/Ped3 gene product of *Arabidopsis thaliana* [17, 18]. Obviously, all these observations do not prove that ALDP has a similar role in the metabolism of VLCFAs, but they do provide a ground of testable hypotheses.

If it is assumed that ALDP is directly involved in the β-oxidation of VLCFAs and if we consider that the sub-cellular compartment where these fatty acids are activated to their CoA thioesters is presently unknown (for a review see [2]), then there are at least three different mechanisms leading to the same outcome: ALDP may be directly

involved in the peroxisomal import of: (1) free VLCFAs, (2) very long-chain acyl-CoAs or (3) a cofactor necessary for the β-oxidation of VLCFAs to occur. In this work, we have addressed the first two possibilities. Our results suggest that ALDP is involved in the translocation of long- and very long-chain acyl-CoAs across the peroxisomal membrane.

Materials and Methods

Materials

Factor Xa protease was obtained from New England Biolabs (#P8010, lot 55). Acetil-CoA and dodecanoyl-CoA were purchased from Roche and Fluka, respectively. Docosanoyl-CoA and tetracosanoyl-CoA were synthesized by American Radiolabeled Chemicals. All the other reagents were from Sigma.

Subcellular fractionation of placenta

A human placenta was obtained from the local maternity. An informed written consent was granted. The tissue was chilled on ice immediately after-birth and transported to the laboratory. The following procedures were carried out at 4ºC. The placenta was washed with SEI buffer (0.25 M sucrose, 1 mM EDTA-NaOH, pH 7.4 and 5 mM imidazole-HCl, pH 7.4) and the adherent membranes were cut off. A portion of approximately 60 g was cut into small pieces and extensively washed in SEI buffer. The tissue was homogenized using a polytron (model PT10/35, Kinematica, Switzerland) in four volumes of SEI buffer supplemented with 0.1 mg/ml PMSF and a mammalian protease inhibitor cocktail (Sigma) at a 1:450 (v/v) final dilution. An organelle fraction was prepared by differential centrifugation. Briefly, the homogenate was centrifuged at 300 x g for 10 min at 4ºC in the SS-34 rotor (Sorvall, model RC-5B PLUS) and afterwards at 3200 x g for 10 min. Finally, the organelle fraction was obtained by centrifugation at 20000 x g for 20 min and resuspended in SEI buffer. Aliquots (20-30 mg/ml) were frozen immediately in liquid nitrogen and stored at -70ºC.

Protease treatment and extraction of membrane proteins

An aliquot of 0.45 mg of placenta organelle protein was incubated at 26ºC for one hour with 9 µg Factor Xa in 0.9 ml of a buffer containing 0.22 M sucrose, 10 mM

Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and 10 mM $MgCl_2$. The protease was inhibited with 0.1 mg/ml PMSF (added from a 50 mg/ml stock solution in ethanol) and 1:200 (v/v) mammalian protease inhibitor cocktail for 5 min on ice. Extraction of membranes with alkaline or low and high ionic strength solutions were performed essentially as described [19, 20]. Briefly, aliquots of 300 µl were diluted to 1.5 ml with 0.16 M sodium carbonate (freshly made) or with a buffer containing 20 mM Tris-HCl, pH 7.0, 1 mM EDTA-NaOH, pH 7.4, 1 mM EGTA-NaOH, pH 8.0, 0.1 mg/ml PMSF and 1:200 (v/v) mammalian protease inhibitor cocktail supplemented or not with 0.67 M NaCl. The samples were sonicated twice for 30 sec each, using a Heat Systems/Ultrasonics sonicator (model W-375) equipped with a microtip and set to a power of 40 W, 50% duty cycle, output 2. After 30 min of incubation on ice the samples were halved. One half was kept on ice for determination of recoveries and the other was subjected to centrifugation at 137000 x g for 1 h at 4ºC in the fixed-angle rotor T1270 (Sorvall, UltraPro 80). Proteins from the total and soluble fractions were precipitated with TCA (10% (v/v) final concentration) and processed for western-blotting analysis.

In vitro synthesis of radiolabeled ALDP and cleavage with Factor Xa

The cDNA encoding the human full-length ALDP transporter was amplified by RT-PCR using the F1 and F8 oligonucleotides exactly as described previously [21] and cloned into the pGEM-T Easy vector (Promega). Subsequently, the cDNA was inserted into the *Eco*RI site of pGEM-4 (Promega). Clones with ALDP cDNA in the correct orientation for further *in vitro* transcription with the Sp6 RNA polymerase were selected. The plasmid was linearized with the *Hind*III endonuclease and transcribed according to the manufacturer's instructions (Roche). ^{35}S-labeled ALDP was synthesized using the translation kit Reticulocyte Type II (Roche) in the presence of ^{35}S-methionine (specific activity >1000Ci/mmol; ICN Biomedicals).

One µl of a reticulocyte lysate containing ^{35}S-labeled ALDP was added to 60 µl of buffer containing 0.25 M sucrose, 40 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$ and 0.2 M NaCl. Then, Factor Xa was added and the reaction extended over 1.30 h at 26ºC.

Preparation of lipids

Stock solutions of acyl-CoA esters were prepared at a final concentration of 0.3 mM in $CHCl_3$:CH_3OH (1:1). These were dried under a stream of nitrogen and resuspended with α-cyclodextrin (10 mg/ml in 20 mM Tris-HCl, pH 8.0) in order to obtain 1 mM final concentration lipid. The suspensions were sonicated for 1 h at room temperature in an ultrasonic water bath (Sonorex super RK255H, Bandelin). After

checking the pH, single-use aliquots were stored at -70ºC. It should be referred that all efforts to solubilize docosanoyl-CoA and tetracosanoyl-CoA in aqueous buffer (e.g., by increasing the ratio of α-cyclodextrin/lipid, increasing the solubilization temperature, extending the sonication time or lowering the lipid concentration to half) were ineffective. Suspensions of these two lipids were stored in aliquots at -70ºC.

In the experiment presented in Fig. 2C, dodecanoic and eicosanoic acids were added directly from a 6.5 mM stock solution in methanol. All the other samples received the same volume of methanol.

Factor Xa cleavage assay

The Factor Xa cleavage assays (60 µl final volume) were performed as follows: 45 µg of placenta organelle protein were incubated in cleavage buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 µM oligomycin (prepared as 10 mM stock solution in ethanol), 0.22 M sucrose, 1 mM ouabain and 0.1 % (v/v) Triton X-100) containing, where indicated, 10 mM nucleotides (added from 100 mM stock solutions, pH 7.5 adjusted with NaOH) and/or 200 µM lipids. After 10 min at 26ºC, Factor Xa (2 µg) was added and the incubation was continued for 45 min at the same temperature. The reaction was stopped with trichloroacetic acid (TCA) and the samples were analyzed by western-blotting.

The intrinsic Factor Xa protease activity, in the presence of dodecanoyl-CoA, eicosanoyl-CoA, ATP, ATPγS, dodecanoyl-CoA plus ATP or ATPγS, and eicosanoyl-CoA plus ATP or ATPγS, was assayed against a chromogenic substrate (Sigma F3301). Factor Xa protease (6 ng) was incubated with 10 mM nucleotides and/or 200 µM acyl-CoAs esters in a final volume of 120 µl cleavage buffer (see above) for 5 minutes at room temperature. The Factor Xa substrate (0.2 mM) was added and the reaction was followed spectrometrically at 405 nm over 30 minutes using a kinetic microplate reader (Sunrise,Tecan, Switzerland). Under these conditions, hydrolysis of the Factor Xa substrate was linear over this period (data not shown).

Miscellaneous

Protein quantification, SDS-PAGE and western-blotting were performed as described before [5]. The anti-ALDP monoclonal antibody (clone 1D6; Euromedex) and the rabbit anti-human ALDP [22] (a kind gift of Prof. Paul Watkins, Kennedy Krieger Institute, Baltimore, USA) were used in the western-blotting analysis at 1:1000 (v/v) dilution. For detection, either chemiluminescent (Super Signal West Dura, Pierce) or

chromogenic substrates (Biorad) were used. Densitometric analysis of western-blots was performed using the UN-SCAN-IT automated digitizing program.

Results and Discussion

Rationale and principle of the method

ABC-transporters are molecular pumps that couple ATP hydrolysis to the vectorial transport of molecules across a biological membrane. Although our knowledge on the mechanism by which this process occurs is still limited, data indicating that these proteins oscillate between different conformations during the catalytic cycle are frequent in the literature (for a review see [23]). The nature and amplitude of these conformational alterations is still a matter of debate. Nevertheless, at least in some cases, a simple protease-based assay can easily reveal such structural rearrangements [24-26]. In this work, we have used this strategy in order to gather data on the type of substrate transported by ALDP. Our premise was that in the presence of the correct substrate, the conformation of ALDP would change and consequently a different susceptibility to proteolysis should be observed.

Thus, our first task was to search for a protease that could allow us to detect substrate-induced conformational alterations on ALDP. In a preliminary set of experiments, a human placenta organelle fraction (see material and methods) was incubated in the presence of either non-esterified VLCFA or the corresponding CoA esters and treated with increasing concentrations of several proteases (data not shown). The behavior of ALDP was monitored by western-blotting analysis using two antibodies directed to different domains of the protein (see below). From the several proteases tested, Factor Xa proved to be the most promising. The results obtained with this protease are described below.

Characterization of the proteolytic pattern of ALDP after Factor Xa cleavage

The characterization of the proteolytic profile of ALDP obtained after Factor Xa cleavage in the absence of exogenous substrates is presented in Fig. 1. After digestion, two different ALDP fragments can be detected: one of these fragments displays an apparent molecular mass of approximately 44 kDa upon SDS-PAGE and is recognized by the monoclonal antibody 1D6 produced against residues 279-482 of the protein [12]; the other fragment displays 33 kDa and is detected by the polyclonal antibody directed to the last 18 carboxy-terminal amino acids of human ALDP [22] (see Fig. 1A).

In order to characterize further these two ALDP fragments, their interaction with the peroxisomal membrane was studied. For this purpose, Factor Xa-treated organelles were subjected to alkaline extraction or were sonicated in the presence of low or high ionic strength buffers. Membrane fractions and extracted (soluble) proteins were then prepared by centrifugation and analyzed by western-blotting. As shown in Fig. 1B, the 44 kDa ALDP fragment is detected in all the membrane fractions, implying that this domain of ALDP is intrinsic to the membrane. In contrast, the 33 kDa fragment displays the properties of an extrinsic membrane protein, since it is readily extracted from the membranes at alkaline pH but not by sonication of the organelles in the presence of low or high ionic strength saline buffers. These results could suggest that, at the concentration used in these assays, Factor Xa cleaves ALDP into two main fragments: one corresponding to the membrane-embedded N-terminal half of ALDP (the 44 kDa fragment) and the other comprising the C-terminal nucleotide binding domain of the protein (the 33 kDa fragment). Indeed, data indicating the existence of a preferred (but not unique) Factor Xa cleavage site in the central region of ALDP was obtained when the ^{35}S-labeled *in vitro* synthesized ALDP was incubated in the presence of the protease. As shown in Fig. 1C, although several ALDP-derived polypeptides can be obtained by treatment with Factor Xa, two major proteolytic

products migrating exactly as the 44 and 33 kDa fragments described above are easily detected (bands a1 and a2, respectively).

It should be noted that the exact Factor Xa cleavage sites on ALDP cannot be located simply using primary structure-based considerations. It is well established that Factor Xa hydrolyses peptide bonds with high efficiency at the carboxyl-side of arginine residues, particularly if these residues are preceded by a glycine. However, as shown recently, several other amino acid residues at the penultimate position of the cleavage site are also allowed [27].

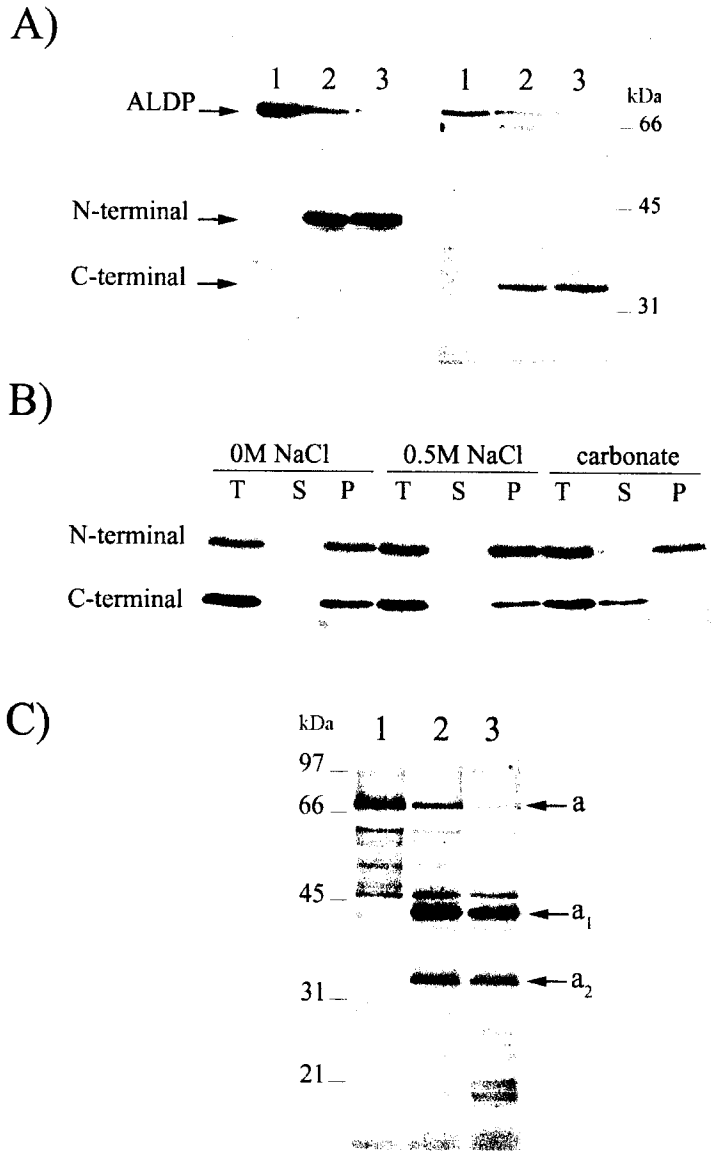

FIG. 1 – Characterization of the proteolytic pattern of ALDP after Factor Xa cleavage
A) Placenta organelles (45 µg of protein) were incubated at 26°C for 45 min in the absence (lane 1) or in the presence of Factor Xa, at final concentrations of 5 and 25 µg/ml (lanes 2 and 3, respectively). After TCA precipitation, the samples were analyzed by SDS-PAGE followed by western-blotting using two different anti-ALDP antibodies: the monoclonal 1D6 (left panel) and a polyclonal directed to the last 18 C-terminal amino acids (right panel). This polyclonal antibody presents a weak cross-reactivity with albumin (faint band at 66kDa). The 44 kDa (N-terminal) and the 33 kDa (C-terminal) ALDP fragments are indicated. Molecular mass markers (kDa) are shown on the right side of the figure. **B)** A placenta organelle fraction, previously digested with Factor Xa, was sonicated in low (0 M NaCl) or high (0.5 M NaCl) ionic buffers or in 0.12 M Na_2CO_3 (carbonate). The samples were halved. One-half was kept on ice as a control (lanes T) and the other half was separated, by centrifugation, into membrane (lanes P) and soluble fractions (lanes S). After TCA precipitation, equivalents portions of each fraction (corresponding to 50 µg of the initial protein) were subjected to SDS-PAGE and analyzed as described above. **C)** ^{35}S-labelled ALDP was incubated at 26°C for 45 min in the absence (lane 1) or in the presence of 5 or 25 µg/ml (final concentration) of Factor Xa (lanes 2 to 3, respectively). The samples were analyzed by SDS-PAGE followed by autoradiography. Full-length ALDP and the two most prominent proteolytic fragments are indicated by arrows a, a_1 and a_2, respectively. The positions of the molecular mass standards are indicated on the left (kDa).

Long- and very long-chain acyl-CoAs increase the sensitivity of the N-terminal domain of ALDP to proteolysis by Factor Xa.

As shown above, treatment of ALDP with Factor Xa results in the production of two major fragments. Virtually the same result is obtained when the protease assay is performed in cleavage buffer (see materials and methods) in the presence of 200 μM reduced CoA acetyl-CoA, octanoyl-CoA or dodecanoyl-CoA (Fig. 2A; compare lanes 2-5 with lane 1). In sharp contrast, when the same concentration of hexadecanoyl-CoA, eicosanoyl-CoA, docosanoyl-CoA or tetracosanoyl-CoA are used in these assays, the amount of the ALDP 44 kDa N-terminal fragment that resists to the protease is highly decreased (Fig. 2A; compare lanes 6-9 with lanes 1-5). No significant variations in the amount of the 33 kDa ALDP C-terminal fragment were detected under the different conditions used in this work (see Fig. 2A; data not shown).

Densitometric analysis of the band corresponding to the 44 kDa ALDP fragment reveals that long- and very long-chain acyl-CoAs decrease the amount of Factor Xa-resistant fragment with different efficiencies: eicosanoyl-CoA > hexadecanoyl-CoA ≈ docosanoyl-CoA > tetracosanoyl-CoA. While the relationship between the effects of eicosanoyl-CoA and hexadecanoyl-CoA is not surprising (the majority of hexadecanoyl-CoA is oxidized in mitochondria *in vivo* [28]), the milder effects observed with tetracosanoyl-CoA were not expected. In fact, this is one of the fatty acyl groups that accumulate in X-ALD patients [29]. Thus, if ALDP is involved in the translocation of very long-chain acyl-CoAs across the peroxisomal membrane (the hypothesis that is being tested here) a larger effect should be observed in the experiment presented in Fig. 2A. It should be noted, however, that several problems were faced when handling docosanoyl-CoA and tetracosanoyl-CoA. In spite of several attempts (see materials and methods) we were unable to obtain true solutions of these compounds. Thus, the real concentrations of these two acyl-CoAs in the protease assay may be lower than intended. In the experiments described below, only

acyl-CoAs presenting the required solubility were used (i.e., CoA thioesters containing acyl groups with 20 or less carbon atoms).

The effects of different concentrations of dodecanoyl-, hexadecanoyl- and eicosanoyl-CoA on the cleavage of the N-terminal fragment of ALDP by Factor Xa were also assessed. As shown in Fig. 2B, a significant increase in the proteolysis of this ALDP domain is already observed at 25 μM eicosanoyl-CoA. At this concentration, hexadecanoyl-CoA promotes only a minor decrease in the amount of Factor Xa-resistant ALDP fragment. Dodecanoyl-CoA has no effect at all concentrations tested, as expected.

Finally, when these protease assays are performed in the presence of 200 μM free eicosanoic or dodecanoic fatty acids, no alteration on the sensitivity of the 44 kDa ALDP fragment to the action of Factor Xa is detectable (see Fig. 2C). Thus, the observed phenomenon is directly related to the presence of acyl-CoAs in the protease assay and not to the corresponding free fatty acids, which, in fact, could be generated in our assays by hydrolysis of the added lipids (e.g, from the action of endogenous thioesterases [30]).

It should be emphasized that the true concentration of free (unbound) lipids in our assays is not known. In vivo, the total intracellular concentration of acyl-CoAs has been reported to be in the range 5-160 μM [31]. However, under these conditions most acyl-CoAs are bound to several proteins (e.g., FABP [31]). The concentration of these lipid-binding proteins in our assays is surely not sufficient to sequester the exogenously added substrates. Nevertheless, our cleavage buffer does contain the lipid-binding reagent, α-cyclodextrin. Thus, the concentration of free lipids in our reactions should also be below their total assay concentration.

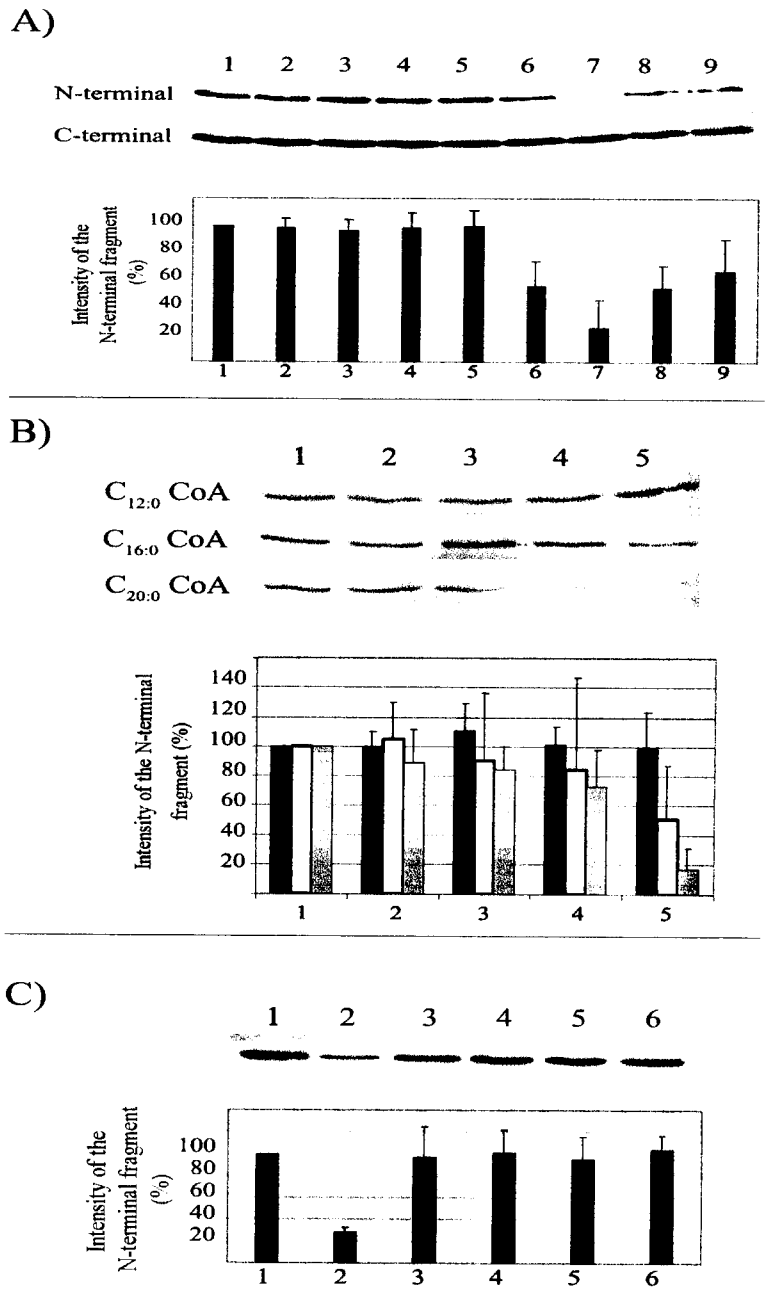

FIG. 2 – Effect of acyl-CoAs esters and free fatty acids on Factor Xa cleavage of ALDP
A) Aliquots of placenta organelles (45 µg of protein) were incubated in cleavage buffer containing 10 mM ATP (lane 1) or 10 mM ATP plus 200 µM of one of the following substances: reduced CoA (lane 2), acetyl-CoA (lane 3), octanoyl-CoA (lane 4), dodecanoyl-CoA (lane 5), hexadecanoyl-CoA (lane 6), eicosanoyl-CoA (lane 7), docosanoyl-CoA (lane 8) or tetracosanoyl-CoA (lane 9). After cleavage with Factor Xa, proteins were analyzed by western-blotting using the monoclonal antibody 1D6 (N-terminal) and the polyclonal antibody directed to the last 18 C-terminal amino acids (C-terminal). A densitometric analysis of the results obtained with the monoclonal antibody 1D6 in three independent experiments is shown (black bars). Values were normalized to the control reaction (lane 1), which was set to 100%. Standard deviations are also shown. **B)** Placenta organelles (45 µg of protein) were incubated in cleavage buffer containing 0, 1, 5, 25 or 200 µM (lanes 1 to 5, respectively) of dodecanoyl-CoA ($C_{12:0}$CoA; black bars in the graphic), hexadecanoyl-CoA ($C_{16:0}$CoA; white bars) or eicosanoyl-CoA ($C_{20:0}$CoA; gray bars). Protein samples were processed as described above using the monoclonal antibody 1D6. **C)** Placenta organelles (45 µg of protein) were incubated in cleavage buffer containing 200 µM of the following substances: dodecanoyl-CoA (lane 1), eicosanoyl-CoA (lane 2), dodecanoic acid (lane 3), eicosanoic acid (lane 4), dodecanoic acid plus reduced CoA (lane 5), eicosanoic acid plus reduced CoA (lane 6). A densitometric analysis of the 44 kDa N-terminal ALDP fragment is shown.

ATPγS reverts the acyl-CoA-induced sensitivity of the N-terminal domain of ALDP to proteolysis

The results presented above are compatible with the possibility that CoA thioesters possessing acyl groups with 16 or more carbon atoms induce conformational alterations on the N-terminal 44 kDa fragment of ALDP increasing its sensitivity to Factor Xa. There are, however, two other possibilities that must be excluded before drawing such conclusion. The first regards the proteases that are present in these assays. Indeed, the activities of Factor Xa or of some endogenous protease(s) present in the organelle fraction used in these experiments could be stimulated by the acyl-CoAs added to the assays. However, when the activity of Factor Xa was determined in our cleavage assay buffer, in the presence of the acyl-CoAs and/or nucleotides used in this work using a specific substrate (see materials and methods) no such effect was observed. Furthermore, when Factor Xa is omitted from the assay mixtures, no degradation of ALDP can be observed at 26ºC (data not shown). Thus, the proteolytic activities are not a variable in our assays. The second possibility that could explain the results described above is related to the physico-chemical properties of the lipids used in this work. Indeed, acyl-CoAs are amphipatic molecules displaying detergent-like properties. Thus, the increased sensitivity of the 44 kDa ALDP fragment to Factor Xa observed in the presence of long- and very long-chain acyl-CoAs could be due, not to conformational alterations of the N-terminal domain of ALDP but rather to physical modifications of its environment. For instance, long- and very long-chain acyl-CoAs could extract some membrane lipids from the N-terminal domain of ALDP increasing its accessibility to Factor Xa. Data suggesting that this is not the case were obtained when the protease assays were performed in the presence of different nucleotides. As shown in Fig. 3, incubation of ALDP in the presence of ATP or ATPγS, alone or in combination with dodecanoyl-CoA, does not increase the sensitivity of the 44 kDa N-terminal fragment to the action of Factor Xa (compare lane 1 with lanes 2-4, 6). It is also evident that the presence of ATP in the

assay mixture does not modify the eicosanoyl-CoA-induced sensitivity of this domain of ALDP to Factor Xa (lane 5). Remarkably, when ATPγS (a poorly hydrolysable ATP analogue) is substituted for ATP, the eicosanoyl-CoA –induced sensitivity of the N-terminal domain of ALDP to Factor Xa is strongly reverted (Fig. 3, compare lanes 5 and 7). Clearly, the presence of ATPγS in the assay mixture blocks the catalytic cycle of ALDP at some step, resulting in a conformation of the 44 kDa N-terminal that is no longer sensitive to Factor Xa. Thus, on one hand, this result indicates the existence of a cross-talk between the C-terminal nucleotide binding domain of ALDP and its N-terminal domain (the lipid-binding domain); on the other hand, it strongly suggests that acyl-CoAs with 16 or more carbon atoms induce conformational (and not environmental) changes on the 44 kDa N-terminal domain of ALDP.

Taken together, our data support the possibility that ALDP plays a direct role in the transport of long- and very long-chain acyl-CoAs across the peroxisomal membrane.

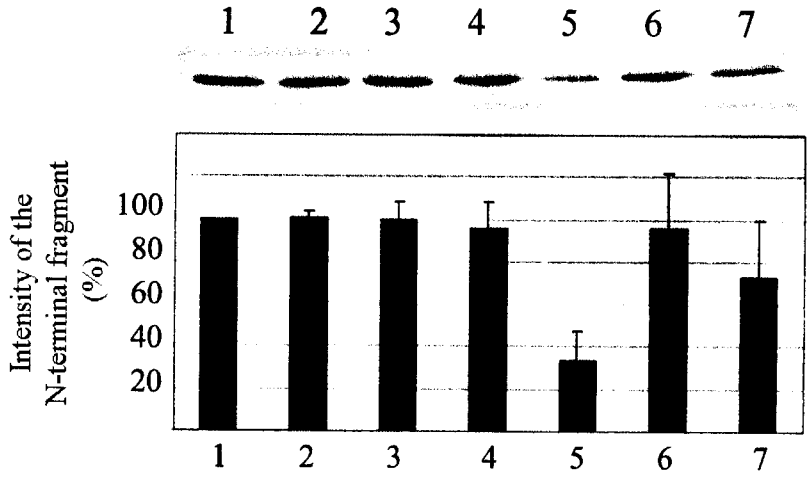

FIG. 3 – ATPγS reverts the acyl-CoA-induced sensitivity of the N-terminal domain of ALDP to proteolysis. Human placenta organelles (45 µg of protein) were incubated in cleavage buffer in the absence of lipids and nucleotides (lane 1) or in the presence of ATP (lane 2), ATPγS (lane 3), dodecanoyl-CoA plus ATP (lane 4), eicosanoyl-CoA plus ATP (lane 5), dodecanoyl-CoA plus ATPγS (lane 6) or eicosanoyl-CoA plus ATPγS (lane 7). Nucleotides and lipids were used at 10 mM and 200 µM final concentrations, respectively. Protein samples were treated with Factor Xa and subjected to western-blotting using the monoclonal mouse anti-ALDP antibody 1D6. A densitometric analysis from three independent experiments is presented. The means and standard deviations are shown. All the values were normalized to the control reaction (lane 1), which was set to 100%.

To conclude, we would like to mention that several attempts were made to obtain additional experimental evidence supporting the observations described above. Specifically, we expressed a N-terminal histidine-tagged human ALDP in *Escherichia coli* using the pQE vector (Quiagen). Highly purified ALDP was obtained from inclusion bodies by chromatography using Ni^{2+}-Agarose in the presence of 6 M guanidine-HCl, pH 7.0 (data not shown). The aim of these experiments was to reconstitute ALDP in liposomes and to determine its ATPase activity in the presence of different acyl-CoAs and free fatty acids. Unfortunately, all efforts to reconstitute the recombinant protein in phosphatidylcoline/phosphatidylethanolamine vesicles using a dilution method, dialysis, or a solid-phase refolding protocols (reviewed in [32]) failed, suggesting that expression of human ALDP in *E. coli* may be a difficult strategy if the aim is to perform functional studies.

Acknowledgements

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DISCUSSÃO GERAL

Epidemiologia genética da X-ALD em Portugal

A X-ALD é a doença peroxissomal mais comum, apresentando uma frequência de 1:21000 indivíduos do sexo masculino (Dubois-Dalcq et al., 1999; Bezman et al., 2001). Como termo de comparação refira-se que por exemplo a fenilcetonúria, cujo diagnóstico é contemplado em rastreio nacional, afecta cerca de 1:12500 indivíduos (Rivera et al., 2000).

Apesar da considerável frequência da X-ALD e apesar do gene *ABCD1* ter já sido clonado em 1993 (Mosser et al., 1993), o estudo molecular desta patologia só foi verdadeiramente implementado em Portugal com o início deste projecto de Doutoramento. Convém referir que a determinação dos níveis de AGCML permite identificar, com elevado grau de confiança, os doentes do sexo masculino (Moser et al., 1999). No entanto, a determinação deste parâmetro bioquímico não permite estabelecer um diagnóstico conclusivo para todas as mulheres em risco, dado que cerca de 15% das portadoras apresenta níveis normais de AGCML no plasma (Moser et al., 1983, Moser, 1999 #21). Nestes casos, a análise molecular é o único método que permite excluir uma condição de heterozigotia (Moser et al., 2004).

A prevenção através do aconselhamento genético e diagnóstico pré-natal, assume um papel muito importante, nomeadamente numa doença para a qual não existe tratamento efectivo. Assim, com o objectivo de implementar o diagnóstico molecular da X-ALD em Portugal, e visando contribuir, de certa forma, para o aumento do conhecimento acerca dos mecanismos moleculares responsáveis pelo aparecimento da doença, iniciou-se este trabalho pela caracterização genética dos doentes afectados com X-ALD e seus familiares, que se encontravam em estudo na Unidade de Enzimologia do Instituto de Genética Médica Jacinto de Magalhães.

Na maioria dos laboratórios onde se realiza o diagnóstico molecular da X-ALD, o protocolo correntemente adoptado consiste na análise do gene *ABCD1* a partir de DNA genómico. No entanto, a identificação das mutações ao nível do transcrito é imprescindível na caracterização de mutações, nomeadamente das que interferem com o correcto processamento do mRNA e cujo efeito poderá ser erroneamente avaliado quando a análise se limita à sequência genómica (Cartegni et al., 2002). Neste quadro engloba-se não só as mutações ditas de *splicing*, mas também as mutações pontuais (por ex. *missense*, mutações neutras) associadas ao mecanismo de *splicing* que afectam sequências exónicas (conhecidas por *exonic slicing enhancers* e *exonic slicing silencers*) (Liu et al., 2001). Atendendo a tudo isto, no trabalho aqui descrito usaram-se dois métodos para identificar e caracterizar mutações no gene *ABCD1*: amplificação por PCR a partir de DNA genómico (descrito por (Boehm et al., 1999)) e amplificação por RT-PCR (método que já havia sido desenvolvido anteriormente para o efeito (Guimaraes, 1998), e que foi optimizado durante este estudo).

Sabendo à partida que a maioria das mutações associadas à X-ALD era não recorrente, e que a mutação mais frequente (c.1415-1416delAG) era encontrada somente em cerca de 8% dos casos (base de dados em http://www.x-ald.nl), iniciou-se o diagnóstico molecular de uma nova família X-ALD recorrendo, sempre que possível, a um doente do sexo masculino. Nestes casos, toda a região codificante do gene *ABCD1* foi rastreada para a presença de mutações, usando-se a estratégia desenvolvida (Trabalho II): amplificação do cDNA por PCR, análise dos fragmentos por CSGE e sequenciação directa do(s) fragmento(s) alterado(s). No entanto, sempre que o estudo de uma nova família teve de ser iniciado por uma portadora (que apresentasse níveis de ácidos gordos aumentados no plasma e/ou fibroblastos de pele em cultura) recorreu-se à amplificação por PCR a partir de DNA genómico (Boehm et al., 1999), seguida de uma análise por CSGE e sequenciação. Esta opção, no caso de portadoras, foi tomada de forma a salvaguardar que mutações que interferissem com

a transcrição fossem detectadas logo na primeira análise, uma vez que estas poderiam resultar em falsos negativos numa abordagem baseada na amplificação por RT-PCR.

A estratégia experimental seguida permitiu, num curto espaço de tempo, identificar em cada uma das 23 famílias X-ALD estudadas pelo menos um defeito genético ao qual se pôde atribuir causalidade. Tal como foi descrito (Trabalho I - tabela 2 e Trabalho II), a elevada heterogeneidade genética já associada anteriormente à X-ALD (Kemp et al., 2001) está bem patente também na população Portuguesa. Note-se que das 22 mutações encontradas, dez são novas e somente duas (c.900G>A e c.1415-1416delAG) foram identificadas em mais do que uma família.

De forma a avaliar o efeito destas mutações na estabilidade da ALDP, determinaram-se os níveis de RNA mensageiro *ABCD1* (por *northern-blotting*) e da respectiva proteína (por *western-blotting*), recorrendo a fibroblastos de pele, em cultura, dos doentes. Tal como foi referido no Trabalho I, a maioria das mutações encontradas não interfere com o processamento do RNA mensageiro, mas sim com os mecanismos de biogénese da proteína. A ideia prevalecente neste campo é que a maioria das mutações (cerca de 78%) resultam na ausência de ALDP (base de dados em http://www.x-ald.nl). No entanto, este valor está seguramente sobrestimado uma vez que só para 20% das mutações descritas se podem encontrar dados relativos à proteína e porque na maioria dos laboratórios se recorre à técnica de imunofluorescência indirecta, que é uma técnica primariamente qualitativa sendo menos sensível do que uma análise por *western-blotting*; note-se que níveis de ALDP equivalentes a 7% dos níveis controlo (doente 2 - Trabalho II) não foram detectados recorrendo a imunofluorescência, enquanto que por *western-blotting* se conseguiu detectar 0.4% dos níveis normais (doente 1 - Trabalho II).

A necessidade de se quantificar os níveis de ALDP no caso das mutações *missense* é discutível, dado que a proteína residual é sempre uma proteína mutada que apesar de se encontrar na membrana pode não ser funcional. No entanto, para as

mutações que afectam locais de *splicing*, mas que ainda assim são compatíveis com a produção de RNA mensageiro da ALDP correctamente processado, a quantificação desta proteína torna-se indispensável. Os dois casos apresentados no Trabalho II são disso um exemplo. Tal como se descreveu neste trabalho, mesmo as mutações de *splicing* supostamente mais severas (por exemplo, a que afecta o doente 1) podem ser compatíveis com a síntese de transcrito normal e consequentemente com a produção de proteína funcional. Neste caso, a quantificação dos níveis de proteína ALDP não só suportou os dados obtidos por RT-PCR, como também sugere que quadros clínicos mais suaves, quando associados a mutações de *splicing*, podem-se dever à presença de um nível residual de ALDP funcional.

Caracterização estrutural dos transportadores ABC peroxissomais

Todos os transportadores ABC conhecidos na membrana do peroxissoma de mamíferos pertencem à classe *half*-ABC, pelo que para formar um transportador funcional devem dimerizar. A determinação do tipo de interacções estabelecidas pela ALDP é extremamente importante no âmbito da X-ALD, dado que se esta proteína formar heterodímeros então poderá estar envolvida no transporte de diversos substratos. Apesar da relevância deste assunto, dados relativos à estrutura dos transportadores ABC peroxissomais são escassos. Um dos principais problemas está relacionado com a natureza hidrofóbica das proteínas, sendo difícil encontrar condições de solubilização que permitam obter um elevado rendimento de extracção das proteínas no estado nativo, o que é agravado pelos baixos níveis de expressão encontrados em condições fisiológicas normais. Talvez por esta razão só existam três trabalhos publicados sobre esta matéria (Liu et al., 1999; Smith et al., 1999; Tanaka et al., 2002). Os resultados obtidos nos três trabalhos sugerem que a ALDP tem capacidade para interactuar com ela mesma e com os outros transportadores ABC peroxissomais. No entanto, e como foi já discutido anteriormente (ver secção 3.1), a

natureza das metodologias usadas não permite inferir que tipo de interacções prevalece *in vivo*. Assim, o objectivo principal que esteve na base do Trabalho III foi dar resposta à seguinte pergunta: "Que tipo de complexos proteicos envolvendo os transportadores ABC peroxissomais podem ser encontrados em condições fisiológicas normais?".

Tal como se descreveu no Trabalho III, o estudo foi iniciado analisando o comportamento dos transportadores ABC peroxissomas de fígado de ratinho por centrifugação em gradiente de sacarose após solubilização com digitonina (Trabalho III - Fig. 1A). A massa molecular aparente verificada nestas experiências (300-400kDa) poderia representar uma propriedade intrínseca destes transportadores ou poderia dever-se à existência de uma proteína acessória aos complexos. De forma a testar a segunda hipótese purificou-se o complexo envolvendo a PMP70, e pelo menos neste órgão, a maioria desta proteína encontra-se sob a forma homomérica. Apesar do número de subunidades que constituem o complexo não ter sido determinado, demonstrou-se que no mínimo o complexo é dimérico. O fígado de ratinho foi o modelo de estudo escolhido por várias razões: (1) pelo menos três transportadores ABC peroxissomais (ALDP, ALDPR, PMP70) coexistem neste órgão; (2) consegue-se isolar peroxissomas com alto rendimento e com elevado grau de pureza, o que se torna uma vantagem para quem pretende purificar uma proteína pouco abundante e (3) o fígado é um dos órgãos que, no caso de doentes e ratinhos modelo para a X-ALD, apresenta acumulação de AGCML (Forss-Petter et al., 1997).

Atendendo a que a PMP70 é o transportador ABC mais abundante na membrana do peroxissoma de fígado de rato (Gouveia et al., 1999), a observação de que a maioria da proteína se encontrava sob a forma homomérica já era previsível, embora nunca tivesse sido demonstrada. No entanto, permanecia por esclarecer que tipo de interacções envolviam um transportador ABC peroxissomal menos abundante, como é o caso da ALDP. Assim, este transportador foi caracterizado através da realização de experiências de imunoprecipitação preparativa, concluindo-se que à

semelhança da PMP70, a maioria da ALDP é homomérica *in vivo*. Saliente-se que os resultados obtidos neste trabalho não permitem generalizar que a homomerização seja a regra para os transportadores ABC peroxissomais. Em primeiro lugar, porque a presença de uma pequena percentagem da PMP70 e ALDP na forma heterodimérica nunca poderá ser determinantemente excluída, uma vez que cada análise tem um limite de detecção associado. Em segundo lugar, porque o estudo foi realizado em fígado de ratinho, não se sabendo se os resultados aqui obtidos podem ser extrapolados para outros órgãos ou tipos celulares. Em terceiro lugar, porque devido à inexistência de anticorpos dirigidos contra a PMP70R, a estrutura quaternária desta proteína não foi estudada. No entanto, se a mensagem do Trabalho III - PMP70 e ALDP, em fígado de ratinho, são maioritariamente transportadores homoméricos - for considerada no contexto da X-ALD então podemos especular que muito possivelmente o número de substratos transportados pela ALDP não é assim tão elevado quanto se supôs após Liu e colaboradores (Liu et al., 1999) terem sugerido que a ALDP apresenta capacidade para homodimerizar e heterodimerizar *in vitro*. Obviamente que como a função da ALDP permanece por determinar, não é de excluir que à semelhança de outros transportadores ABC, a ALDP mesmo sendo homodimérica apresente especificidade para mais do que um tipo de substrato.

Caracterização funcional da ALDP

Uma das principais questões em aberto na área da X-ALD é sem dúvida a determinação do papel fisiológico da ALDP. Uma vez que é indiscutível que defeitos no gene *ABCD1* são os responsáveis pelo aparecimento da X-ALD e que os AGCML acumulam na presença de mutações neste gene, permanece por esclarecer de que forma a ALDP está relacionada com o aumento da concentração destes lípidos em particular.

Com o intuito de verificar se os AGCML são substratos para a ALDP, desenvolveu-se a metodologia descrita no Trabalho IV. A abordagem experimental usada é vulgarmente direccionada para o estudo do mecanismo de acção de outros transportadores ABC (Wang et al., 1998; Julien and Gros, 2000; Kashiwayama et al., 2002; Manciu et al., 2003) de forma a avaliar a dependência energética do processo de transporte, recorrendo ao uso de vários nucleótidos e/ou drogas hidrosolúveis.

Tal como foi descrito no Trabalho IV, os resultados mostram que existe uma alteração da susceptibilidade da ALDP a proteólise somente quando os ácidos gordos saturados de cadeia longa e muito longa activados com Coenzima A são adicionados ao ensaio. No entanto, a natureza química destes substratos (moléculas anfipáticas) levanta dúvidas relativamente ao significado das observações. Assim, o novo padrão proteolítico pode dever-se à exposição de resíduos da ALDP que se encontravam protegidos na camada lipídica antes da solubilização, e não propriamente à alteração da sua estrutura conformacional como resposta à presença do substrato adicionado. Na tentativa de esclarecer este ponto realizou-se o ensaio na presença de ATPγS (um análogo de ATP, que não é facilmente hidrolisável) com o objectivo de bloquear o mecanismo de transporte. Nestas condições, seria de esperar um efeito diferencial na proteólise da ALDP, o que de facto se veio a comprovar (Trabalho IV- Fig. 3). Este resultado permite sugerir que a ALDP transporta ácidos gordos saturados de cadeia longa e muito longa activados com Coenzima A.

Na tentativa de refutar a ideia de que o fenómeno observado fosse simplesmente resultado de um processo de solubilização, recorreu-se às linhas celulares (fibroblastos) dos doentes nas quais se identificaram previamente mutações que eram compatíveis com a síntese de níveis normais de ALDP (S108L, G266R e R591W; Trabalho I). O comportamento destas proteínas em gradientes de densidade de sacarose, na presença de digitonina, é semelhante ao observado para linhas celulares controlo (300-400kDa tal como em fígado de ratinho - Trabalho III; resultados não publicados) concluindo-se que estas mutações não interferem com os

mecanismos de biogénese do transportador ALDP, mas sim com a sua função. A ideia que esteve na base destas experiências assumia que alguma das formas mutadas da ALDP (cuja função está comprometida) pudesse, na presença dos substratos, apresentar um padrão proteolítico distinto do apresentado pela ALDP funcional. No entanto, esta hipótese não se comprovou (resultados não publicados) pelo que estas linhas celulares não permitiram esclarecer a dúvida inicial. Uma justificação plausível para a obtenção destes resultados negativos é a de que as mutações podem interferir somente com a cinética de reacção e não com a ligação do lípido à proteína. Por outro lado, também se poderá assumir que, de facto, a ALDP mutada fica bloqueada numa etapa do transporte mas que, nas condições de ensaio actuais, não se consegue distinguir a nova conformação adoptada pela proteína.

Inevitavelmente, a resposta à questão colocada requer experiências de reconstituição da ALDP em lipossomas. Tal como foi já foi referido (Trabalho IV), algumas tentativas neste sentido ainda foram realizadas, mas sem sucesso.

PERSPECTIVAS FUTURAS

Apesar dos inúmeros estudos bioquímicos e dos avanços na determinação da estrutura e do mecanismo de acção dos transportadores ABC, são ainda várias as lacunas no nosso conhecimento acerca destas proteínas. Esta constatação é válida mesmo para os transportadores nos quais se observa a existência de um maior investimento, como é o caso da glicoproteína P. Assim, e atendendo ao facto dos transportadores ABC constituírem uma das maiores famílias de proteínas conhecidas, não é de admirar que existam transportadores sobre os quais ainda pouco ou nada se sabe. Tal como foi discutido neste trabalho, os transportadores ABC existentes na membrana do peroxissoma de mamíferos são disso um exemplo.

Actualmente, uma das razões que impede a evolução na área de estudo dos transportadores ABC peroxissomais, é a falta de observações conclusivas que permitam estabelecer novas linhas de investigação. Uma das causas subjacentes a este problema relaciona-se com a falta de recursos materiais de análise. Assim, um esforço concentrado na obtenção de anticorpos específicos contra várias partes das proteínas, e na exploração de sistemas de expressão eficientes seria certamente bastante recompensador a médio prazo. Por exemplo, a disponibilidade de bons anticorpos permitiria quantificar os níveis dos quatro transportadores ABC peroxissomais, em diferentes células e tecidos de forma a esclarecer a problemática da heterodimerização; até ao momento, praticamente todos os dados relativos ao padrão de expressão temporal e espacial destas proteínas foram obtidos de uma forma indirecta, recorrendo à análise do RNA mensageiro (por *northern-blotting* e/ou amplificação por RT-PCR).

Tal como acontece no plano estrutural, a compreensão do papel fisiológico da ALDP é fundamental quando se pretende elucidar os mecanismos patogénicos responsáveis pelo aparecimento da X-ALD. De forma a esclarecer definitivamente a

função da ALDP, a proteína deverá ser reconstituída em lipossomas. Este tipo de trabalho é vulgarmente realizado com os transportadores ABC presentes na membrana plasmática, tomando-se em consideração que o estímulo da actividade de ATPase corresponde à aceitação do substrato (exemplos, (Ambudkar, 1995; Scarborough, 1995)). No entanto, as metodologias empregues nestes estudos não podem ser directamente transponíveis para o caso da ALDP. A principal razão está relacionada com a difícil obtenção de proteína pura, em quantidade suficiente, para a realização destas experiências. Assim, seria importante identificar ou estabelecer uma linha celular que expressasse abundantemente a ALDP. O desenvolvimento de um protocolo de reconstituição seria útil, não só na determinação do tipo de substrato(s) aceite(s) pela proteína, mas também na obtenção de dados relativos ao mecanismo de transporte.

Um dos campos de investigação que ainda permanece por explorar está relacionado com a determinação dos mecanismos de biogénese destes transportadores. Alguns grupos têm já tentado abordar este assunto, através da determinação das regiões e/ou resíduos importantes para o correcto endereçamento da ALDP (Landgraf et al., 2003) e PMP70 (Biermanns and Gartner, 2001) para o peroxissoma. Com vista a obter dados mais abrangentes, relativos não só ao endereçamento correcto mas também à correcta inserção na membrana e aquisição de estrutura funcional, seria vantajoso desenvolver um sistema de importação peroxissomal *in vitro*, específico para estas proteínas. Tal abordagem experimental foi já referenciada para a PMP70 (Imanaka et al., 1996). No entanto, a metodologia e os critérios usados na avaliação da ocorrência de importação são questionáveis e pouco informativos, pelo que esse sistema nunca mais foi explorado. Recentemente, foi descrito um novo ensaio orientado para o estudo do receptor peroxissomal Pex5p (Gouveia et al., 2003b) e que tem permitido analisar o mecanismo de translocação proteica peroxissomal (Gouveia et al., 2003a; Oliveira et al., 2003; Costa-Rodrigues et

al., 2004). A aplicação de um sistema deste tipo ao estudo dos transportadores ABC peroxissomais, possibilitaria a determinação dos factores necessários nas diferentes etapas do processo de importação (dependência de ATP, pH, etc.), bem como a identificação dos domínios e/ou resíduos importantes nos mecanismos de biogénese (endereçamento, inserção membranar e dimerização) destas proteínas. Neste caso, o efeito patogénico das mutações pontuais, que ocorrem naturalmente no gene *ABCD1*, e que se encontram associadas a formas instáveis de proteína, poderia ser avaliado.

Relativamente ao enquadramento do peroxissoma no contexto celular espera-se, com alguma expectativa, que a extensa caracterização dos ratinhos *knock-out*, actualmente disponíveis para a ALDPR e PMP70 (Jimenez-Sanchez et al., 1997; Berger et al., 2003) permita identificar a função biológica destas proteínas. Os resultados teriam consequentemente um grande impacto na saúde humana, facilitando o diagnóstico de novas patologias e originando novas linhas de investigação, nomeadamente no desenvolvimento de terapias para a X-ALD.

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