厚生労働科学研究費補助金（長寿科学総合研究事業）
分担研究報告書

在宅人工呼吸器装着 ALS 患者の実態調査

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研究要旨　JaCALS 推進の基礎資料として、在宅人工呼吸器装着 ALS 患者の現状調査を実施した。気管切開、人工呼吸器装着 ALS 患者のうち在宅療養中または在宅療養経験のある 17 名を直接訪問し、合併症、コミュニケーション、外出、介護体制、介護に関する要望などについて聞き取りを行った。積極的に外部とコミュニケーションをとり外出もしている患者がいる反面、6 割の患者は複雑な内容のやりとりができず、65%の患者は全く外出をしていなかった。約半分の患者は 1 年以内に入院を要する合併症があり、呼吸器系疾患が多かった。介護者からは時間的拘束の緩和を求める切実な要望があった。今後はコミュニケーション装置の改良、装置使用についての援助や指導、喀痰吸引のできるヘルパーやショートステイ施設の確保、ヘルパーやケアマネージャーへの難病に関する教育が必要と考えられた。

A. 研究背景・目的

全国の在宅療養中の人工呼吸器装着 ALS 患者数について、正確な統計資料はないが、人工呼吸器などの器具の改良に伴い、徐々に増加していると考えられる。しかしながらその実際について十分な調査は行われていない。

今後の療養環境整備および JaCALS 推進のための基礎情報把握の一環として、我々は気管切開・人工呼吸器装着 ALS 患者の在宅療養の実状につき調査を行った。

B. 研究方法

在宅療養中または在宅療養経験のある気管切開・人工呼吸器装着 ALS 患者 17 名を直接訪問し、合併症、コミュニケーション、外出、介護体制、介護に関する要望などについて聞き取り調査を行った。訪問にあたっては事前に主治医から趣旨の説明と訪問の是非の打診を行った。同意を得た例について調査を行った。

C. 研究結果

患者は男性 12 名、女性 5 名、年齢は 40 才～79 才、平均 59 才、ALS 罹患年数は 1.5 年～15 年、平均 6.3 年、気管切開後の年数は 0.5 年～10.3 年、平均 3.5 年であった。

24%の患者が手紙、電子メールなどで積極的に自宅外の人々とコミュニケーションをとっていた。しかしながら文字盤の活用は 29%、コンピュータの活用は 24%にとどまり、59%の患者は複雑な内容のやりとりができなかった。（図 1、図

2)

1 週間に 1 回以上外出している患者が18%あったが、65%の患者は過去1年間、医療機関に行く以外は全く外出していなかった。(図 3)

47%の患者が 1 年以内に 1 回以上入院を要する合併症を経験しており、内訳は肺炎 2 例、結核 2 例、気胸 2 例、胸膜炎 1 例、心筋梗塞 1 例、不整脈 1 例、褥瘡 1 例、肺出血 1 例であった。(図 4) 41%の患者が過去に褥瘡を経験しており、大部分は仙骨部だった。(図 5)

82%で主介護者は 1 人であり、その大部分は配偶者だった。(図 6) 76%の主介護者が拘束時間を問うと 24 時間×週 7 日と回答した。Care Strain Index[1] の質問表のうち、81%の介護者が「介護に時間が拘束され外出できない」、76%が「ぐっすり眠れない」に Yes と回答した。(図 7) オープンクエスチョンで要望を尋ねたところ「留守番をお願いできる人がほしい」(5 名)、「喀痰吸引できるヘルパーを確保できるようにしてほしい」(4 名)、「ヘルパー、ケアマネージャーに技術、病気の知識を教育してほしい」(4 名)、「コミュニケーション手段を改善してほしい」(4 名)といった声が多かった。

D. 考察

コミュニケーションツールの活用は十分と言えず、装置の改良、装置使用指導などの援助が望まれる。また介護者の時間的拘束を緩和する必要があり、喀痰吸引などの技術と知識がある介護者の確保やショートステイ施設の確保が必要である。ヘルパーやケアマネージャーに対して、難病に関する教育を行うことも重要である。

E. 結論

人工呼吸器装着 ALS 患者の在宅療養について、特にコミュニケーションと介護について問題点が多くあり、今後も継続した支援への努力が必要である。

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Widespread nuclear and cytoplasmic accumulation of mutant androgen receptor in SBMA patients.

Brain. 2005 Mar;128(Pt 3):659-70.

H. 知的財産権の出願・登録状況　なし

図1　コミュニケーション

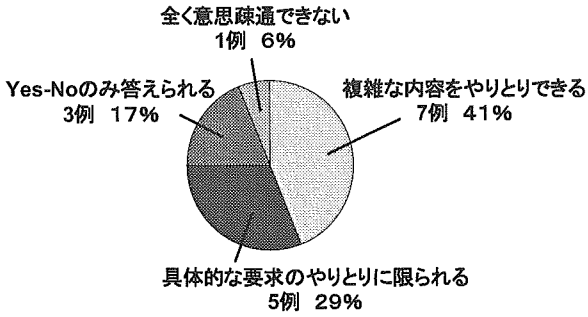


図2　コミュニケーション手段

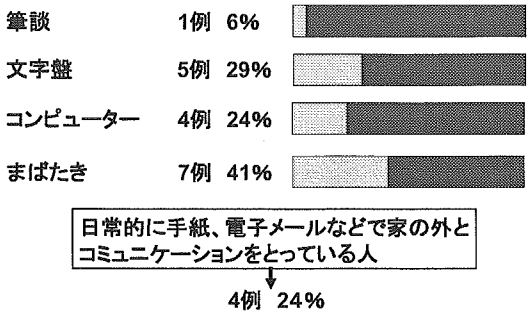


図3　患者の外出

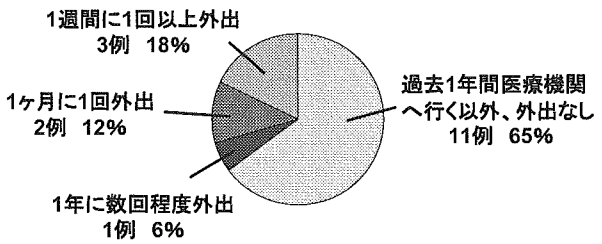


図4　合併症

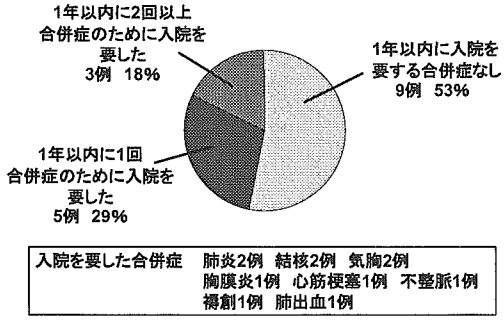


図5　褥創

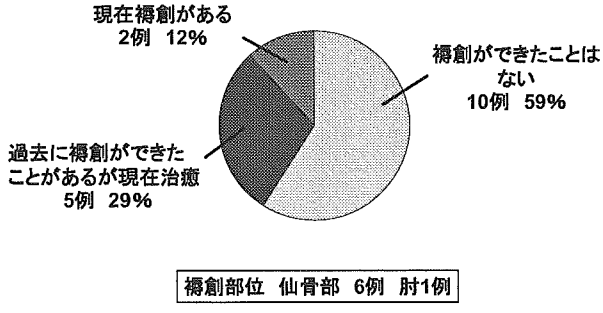


図6　介護体制

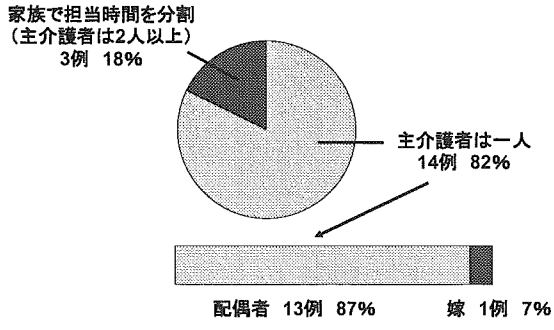
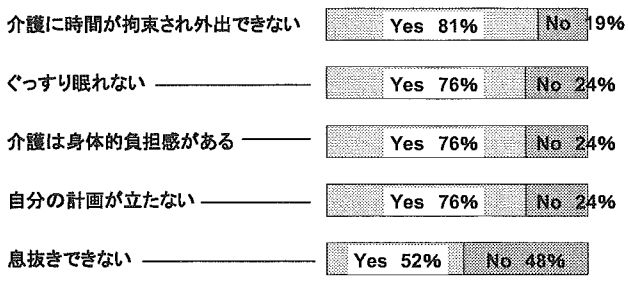


図7　主介護者のCare Strain Index
質問表の抜粋



厚生労働科学研究費補助金（長寿科学総合研究事業）
分担研究報告書

日本語版 ALSFRS-R 電話調査の妥当性

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研究要旨　JaCALS 実施にあたり、前向きの重症度把握を多くの ALS 患者で実施するために臨床研究コーディネーター(Clinical Research Coordinator: CRC)からの電話による ALSFRS-R 調査を計画した。名古屋大学神経内科においてすでに JaCALS に登録した 22 例について CRC からの電話調査による ALSFRS-R スコアと医師による直接診察でのスコアを比較して、その妥当性を検証した。ALSFRS-R スコア総点の相関係数は 0.965(Pearson)と良好であり、各項目についての κ 統計量も 0.53 から 0.86 と良好な一致を示した。JaCALS における CRC による ALSFRS-R 電話調査は十分な信頼性を持つと考えられる。現在のところやや症例数が少なく、今後さらに例数を増やして、再検証を行う予定である。

研究背景

ALSFRS（ALS Functional Rating Scale）は ALS 患者の日常生活機能(ADL)がどの程度損なわれているかを把握するために米国で作成された評価尺度である。1990 年代はじめから使われ始め、呼吸器系の項目が追加されて改訂版となり現在は ALSFRS-R として使用されている。米国で信頼性検討[1]が行われており、日本語版 ALSFRS-R についても信頼性検討[2]がおこなわれている。

現在行われている治療介入研究の多くで、primary outcome としてこの ALSFRS-R の変化量が用いられている。

我が国の ALS 患者前向き臨床像を把握し、今後の治療介入研究推進の基礎資料とするために、全国規模、多数例の ALS 患者について ALSFRS-R の経時的変化を把握することは重要である。

JaCALS において、登録患者の経時的情報を前向きに調査するにあたり、いかに脱落例を少なくするかが課題である。ほとんどの場合、ALS の診断、治療方針決定には専門医の関与が必須であり、JaCALS を構成するような地域の基幹医療施設を一度は受診する患者が多い。しかし症状の進行に伴い、自宅に近い病院などに転医していくケースが多く、登録施設で全経過を追えない場合が稀ならずある。また診療の現場で医師は多忙であり、研究に関する負担を多く課すことはできない。

JaCALS では看護師、薬剤師など法律上の守秘義務を有する医療職の資格を持った臨床研究コーディネーター(CRC: Clinical Research Coordinator)からの定期

的な電話調査により、前向きのALSFRS-R経時的変化を把握することを計画した。

英語版のALSFRS-Rについては、すでに介護者への電話調査で十分な精度を持って調査が可能であるとする報告[3]がなされている。JaCALSを進めるにあたり、日本語版ALSFRS-R電話調査について検証する必要がある。

A. 研究目的

JaCALSにおける基礎資料として、CRCによる日本語版ALSFRS-R電話調査の検証を行う。

B. 研究方法

CRC業務マニュアルにおいて、電話調査における話し方、記録の仕方とレポート作成法などについて定めた。またALSFRS-Rの各項目について、点数を定めるフローチャートを作成し、これを見ながら電話をすることとした。担当CRCに対して、ALSの臨床像、疫学、病態、治療、ALS患者および介護者に対して行うべき配慮、JaCALSの背景、目的、関連する倫理指針および電話調査にあたっての具体的な手順に関する研修を実施した。

名古屋大学神経内科において、JaCALSの登録を行った22例のALS患者について、医師が診察室で直接本人を診察して記載したALSFRS-Rスコア（医師スコア）とCRCが電話調査で点数を定めたALSFRS-Rスコア（電話スコア）の比較検討を行った。

ALSFRS-R総点の相関についてPearson相関係数を算出し、各項目(Table 1)についてκ統計量による一致性の検討を行った。

統計解析にはMicrosoft Excel 2002 SP3およびDr.SPSS II for Windows (SPSS Japan Inc. Tokyo Japan)を用いた。

倫理面への配慮

すべての解析対象の患者から、JaCALS参加について十分な文書でのインフォームドコンセントを得た。JaCALS研究計画および説明書・同意書は名古屋大学医学部倫理委員会の承認を得た。

C. 研究結果

22例のうち電話調査に対して、本人が回答した例が9名、主介護者が回答した例が13名であった。

医師スコアと電話スコア総点の散布図と回帰直線をFigure1に示す。相関係数は0.965(p<o.oo1)と良好な相関を示した。各項目のκ統計量をTable2に示す。κ統計量は1の場合完全な一致を示し、0.75以上でexcellent agreement、0.4以上0.75未満でfairly to good agreement、0.4未満でpoor agreementと判定される。今回のκ統計量は0.53から0.86の間で分布し、良好な一致を示した。

D. 考察

日本版ALSFRS-Rについて、患者と直接対面した評価での検者間の一致が検討[2]されている。この報告では総点の相関係数は0.97であり、各項目のκ統計量は0.52から1.0の間で分布し、κの平均は0.67であった。今回の電話調査の妥当性検討ではκの平均は0.73となり、総点の相関係数(0.965)と合わせて直接対面での検者間の一致と比較しても遜色のないレ

ベルの一致を示した。アメリカ合衆国における ALSFRS-R 電話調査の報告[3]における診察室でのスコア総点と電話調査でのスコア総点間の相関係数も 0.966 と類似した値であった。

これらの値からは、JaCALS における ALSFRS-R 電話調査は十分な信頼性を持つと考えられる。

今後さらに、症例数を増やしたうえで、CRC 間での一致の検証、回答者が本人か介護者かでの相違の有無についての検討を進めていく予定である。

E. 結論

CRC による ALSFRS-R 日本語版電話調査は十分な信頼性があるものと考えられる。

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Brain. 2005 Mar;128(Pt 3):659-70.

H. 知的財産権の出願・登録状況 なし

Figure 1. ALSFRS-R 医師スコアー電話スコア 散布図

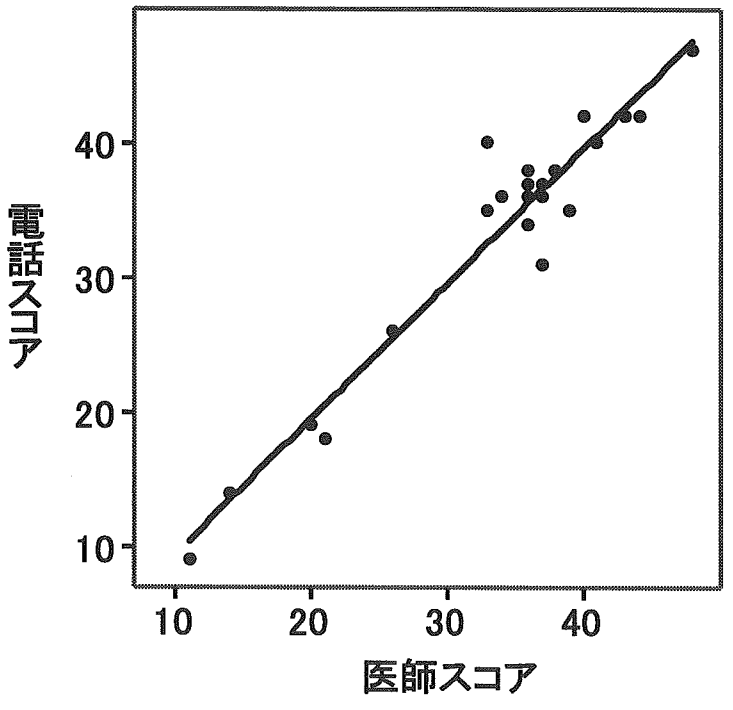

Table1.　日本語版 ALSFRS-R

言語
4　会話は正常
3　会話障害が認められる
2　繰り返し聞くと意味がわかる
1　声以外の伝達手段と会話を併用
0　実用的会話の喪失

唾液分泌
4　正常
3　口内の唾液はわずかだが、明らかに過剰（夜間はよだれが垂れることがある）
2　中等度に過剰な唾液（わずかによだれが垂れることがある）
1　顕著に過剰な唾液（よだれが垂れる）
0　著しいよだれ（絶えずティッシュやハンカチを必要とする）

嚥下
4　正常な食事習慣
3　初期の摂食障害（時に食物を喉につまらせる）
2　食物の内容が変化（継続して食べられない）
1　補助的なチューブ栄養を必要とする
0　全面的に非経口性または腸管性栄養

書字
4　正常
3　遅い、または書きなぐる（すべての単語が判読可能）
2　一部の単語が判読不可能
1　ペンは握れるが、字を書けない
0　ペンが握れない

摂食動作（胃瘻設置の有無により（1）、（2）のいずれか一方で評価する）
（1）食事用具の使い方（胃瘻設置なし）
4　正常
3　幾分遅く、ぎこちないが、他人の助けを必要としない
2　フォークは使えるが、はしは使えない
1　食物は誰かに切ってもらわなくてはならないが、何とかフォークまたはスプーンで食べる事ができる
0　誰かに食べさせてもらわなくてはいけない

（2）指先の動作（胃瘻設置患者）
4　正常
3　ぎこちないが全ての手先の作業ができる
2　ボタンやファスナーを留めるのにある程度手助けが必要
1　看護者にわずかに面倒をかける
0　全く何もできない

着衣、身のまわりの動作
4　正常
3　努力して（あるいは効率が悪いが）独りで完全にできる
2　時折手助けまたは代わりの方法が必要
1　身の周りの動作に手助けが必要
0　全面的に他人に依存

寝床での動作
4　正常
3　幾分遅く、ぎこちないが助けを必要としない
2　独りで寝返りをうったり、寝具を整えられるが非常に苦労する
1　寝返りを始めることはできるが、独りで寝返りをうったり、寝具を整えることができない
0　自分ではどうすることもできない

歩行
4　正常
3　やや歩行が困難
2　補助歩行
1　歩行は不可能
0　脚を動かすことができない

階段登り
4　正常
3　遅い
2　軽度の不安定または疲労
1　介助が必要
0　登れない

呼吸（呼吸困難、起座呼吸、呼吸不全の３項目を評価）
（1）呼吸困難
4　なし
3　歩行中に起こる
2　日常動作（食事、入浴、着替え）のいずれかで起こる
1　座位または臥位のいずれかで起こる
0　極めて困難で呼吸補助装置を考慮する

（2）起座呼吸
4　なし
3　息切れのため夜間の睡眠がやや困難
2　眠るのに支えとする枕が必要
1　座位でないと眠れない
0　全く眠ることができない

（3）呼吸不全
4　なし
3　間欠的に呼吸補助装置（bipap)が必要
2　夜間に継続的に呼吸補助装置（bipap)が必要
1　１日中呼吸補助装置（bipap)が必要
0　挿管または気管切開による人工呼吸が必要

Table2.　ALSFRS-R 各項目別 κ 統計量

項目	言語	唾液分泌	嚥下	書字	摂食動作	着衣	寝床	歩行	階段のぼり	呼吸困難	起座呼吸	呼吸不全
κ	0.81	0.53	0.86	0.74	0.68	0.77	0.59	0.62	0.64	0.81	0.83	0.82

Ⅲ.研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ.研究成果の刊行物・別刷

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CALCIUM SENSING RECEPTOR UBIQUITINATION AND DEGRADATION MEDIATED BY THE E3 UBIQUITIN LIGASE DORFIN

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Running Title: *Dorfin-mediated Calcium Sensing Receptor Degradation*
Key words: Calcium sensing receptor, dorfin, ubiquitination, endoplasmic reticulum-associated degradation pathway, proteasome, protein degradation

Calcium-sensing receptors (CaR) contribute to regulation of systemic calcium homeostasis by activation of G_q- and G_i-linked signaling pathways in the parathyroids, kidney, and intestine. Little is known about the mechanisms regulating CaR synthesis and degradation. Screening of a human kidney yeast two-hybrid library identified the E3 ubiquitin ligase dorfin as a binding partner for the intracellular carboxyl terminus of CaR. Interaction between CaR and dorfin was confirmed by coimmunoprecipitation from HEK293 cells. Ubiquitination of CaR was observed in the presence of the proteasomal inhibitor MG132; mutation of all putative intracellular loop and carboxyl terminal lysine residues abolished ubiquitination of CaR. Coexpression with dorfin decreased the amount of total CaR protein and increased CaR ubiquitination, while a dominant negative fragment of dorfin had opposite effects. The AAA-ATPase p97/valosin-containing protein (VCP) associates with both CaR and dorfin in HEK293 cells. Treatment with tunicamycin, an inhibitor of N-linked glycosylation, induced the appearance of the unglycosylated 115 kD CaR form, which was further increased by exposure to MG132, or upon transfection with a dorfin dominant negative construct, suggesting that dorfin-mediated proteasomal degradation of immature CaR occurs from the endoplasmic reticulum. Since endogenous CaR in MDCK cells is also subject to degradation from the endoplasmic reticulum, dorfin-mediated ubiquitination may contribute to a general mechanism for CaR quality control during biosynthesis.

The calcium sensing receptor (CaR) contributes to maintenance of systemic Ca^{2+} homeostasis, regulating parathyroid hormone secretion, absorption/resorption of Ca^{2+} by the intestine and kidney, and may also have effects in bone (1). CaR belongs to family C of the GPCR

superfamily, having structural similarities to metabotropic glutamate receptors, $GABA_B$ receptors, and some putative pheromone/taste receptors (1,2). Common to all members of family C is a large extracellular domain of more than 600 amino acids containing the agonist binding site, a heptahelical transmembrane domain, and a large intracellular carboxyl terminal tail of more than 200 amino acids (1,2). Members of Family C, including CaR, function as dimers, stabilized either by a disulfide bond or non-covalent interactions (3,4,5). The CaR extracellular domain contains N-linked glycosylation sites (2,6,7), and is stabilized by multiple intramolecular disulfide bonds, as well as one intermolecular disulfide bond between monomers in the dimer (8,9,10). Upon agonist stimulation, CaR activates diverse signaling pathways leading to changes in hormone secretion, cell proliferation, differentiation and/or apoptosis (1). While considerable progress has been made in understanding the structure, activation, and signaling of CaR, the biosynthesis, trafficking, targeting and turnover mechanisms regulating CaR remain largely unexplored.

To identify novel proteins that might regulate trafficking and/or targeting of CaR, the intracellular carboxyl terminus of CaR was used as bait in a yeast two-hybrid (Y2H) screen of a human kidney cDNA library. One of the proteins identified in the screen was the E3 ubiquitin ligase dorfin (double-RING finger protein) (11). Dorfin was originally cloned from human spinal cord and is expressed in many organs, including kidney, liver, intestine, and the central nervous system (11). Dorfin localizes to a region near the centrosome in an aggresome-like structure in cultured cells (11). In the nervous system, dorfin ubiquitinates superoxide dismutase-1 (12) and synphilin-1 (13), and is a component of Lewy bodies observed in Parkinson's and other neurodegenerative diseases (12,13,14). Ubiquitination results in the attachment of ubiquitin, a highly conserved 76-amino acid polypeptide, to the ε-amino group of lysine residues of target proteins, and requires the sequential actions of three enzymes. Final transfer of activated ubiquitin to target proteins is coordinated by the E3 ligase, which specifically interacts with both E2-ubiquitin and the target protein (15). Dorfin contains two RING domains at its amino terminus, which function as recruiting motifs for specific E2s (11). The carboxyl terminus of dorfin has no identifiable motifs, but has been shown to confer specificity of binding to synphilin-1 (13). Dorfin interacts directly with VCP (valosin-containing protein, also called p97 or Cdc48 homologue) (14), an AAA-ATPase proposed to have a role in endoplasmic reticulum-associated protein degradation (ERAD). VCP assists in translocation of ubiquitinated proteins from the ER and acts as a chaperone, targeting ubiquitinated proteins to the proteasome for degradation (16,17,18,19).

In this report, we characterize the functional interactions between CaR and dorfin in HEK293 cells, and demonstrate that dorfin mediates CaR ubiquitination, leading to degradation by the proteasome. Both dorfin and CaR interact with VCP in HEK293 cells, and a dominant negative fragment of dorfin protects immature forms of CaR from degradation. Finally, endogenous CaR in MDCK cells is also

subject to ER-associated degradation, suggesting a common mechanism may regulate quality control of both exogenously and endogenously expressed CaR. These results suggest that dorfin may recognize misfolded or non-functional CaR at the endoplasmic reticulum, leading to ubiquitination and proteasomal degradation.

EXPERIMENTAL PROCEDURES

Materials. Human kidney cDNA library and all the materials for the Y2H screen were purchased from Clontech. HEK293 and MDCK cells were from the American Tissue Culture Collection and used through lab passage number 30. Restriction enzymes were from New England Biolabs and Promega. The EGFP-dorfin plasmid (dorfin chimera with EGFP at amino terminus) and rabbit polyclonal antibody against dorfin (D-30) were generated as described (11). The HA-ubiquitin plasmid was generously provided by Dr. Richard JH Wojcikiewicz (SUNY Upstate Medical University, Syracuse, NY). Monoclonal antibodies were from various sources (anti-Flag M2 and anti-actin antibodies, Sigma; anti-HA antibody, Roche Diagnostics; anti-VCP antibody, Research Diagnostics; anti-GFP antibody, Molecular Probes). Rabbit polyclonal antibody against CaR (LRG) was generated as described (20). Anti-phospho-ERK1/2 (p42/44) polyclonal antibody was from Cell Signaling Technology. ECL anti-mouse and anti-rabbit, horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham. MG132 and tunicamycin were purchased from Sigma.

Plasmid construction. CaR with an amino terminal Flag epitope (Flag-CaR) was generated as described (21). CaR carboxyl terminal (CaR-CT) fragments in the Y2H bait vector pGBKT7 were prepared by PCR using primers containing NdeI and SalI sites, using Flag-CaR as template. PCR products were digested with NdeI/SalI, and subcloned into NdeI/SalI-digested pGBKT7. Dorfin carboxyl terminal fragments in the Y2H prey vector pACT2 were prepared similarly, using NcoI and XhoI sites. The dominant negative construct of dorfin was generated as a chimera with EGFP at the carboxyl terminus (DCT-EGFP). The DCT fragment containing dorfin residues 561-838 was prepared by PCR using primers containing BglII and SalI sites. The PCR product was digested with BglII/SalI and subcloned into BglII/SalI-digested PEGFP-N1 vector (Clontech). Another version of dominant negative dorfin (DCT-*c-myc*) was prepared similarly by PCR the DCT fragment using primers containing XbaI and HindIII sites. The PCR product was digested with XbaI/HindIII and subcloned into XbaI/HindIII-digested pcDNA3.1A(-) vector (Clontech). Another construct of dorfin (DNT-EGFP) containing the N-terminal RING-finger domains of dorfin from residue 1-367 was prepared by PCR using primers containing SacI and KpnI sites. The PCR product was digested with SacI/KpnI and subcloned into SacI/KpnI-digested pEGFP-N1 vector (Clontech). Point mutations were generated by a modified inverse PCR mutagenesis method (22). All PCR reactions used *Pfu* DNA polymerase (Stratagene). All constructs were verified by dideoxy-DNA sequencing (DNA Sequencing Facility, Cornell University, Ithaca, NY). Sequences of primers

provided upon request.

Y2H assay screening. Gal4-based Y2H library screening was performed by yeast mating as recommended in the manufacturer's instructions (Clontech). Yeast *Saccharomyces cerevisiae* MATa strain AH109 was transformed with bait plasmid containing CaR-CT (aa 866-1078) and incubated with yeast *Saccharomyces cerevisiae* MATα strain Y187 pretransformed with human kidney cDNA library (Clontech) in 2xYPDA/Kan at 30°C for 24 hours. The mixture was plated on SD/-Ade/-His/-Leu/-Trp plates to screen for ADE2$^+$ and HIS3$^+$ clones. Plates were incubated at 30 °C until colonies appeared. Colonies were restreaked on SD/-Ade/-His/-Leu/-Trp + X-α-gal plates to screen for MEL1$^+$ clones. Clones that activated three reporter genes, ADE2, HIS3 and MEL1, were considered positive and identified by purifying plasmids and sequencing inserts.

Directed Y2H studies were performed by cotransformation using the lithium acetate method (23). Bait plasmids containing CaR-CT fragments and prey plasmids containing dorfin carboxyl terminal fragments were cotransformed into AH109 and plated on SD/-Ade/-His/-Leu/-Trp + X-α-gal plates. An interaction was considered positive when three reporter genes (ADE2, HIS3 and MEL1) were activated.

Cell culture and transfection. HEK293 and MDCK cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin (37 °C, 5% CO_2). HEK293 cells were transiently transfected with Novafector (Venn Nova LLC, Pompano, FL) according to manufacturer's instructions; experiments were done seventy-two hours after transfection. For inhibitor studies, cells were treated with MG132 and/or tunicamycin (solubilized in DMSO) for twelve hours prior to lysis. Comparable levels of DMSO had no effect on measured parameters.

SDS-PAGE and western blotting. Transfected HEK293 cells or MDCK cells were washed with PBS-EDTA and lysed on ice with PBS containing 5 mM EDTA, 0.5% Triton X-100, 10 mM iodoacetamide, plus protease inhibitor mixture (Roche Applied Science). Cell lysates were agitated for 30 min at 4°C and cleared by centrifugation. Supernatants were mixed with loading buffer (12 M urea, 4% SDS, 0.01% bromophenol blue, 100 mM β–mercaptoethanol in 200 mM Tris) and separated on 4-15% SDS-polyacrylamide gels (Bio-Rad) before transfer to nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies (GFP, 1:500; LRG, 1:1000; HA, 1:1000; actin, 1:1000; D-30, 1:3000; or VCP, 1:1000) overnight at 4°C. Membranes were incubated with secondary antibody coupled to horseradish peroxidase (1:5000) at room temperature for one hour. Proteins were visualized by enhanced chemiluminescence (Super West Pico Chemiluminescent Substrate, Pierce). Assay of ERK1/2 phosphorylation was as previously described (21). When the same blot was probed for the presence of coprecipitated proteins, nitrocellulose membranes were stripped in Restore Western Blot Stripping Buffer (Pierce) and probed with a second primary antibody.

Immunoprecipitation. Transfected HEK293 cells were washed with PBS and lysed as described. After sonication on ice, samples were agitated for 30 min at 4°C and incubated with 10 µl protein G-agarose (Invitrogen) to minimize nonspecific binding. Samples were centrifuged and supernatants incubated with antibody for 3 hrs at 4°C. 15 µl protein G-agarose was then added, and samples rotated overnight (4°C). Precipitates were incubated in loading buffer for 30 min at (25°C), and run on SDS-polyacrylamide gels as described. To detect specific ubiquitination, a two-step immunoprecipitation was performed. After the first immunoprecipitation as described, pellets were washed three times with lysis buffer and incubated with 1% SDS/PBS for 30 minutes (25°C) to disrupt non-covalent interactions. Samples were centrifuged and supernatants were diluted with lysis buffer, followed by a second immunoprecipitation. The resultant precipitates were incubated in loading buffer and separated on SDS-polyacrylamide gels as described.

Densitometry and statistics. Blots were digitized using an Epson Expression 800 Photo scanner and quantified by densitometry using AlphaEaseFC StandAlone Software (San Leandro, CA). Results were mean ± S.D. of at least three independent experiments; graphs were generated using Sigma Plot 2000. Student's t-test (two comparisons) was performed, $p < 0.05$ was considered significant.

RESULTS

Y2H screen identifies the E3 ubiquitin ligase dorfin as a CaR-binding protein. The cytoplasmic carboxyl terminus of CaR (CT, residues 866-1078) was used as bait to screen a human kidney cDNA library (Figure 1A). Positive clones were selected by activation of three reporter genes, ADE2, HIS3, and MEL1. Blast searches of the NCBI database with positive clones identified in the screen yielded a fragment corresponding to the carboxyl terminus (residues 561-838) of the E3 ubiquitin ligase dorfin (double-RING finger protein), an 838 amino acid protein (Figure 1B) (11).

To localize the dorfin interaction site on the CaR carboxyl terminus, various truncations of the CaR carboxyl terminus (in pGBKT7), illustrated in Figure 1A, were screened against the carboxyl terminus of dorfin (residues 561-838) (in pACT2) after cotransformation in the AH109 strain. The full CaR-CT (residues 866-1078) interacted with dorfin, confirming the results of the initial screen. Truncations of the carboxyl end of CaR-CT were well tolerated, displaying positive interactions with dorfin. Only the smallest fragment, containing residues 866-886, did not interact with dorfin. Truncations from the amino terminus of the CaR-CT narrowed the region for interaction with dorfin to residues 880-900 (Figure 1A).

Complementary directed Y2H screens were performed to localize the CaR-CT interaction site on the carboxyl terminus of dorfin (residues 561-838). Any truncations from the carboxyl terminus of dorfin inhibited interactions with CaR-CT, while interaction with CaR-CT was retained upon removal of up to 100 residues from the amino terminus of the dorfin fragment (residues 561-660). The minimal fragment of the dorfin carboxyl

terminus required for interaction with CaR-CT contains residues from 660-838 (Figure 1B).

Coimmunoprecipitation of CaR and dorfin from HEK293 cells confirms their interaction. Human CaR having an amino terminal Flag epitope (Flag-CaR) was transfected into HEK293 cells, and Flag-CaR was immunoprecipitated with anti-Flag antibody. The immunoprecipitate was separated on a 4-15% SDS-PAGE reducing gel, blotted to nitrocellulose and probed with anti-dorfin antibody D-30. Endogenous dorfin was observed as a doublet with molecular weight approximately 100 kD in the presence (lane 1) but not in the absence (lane 2) of transfected Flag-CaR (Figure 2A, top panel). The two lower panels in Figure 2 illustrate the expression of endogenous dorfin (probed with anti-dorfin antibody D-30) or Flag-CaR (probed with anti-CaR LRG antibody) in cell lysates. These results confirm the interaction of full length CaR and dorfin in HEK293 cells.

To confirm that the dorfin fragment identified in the Y2H studies is required for the interaction of dorfin and CaR in mammalian cells, Flag-CaR and full length dorfin (EGFP-dorfin) or dorfin truncations (DNT-EGFP or DCT-EGFP) were tested for coimmunoprecipitation from HEK293 cells. DNT-EGFP contains the amino terminal RING-finger domains of dorfin from residue 1-367; DCT-EGFP contains the carboxyl terminal domain of dorfin from residue 561-838. Anti-Flag antibody was used to immunoprecipitate Flag-CaR, and blots were probed with anti-GFP antibody to detect dorfin species. EGFP-dorfin (130 kDa) and DCT-EGFP (60 kDa) coprecipitated with Flag-CaR (Figure 2B, top panel), but DNT-EGFP (68 kDa) did not. Middle and bottom panels of Figure 2B illustrate expression of the indicated constructs in cell lysates when probed with anti-GFP (middle) or anti-CaR LRG (bottom) antibodies. These results confirm that the domain of dorfin mediating the interaction with CaR in yeast, i.e., the carboxyl terminus, is also required for the interaction of dorfin with CaR in mammalian cells.

CaR is ubiquitinated. Interaction between CaR and dorfin suggests that CaR may be ubiquitinated. To test this possibility, Flag-CaR and amino terminal HA-tagged ubiquitin (HA-Ub) were cotransfected into HEK293 cells, and a two-step, denaturing protocol was used to immunoprecipitate Flag-CaR. Briefly, anti-Flag antibody was used to immunoprecipitate CaR, followed by treatment of the pellet with 1% SDS/PBS to disrupt non-covalent interactions between CaR and its associated proteins. The supernatant was diluted with lysis buffer and subjected to a second round of immunoprecipitation with anti-Flag antibody. The western blot was probed with anti-HA antibody to detect ubiquitinated species. Ubiquitination of CaR was observed in the presence of the proteasomal inhibitor MG132, appearing in the range from 150 kD to more than 250 kD, while ubiquitination was barely detectable in the absence of MG132 (Figure 3A, upper panel, lanes 1-2). MG132 increased the amount of CaR protein (Figure 3A, lower panel, lanes 1-2), suggesting that ubiquitination followed by proteasomal degradation contributes to regulation of CaR.

Ubiquitin ligases covalently conjugate ubiquitin to lysine residues of target

proteins. To confirm that CaR is ubiquitinated, we mutated intracellular lysine residues to arginine. There are nine lysine residues within the CaR carboxyl terminus (residues 863, 882, 897, 917, 931, 963, 965, 984 and 1002) and seven lysine residues within the three intracellular loops (residues 636, 644, 709, 717, 793, 796 and 805). When each lysine residue was mutated to arginine individually, the resulting mutants were still heavily ubiquitinated (data not shown). Since single point mutations did not abolish ubiquitination, CaR must be ubiquitinated at more than one lysine residue. All sixteen lysine residues were therefore mutated to arginine simultaneously, and ubiquitination of the mutant, termed Flag-CaR(0K), determined in the absence or presence of MG132 (Figure 3A, upper panel, lanes 3-4). Flag-CaR(0K) was not ubiquitinated to a significant extent even in the presence of MG132. In addition, the amount of Flag-CaR(0K) was not significantly changed upon addition of MG132 (100% in the absence versus 107.6 ± 10.7% in the presence of MG132) (Figure 3A, lower panel, lanes 3-4), while wild type Flag-CaR was sensitive to MG132 treatment (100% in the absence versus 148.4 ± 4.7% in the presence of MG132) (Figure 3A, lower panel, lanes 1-2). Both Flag-CaR and Flag-CaR(0K) achieved mature glycosylation consistent with plasma membrane localization (Figure 3A, lower panel) (6,7), and had a comparable ability to stimulate ERK1/2 phosphorylation upon exposure of cells to 5 mM Ca^{2+} (Figure 3B). The absence of Flag-CaR(0K) ubiquitination is not the result of an inability to associate with dorfin, since immunoprecipitation with anti-Flag antibody of either Flag-CaR or Flag-CaR(0K) results in coprecipitation of comparable levels of endogenous dorfin (Figure 3C, top panel). Also illustrated in Figure 3C is the presence of endogenous dorfin (middle panel) or transfected Flag-CaR (bottom panel) in cell lysates. These results confirm that CaR is ubiquitinated at multiple lysine residues and degraded by the proteasome.

Dorfin mediates CaR ubiquitination. Dorfin is an E3 ubiquitin ligase and interacts with CaR, and therefore likely mediates CaR ubiquitination. To test this possibility, Flag-CaR and HA-Ub cDNAs were cotransfected into HEK293 cells, without or with EGFP-dorfin cDNA. Anti-Flag antibody was used to immunoprecipitate CaR; western blots were probed with anti-HA antibody to detect ubiquitinated species. In the absence of dorfin, a low level of CaR ubiquitination was detected; cotransfection with dorfin dramatically increased CaR ubiquitination (Figure 4A). HEK293 cells express endogenous dorfin (data not shown, but see eg. Figure 2A or 3C), and thus basal CaR ubiquitination (in the absence of cotransfected dorfin) might be catalyzed by endogenous dorfin. To test this possibility, we used DCT-EGFP as a dominant negative to interfere with ubiquitination mediated by endogenous dorfin. This construct cannot catalyze ubiquitination of substrates, since it does not contain the amino terminal RING domains which are essential for interaction with ubiquitin-conjugating enzymes (11). Cotransfection of HEK293 cells with Flag-CaR, HA-Ub and DCT-EGFP resulted in a reduction in CaR ubiquitination, compared with ubiquitination mediated by endogenous dorfin (-DCT) (Figure 4B). These results suggest that the E3 ubiquitin ligase dorfin

mediates ubiquitination of CaR.

Dorfin regulates the amount of CaR protein in HEK293 cells. Since dorfin mediates CaR ubiquitination, it must contribute to regulation of total cellular CaR protein. Flag-CaR cDNA was transfected into HEK293 cells with increasing amounts of EGFP-dorfin cDNA (Figure 5A). Total cDNA was kept constant with pcDNA3.1. The expression of CaR and dorfin were characterized by immunoblotting lysates from HEK293 cells with either anti-CaR LRG antibody (Figure 5A, top blot) or anti-GFP antibody (Figure 5A, bottom blot). Actin was used as a loading control (Figure 5A, middle blot). When dorfin was increased, the amount of CaR decreased in a dose-dependent manner (Figure 5A). The graph in Figure 5A shows averaged results for 3 independent experiments, and demonstrates a significant decrease in CaR protein as dorfin protein is increased.

When CaR cDNA was transfected into HEK293 cells with increasing amounts of the dominant negative DCT-EGFP cDNA, CaR protein in cell lysates increased as a function of DCT protein (Figure 5B, top blot). The graph illustrates averaged results from 3 independent experiments (Figure 5B). To confirm that EGFP made no contributions to the observed responses, a DCT construct containing a carboxyl terminal *c-myc* epitope was also generated. DCT-*c-myc* protected CaR against dorfin-mediated degradation in a manner comparable to DCT-EGFP (data not shown), indicating that the dorfin fragment specifically competed with endogenous dorfin to protect against CaR degradation. These results suggest that endogenous dorfin regulates CaR degradation.

To test if dorfin mediates degradation of CaR in a proteasome-dependent manner, the effect of the proteasomal inhibitor MG132 on dorfin-mediated CaR degradation was examined. When cells cotransfected with Flag-CaR and EGFP-dorfin were treated with MG132, the dorfin-dependent decrease of CaR protein level was abrogated (Figure 5C), suggesting that a proteasome-dependent mechanism underlies dorfin-mediated degradation of CaR.

VCP interacts with both CaR and dorfin. Dorfin interacts directly with VCP, an AAA-ATPase proposed to have a role in endoplasmic reticulum-associated degradation (ERAD) of proteins (14). In HEK293 cells transiently transfected with Flag-CaR, an antibody against endogenous VCP immunoprecipitated both Flag-CaR and endogenous dorfin (Figure 6, lane 1). In the absence of Flag-CaR, the anti-VCP antibody pulled down endogenous dorfin (Figure 6, lane 2), as has previously been shown (14). Lower blots of Figure 6 indicate endogenous expression of VCP and dorfin, as well as Flag-CaR in lysates of transfected cells. Since both dorfin and CaR can interact with VCP, it is likely that dorfin-mediated ubiquitination and degradation of CaR is occurring at the ER via a VCP-facilitated ERAD pathway.

All forms of CaR are degraded via the proteasome. If CaR interacts with dorfin and VCP at the ER, it is possible that misfolded or unfolded, immature CaR is targeted for proteasomal degradation by dorfin. To test this possibility, we examined the effects of tunicamycin, an