であるユビキチンリガーゼであること，loss-of-function 型効果により疾患が発症することより今後もっとも重要な課題は基質同定である．現在9つの基質が報告されているが，われわれの yeast two hybrid 法による結合蛋白は13クローン単離されており，基質は他にも存在することが予想される．また各基質間の関係も明らかにする必要があろう．一方で，基質同定のみのアプローチでは変性機序を明らかにすることは困難と考えており，基質同定と平行して発症機序や parkin の制御機構などを明らかにしていくことが必要であろう．

また劣性遺伝性より parkin ノックアウトマウスが病態解明に良きモデルとなることは容易に予想できる．今後の詳細な解析には遺伝子組み換えマウスモデルの作製が欠かせない．Parkin 蛋白の機能については parkin-related disease の病態解明のみならず孤発型 PD の病態解明に繋がる可能性が高い．なぜなら，正常 parkin が Lewy 小体形成の上で必須因子である可能性が高い．事実，αSpn22 や synphilin-1 が基質候補として検討されていることからも parkin 蛋白の詳細な機能が証明されれば孤発型 PD の Lewy 小体形成のメカニズムを明らかにすることが可能と考えている．Parkin-related disease では一般に若年発症が特徴であり Lewy 小体をみとめないことより，Lewy 小体自体が細胞毒性を持つという仮説は parkin-related disease の存在がある以上否定的かもしれない．

現在 Park1，Park2，Park5，Park7 の原因遺伝子が同定されている．最近になり Park4 が α-synuclein をふくむ 1.5 Mb の領域の triplication であることが報告された[43]．Park4 は，家族性 DLBD の臨床型をとっていることより α-synuclein の overproduction がその原因であるとしている．この考えは家族性 AD と共通しており，遺伝性 PD の共通機構に α-synuclein の overproduction が共通カスケードを形成しているかもしれない．孤発型 PD は α-synuclein の分解能の低下の結果かもしれない．一連の遺伝性 PD の原因遺伝子が単離され，機能が解明されれば必ずや孤発型 PD の原因が明らかにされると信じている．PD の多様性からすれば現在マップされた遺伝性 PD 以外にも存在している可能性があり，事実，新規遺伝性 PD の連鎖解析をおこなっている．更にわれわれの研究室では parkin 遺伝子変異陰性例が存在することより劣性遺伝性である Park6 に連鎖する家系が少なからず存在しており，原因遺伝子単離に向けて解析中である．Park 7 にもハプロタイプからは連鎖している可能性の高い家系が存在している．その原因遺伝子 DJ-1 について遺伝子変異解析をおこなっている．

これら家族性 PD の原因遺伝子が単離されれば黒質変性の機序が明らかにされることが予想され，単一遺伝子異常から孤発型 PD 解明のアプローチが本格的に始めることができるものと信じている．

III. Park1 に連鎖する家系おける α-Synuclein 遺伝子のハプロ不全

Park1 の原因遺伝子 α-synuclein 変異は Al53Thr[44]，Ala30Pro[45]の2種であり，しかもその変異を持つ家系は，きわめて少ない．しかしながら，α-synuclein は Lewy 小体のもっとも主要な構成成分であることが証明された．またこの遺伝子を overexpression させた mice や fruit fly では Lewy 小体の形成が証明されている．Fruit fly に関しては運動機能低下が報告されている．最近になり Ala53Thr 変異を over expression させた mice で運動機能と Lewy 小体の形成が証明されている．したがって，α-synuclein の機能解析をふくめた研究は孤発型 PD の解明に繋がる可能性がある．近年，Markopoulou らにより，α-synuclein の Ala53Thr 変異をもつ家族性 PD の家系で，mutant allele の mRNA レベルが発現していないハプロ不全という状態が関与することが報告された[46]．われわれは，ネブラスカ大の Katerina Markopoulou 先生，メーヨクリニックの Zbigniew Wszolek 先生，そしてチュービンゲン大の Olaf Riese 先生との共同研究で，この家系とは別の Al53Thr 変異を持つアメリカ在住ギリシャ人家系と Ala30Pro の変異を持つドイツ人家系についてハプロ不全の関与について検討した．患者由来のリンパ芽球をもちいて DNA および RNA を抽出し，それをもちいて PCR，RT-PCR をおこない，各制限酵素で消化し，変異の有無を確認した．DNA では確かに各変異が存在していることを確認した．この方法では定量が不可能なので，TaqMan PCR にて定量した．この方法では TaqMan probe と呼ばれる 20〜30 塩基ほどの両端に蛍光ラベルした oligonucleotide を使う．この probe の両端には reporter (5′末端に Fluorescein 系の蛍光色素)，quencher (3′末端に Rhodamine 系の蛍光色素) と呼ばれる2つの蛍光色素がラベルされており，この状態では蛍光共鳴エネルギーの移動現象により reporter の蛍光は抑制される．PCR の伸長反応が進むと，probe が加水分解され reporter の遊離にともない蛍光強度が増加し，指数関数的に増幅された PCR 産物を蛍光強度から real time に定量測定ができる．

われわれは Wild と Mutant を特異的に認識するように 3′-end specific primer を設定し，mutant と wild を区別できる条件を決め，α-synuclein mRNA の定量をおこなった．各 mutant allele について定量した結果，ドイツ家系では罹患期間が長い症例で mutant allele の発現がほとんどみとめなかった．アメリカ-ギリシャ家系では痴呆症状のある患者で mutant allele の発現をみとめなかった．各変異については RT-PCR で確認しており，mutant allele の発現のみとめられなかった患者では直接塩基決定法でも wild type が配列として示されていた．Wild の量と Mutant の量の比では，臨床症状の重症度に相関関係がみとめられた (Fig. 17A〜C)．以上のことから α-synuclein に変異を持つ家系では，リンパ芽球をもちいた mRNA 定量にて臨床症状と mutant allele の発現が低下するハプロ不全に相関が，ことなる二種の変異型を持つ家系でみとめられた[47]．ハプロ不全は蛋白レベルでもおこっており，HPLC/Mass spectrometry で解析したところ確かに mutant allele にハプロ不全をみとめるリンパ芽球では wild type の α-synuclein のみが発現していた．つまり蛋白レベルでハプロ不全が生じていることが証明されたわけである．リ

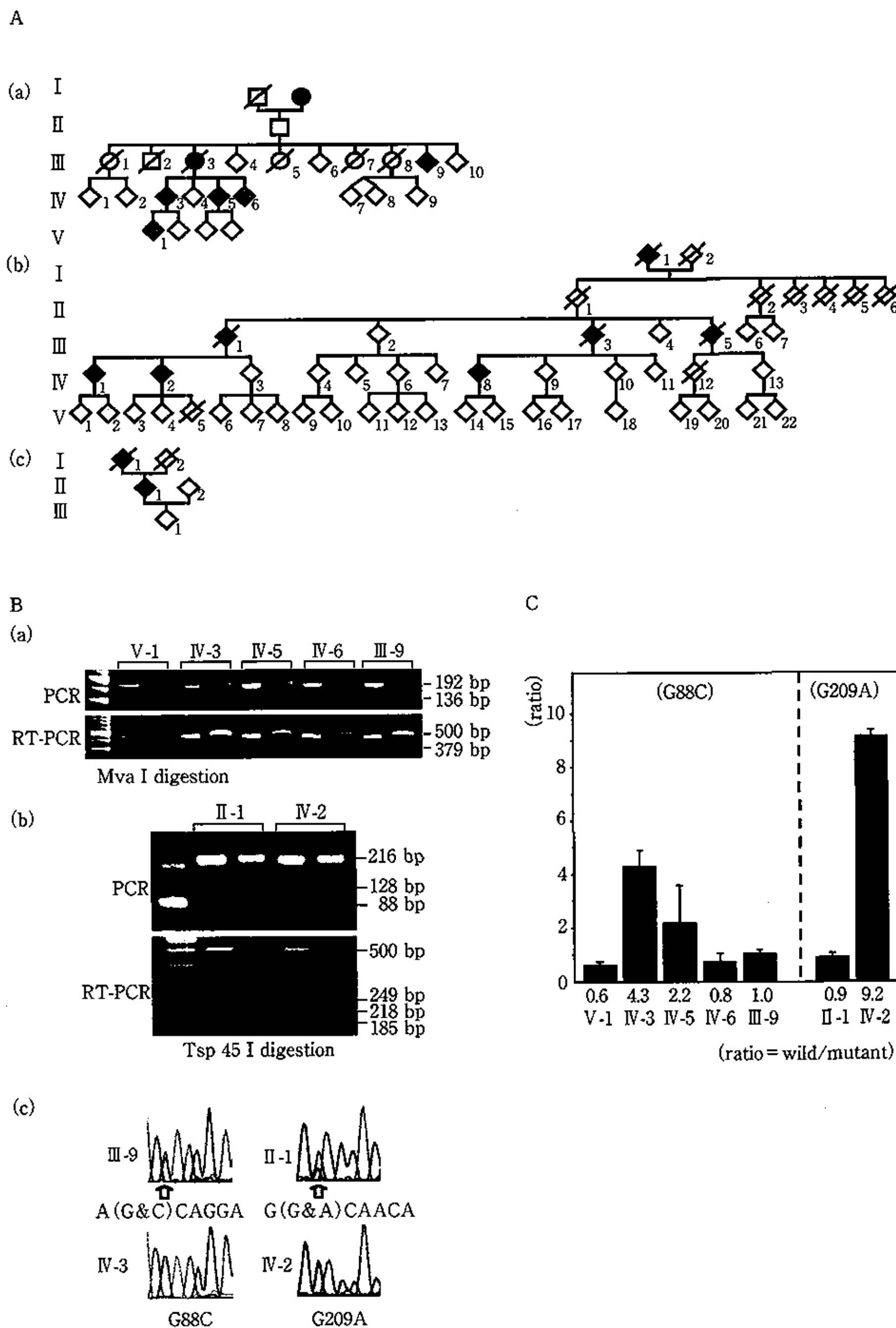

Fig. 17　A（a）はドイツ家系（Ala30Pro[G88C]変異）．（b〜c）はギリシャ-アメリカ家系でAla53Th[G209A]r変異を持つ．B-(a, b)：すべての発症者は変異を持つことが確認された（PCR-RFLP）．RT-PCRではIV-3の罹患者はDNA上変異を持つが，RT-PCRでは変異アリルが発現していない．B-(c)：直接塩基決定法では，やはり変異アリルの発現を観察できない．C：変異アリルと正常アリルの比を検討した．IV-3はもっとも症状が重く，また罹患期間も長い．IV-2は症状も重症だが痴呆をみとめる．変異アリルの発現は臨床症状と逆相関にあり，ハプロ不全は臨床症状を反映している．蛋白レベルでも変異アリル由来の変異蛋白は発現していない．

ンパ芽球でハプロ不全がみとめられたが，脳での状態を反映しているかは不明である．しかしながら，臨床症状との関連で注目すべき現象であり，α-synuclein変異を持つ家族性PDに共通した現象と捉えられる．このハプロ不全については，parkin遺伝子変異をヘテロ接合体で持ち発症している患者の機序についても説明可能である．したがって家族性PDの発症にこのハプロ不全は共通した機序として存在する可能性もある．

IV. 家族性パーキンソン病から孤発型パーキンソン病へ

(1) パーキン遺伝子多型と孤発型パーキンソン病

われわれは孤発型PDのミトコンドリア研究からAR-JPの原因遺伝子単離に成功した．この単一遺伝子異常である家族性PDの研究からユビキチン・プロテアソーム系が重要であることが推定された．Lewy小体はユビキチン陽性の細胞内封入体であり，ここでユビキチン・プロテアソーム系の関与がクローズアップされたといって過言でない．そこでわれわれはPark2，Park5の原因遺伝子パーキン遺伝子とUbiquitin carboxy-terminal-L1遺伝子多型に焦点をあて，孤発型PDのリスクファクターに成るか否か検討した．まずわれわれはパーキン遺伝子の変異解析の過程で，アミノ酸置換のある三種の遺伝子多型をみいだした[48]．みいだされた遺伝子多型は，S/N167，R/W366，そしてV/L380の三カ所で，年齢分布が一致するコントロール群と家族歴のない孤発型PD群で，各遺伝子多型の頻度を検討した．S/N167，V/L380については両群で有意差をみとめなかったが，R/W366については，孤発型PD vs. コントロールが1.2 vs. 4.4%と孤発型PD群で頻度が低かった．このことよりこのR/W366遺伝子多型はPD発症に関して保護的に作用している可能性が推定された．この結果は，台湾人のPD患者でも再現されており，少なくともアジア系PD患者では保護的に作用している可能性がある．

UCH-L1遺伝子は優性遺伝性PDの原因遺伝子であるが，現在まで病的変異は1家系のみである．われわれの優性遺伝性PDがうたがわれる家系で変異を解析したが，変異はみいだせなかった[49]．しかしながら，この遺伝子に遺伝子多型が存在していたので，みいだされた遺伝子多型について検討をおこなった．UCH-L1遺伝子にS/Y18の遺伝子多型をみいだしたので，そのS/Y18の頻度について検討した．この研究は白人患者についても解析するためにイギリスの神経学研究所のNicholas Wood先生，Novartis社のMichael Polymeropoulos先生の協力をえておこなった．多型であるY型が日本人で多く，wild typeとされているS型が少ない傾向にあった．日本人患者では約30%と発症をおさえる保護的作用をみとめた[50]．しかしながら，白人では保護作用がなく人種間における遺伝子多型頻度を考慮すべき問題として上げられた．しかしながら，解析数を増やすとこの多型は保護的作用を持っている可能性が指摘されはじめており，世界規模で統計学的処理をおこなっている．

現在も真の危険因子の同定を目指して様々な遺伝子多型について検討している．遺伝性PDが多様性を示しているように孤発型PDもまた多様性をもった集団と考えるのが妥当と考える．現在オーダーメード医療を目指してあらゆる臨床症候と遺伝子多型について国立精神・神経センター村田美穂先生，香川県立中央病院の山本光利先生，大阪大学臨床遺伝学戸田達史先生との共同研究で推進している．

(2) 臨床マーカーの開発を向けて：尿内のフリーラジカルの測定

先に触れたMPTPの黒質神経細胞死の機序は，直接ミトコンドリアのエネルギー産生系を阻害することにより細胞死を惹起させることがわかっている．またエネルギー産生系だけでなく活性酸素種を発生させることもわかっており，PDの発症機序を考えるうえでミトコンドリア機能低下と酸化的ストレスの関与は重要である．そこでこの酸化ストレスの関与を示すようなマーカーの開発を検討した．PDでは脳内の8-OHdGが有意に上昇していることが報告されていたので，この8-OHdGに注目して検討している．尿中の8-OHdGは，患者に対する侵襲が少なく簡単に検査できることより有効なマーカーになりうるかもしれない．現在，MAO-inhibitorであるDeprenylの神経保護作用と併せて臨床マーカーになりうるか検討中である．

(3) 孤発型パーキンソン病と家族性パーキンソン病の共通メカニズムそして新薬開発に向けて

家族性PDの原因遺伝子産物であるparkin蛋白とUCH-L1はユビキチン・プロテアソーム系の直接分子であり，α-synunclein はユビキチン陽性であるLewy小体の主要成分である．このことはユビキチン・プロテアソーム系がPDの発症メカニズムにおいて主要なカスケードであることを示す．更にparkin-related diseaseの病理にLewy小体をみとめないこともその系の関与を推定させるものと考えている．一方，cell biologyを使った系でのα-synucleinの凝集に関する研究では，その凝集にミトコンドリア機能低下が必要とされている．26S proteasomeはATP依存性の蛋白分解をおこなうことよりミトコンドリア機能低下はユビキチン・プロテアソーム系の機能障害をひきおこす可能性が考えられる．つまりミトコンドリア機能低下とユビキチン・プロテアソーム系は密接な関係にあると推定される．

われわれがおこなってきたPDの発症機序に関する研究は，ミトコンドリア研究にはじまり，その研究結果からえられたヒントをもとに家族性PDの原因遺伝子parkinの単離に繋がった．更にparkin蛋白の機能がユビキチン・プロテアソーム系の構成酵素であるユビキチンリガーゼであることを明らかにすることができた．Parkin遺伝子の発見と機能解析の成功は，多くの神経変性疾患で観察されている封入体の形成機構を考える上で重要な情報を提供することになった．とくにPDではLewy小体の細胞毒性の有無については今後の検討が必要であるが，少なくともその形成過程が細胞毒性を示すのはまちがいないと考えている．したがってPDにおいてはLewy小体の形成を阻止できれば神経細胞死を抑制できる可能性が高い．さらに封入体形成はPDのみならず他の多くの神経変性疾患でも共通にみとめられており，この封入体形成を阻止できる薬物が開発されれば多くの神経変性疾患の治療に利用できると予想される．従来の治療方法はドパミンの補充に重点がおこなわれてきた．しかしながら，parkin蛋白の機能解明からのヒントからユビキチン・プロテアソーム系が神経変性に関与していることが推定されている．更にユビキチン・プロテアソーム系の関与従来の治療方法とはこ

となる新しい方向性を提供している．事実，ショウジョウバエのPD modelの解析からはシャペロンが，新薬としての可能性が指摘されている．われわれのおこなってきた研究は更にLewy小体形成メカニズム解明へとシフトさせ，Lewy小体形成の分子機構を解明すると共にシャペロン機能を代償することが可能な薬剤の開発やユビキチン・プロテアソーム系の機能促進因子の開発などが次なる課題である．

謝辞：本研究は，ミトコンドリア研究をはじめparkin遺伝子やparkin蛋白のユビキチンリガーゼの発見，その基質同定まで多くの共同研究者により遂行されてきたものであります．ここに多くの共同研究者の方々に深甚の謝辞を表したいと思います．またここに記した研究成果には，水野神経学教室の多くの教室員，大学院生，研究員，技術員の方々が参加しております．この成果は水野神経学教室のチームワークの賜であります．最後に特別論文の寄稿の機会を与えていただいた神経学会運営委員会および編集委員会の先生方に深甚の謝辞を表したいと思います．

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Abstract

Etiology and Pathogenesis of Parkinson's disease : from mitochondrial dysfunctions to familial Parkinson's disease

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Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. It is urgently needed to elucidate the cause of the disease and to establish neuroprotective treatment.

We have been working on the etiology and pathogenesis of PD for many years and we found selective loss of mitochondrial complex I and the alpha-ketoglutarate dehydrogenase complex in the nigral neurons of patients with PD. Our observation firmly established mitochondrial defects in PD. Mitochondrial respiratory failure induces oxidative damage in neurons, and we found increase in hydroxynonenal and 8-oxo-deoxyguanine, indices of oxidative damage, in the nigral neurons of PD. These abnormalities can trigger apoptotic cell death.

The primary events which induce mitochondrial failure and oxidative damage are not known, however, it has been postulated that the interaction of genetic risk factors and environmental factors would initiate the degenerative process. Based on this assumption, we conducted genetic association studies by the candidate gene methods. We found that polymorphic mutations of superoxide dismutase-2 and 24-kDa subunit of mitochondrial complex I were associated increased risk of developing Parkinson's disease.

While we were doing this genetic association study, we found a family, in which parkinsonian phenotype completely segregated with a polymorphic mutation of the superoxide dismutase-2 gene. In this family, 4 out of 6 siblings were affected with early onset parkinsonism and the parents were apparently normal. Thus the mode of inheritance appeared to be autosomal recessive and this type is now called as AR-JP or Park2. We confirmed the linkage of this type of familial Parkinson's disease to the superoxide dismutase loci that is located in the telomeric region of chromosome 6 by the linkage analysis using microsatellite markers in this region.

Then we found another family, in which an affected patient showed lack of one of the microsatellite markers (D6S315), which we were using in the linkage analysis. This observation prompted us to initiate the molecular cloning of the disease gene utilizing D6S315 as the initial probe. The molecular cloning was done with the collaboration with Professor Nobuyoshi Shimizu of Keio University. We identified a novel gene and confirmed that mutations of this novel gene were found only in the patients with autosomal recessive Parkinson's disease. The novel gene was named parkin.

We conducted mutational analysis on more than 700 families with Parkinson's disease. We also established a method to detect compound heterozygotes of parkin mutations. Mutinous of the parkin gene were found in approximately 50% of autosomal recessive families. Many kinds of exonic deletions and point mutations were found. This type of familial Parkinson's disease had been considered to be unique among Japanese, but since we started mutational analysis of the parkin gene, we confirmed the world wide distribution of parkin gene mutations.

Then we analyzed functions of parkin protein with the collaboration with Dr. Keiji Tanaka of Tokyo Metropolitan Institute of Medical Sciences. We found that parkin protein was a ubiquitin-protein ligase of the ubiquitin system. Now we are working on the candidate substrates of parkin protein as a ubiquitin ligase. We found that CDCrel-1, a synaptic vesicle protein, was a candidate substrate of parkin protein. In addition, we found two additional candidate proteins, i.e., alpha-synuclein 22 and PAEL receptor, with the collaboration of Professor Denis Selkoe of Harvard Medical School and Dr. Ryosuke Takahashi of RIKEN, respectively. Accumulation of PAEL receptor in the endoplasmic reticulum causes endoplasmic reticulum stress and apoptotic cell death. We found evidence to indicate accumulation of PAEL receptor and the presence of endoplasmic reticulum stress in a patient with AR-JP (Park2).

Thus our studies firmly established that a genetic defect of an enzyme in the ubiquitin-proteasome system induces selective nigral neuronal death. We indicated the important role of the ubiquitin-proteasome system in neurodegeneration in general. In many other neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, Machado-Joseph disease, dentatorubral-pallidoluysian atrophy, and ALS, ubiquitinated proteins are accumulated in neurons. Thus protein handling in the ubiquitin-proteasome system appears to be affected in these neurodegenerative disorders despite the difference in the primary defects. Our studies also suggest many potential approaches for the discovery of neuroprotective treatment for not only Parkinson's disease but also other neurodegenerative disorders.

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Review

Ubiquitin, proteasome and parkin

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Abstract

The ubiquitin–proteasome system (UPS) is important for intracellular proteolysis, and is responsible for a diverse array of biologically important cellular processes, such as cell-cycle progression, signaling cascades and developmental programs. This system is also involved in the protein quality control, which maintains the health of the cell. Thus, the UPS provides a clue for understanding of the molecular mechanisms underlying various neurodegenerative diseases. In the last decade, we witnessed a tremendous progress in uncovering the mechanisms of Parkinson's disease (PD). Of the several genes that can cause familial PD, parkin, the causative gene of autosomal recessive juvenile parkinsonism (ARJP), is of a special interest because it encodes an ubiquitin-protein ligase, which covalently attaches ubiquitin to target proteins, designating them for destruction by the proteasome. This review summarizes recent studies on the UPS pathway with a special reference to parkin, focusing on how parkin is linked to the pathogenesis of ARJP.
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Keywords: Neurodegeneration; Parkin; Parkinson's disease; Proteasome; Quality control; Ubiquitin

1. Introduction

Numerous studies have recently emphasized the biological importance of the ubiquitin–proteasome system (UPS), which is capable of catalyzing rapidly, timely, and unidirectionally a multitude of biological reactions including cell-cycle progression, DNA repair, cell death (e.g., apoptosis), signal transduction, transcription, metabolism, and immunity [1–3]. In addition to regulating the functions of divergent proteins, UPS plays a major role in stress response and in protein homeostasis, i.e., protein quality control, not only in the endoplasmic reticulum but also in the cytosol of eukaryotic cells [4,5]. There is sufficient evidence at present to indicate that the function of UPS is closely related to the etiology of neurodegenerative disorders, particularly Parkinson's disease (PD). Specifically, dopaminergic neurons are vulnerable to the failure of UPS-mediated protein catabolism and accumulation of as yet unidentified protein(s) caused by dysfunction of certain pathway(s) of UPS causes the loss of dopaminergic neurons. Studies of this topic have focused on the role of UPS in the pathogenesis of both familial and sporadic PD.

2. Ubiquitin

Ubiquitin (Ub), consisting of 76 amino acid residues, is a highly conserved small protein that acts as a degradation marker for a wide spectrum of cellular proteins and a unique molecule of intracellular proteolysis [1]. It is first activated ATP-dependently by an E1 (activating enzyme), forming a high-energy thioester bond between ubiquitin and an E1, and the activated ubiquitin is then transferred to an E2 (conjugating enzyme), forming a similar thioester linkage between ubiquitin and an E2 (Fig. 1). In some cases, E2 directly transfers ubiquitin to the target proteins, but the reaction often requires the participation of an E3 (ligating enzyme, and thus referred as ubiquitin-protein ligase). Through a cascade of enzymatic reactions, ubiquitin is covalently attached through its C-terminal Gly residue to the ε-NH_2 group of the Lys residue on the target proteins. Finally, a polyubiquitin chain is formed by repeated reactions through which another ubiquitin links a

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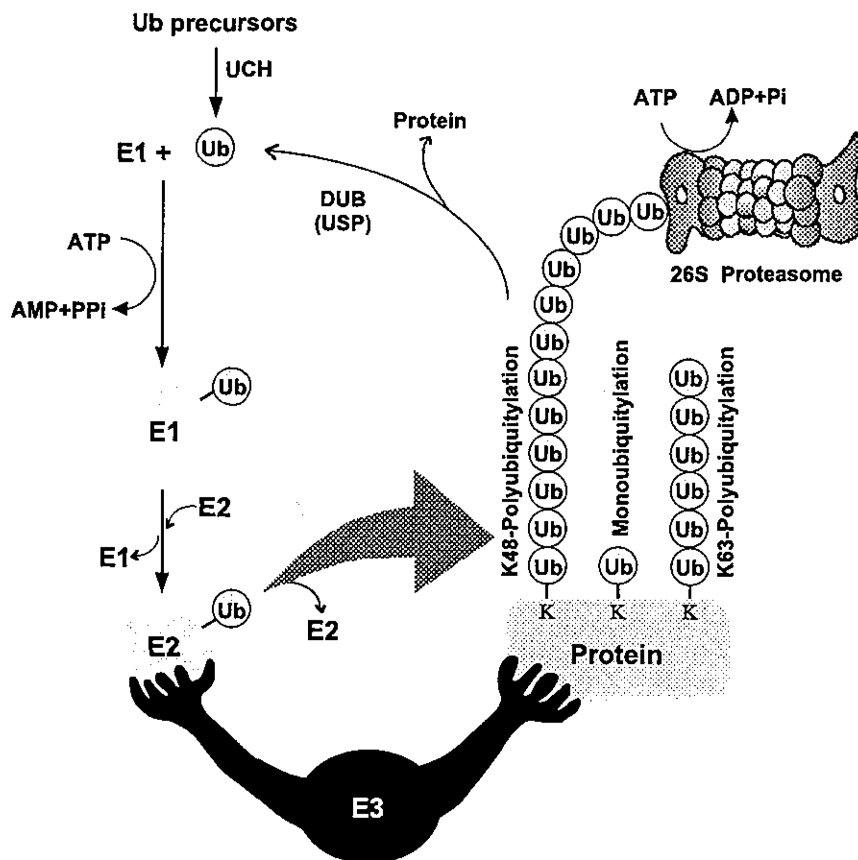

Fig. 1. The ubiquitin–proteasome system. Ub, ubiquitin; E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes; DUB, deubiquitylating enzyme; USP, ubiquitin-specific protease; HCH, ubiquitin C-terminal hydrolase. The 26S proteasome is a eukaryotic ATP-dependent, multi-subunit proteolytic complex. See text for details.

Lys residue at position 48 within one ubiquitin associated with the target protein (Fig. 1). Ubiquitin has seven Lys residues, which are all used for polymerization catalyzed by this ubiquitin-modifying system [2,6], but a polyubiquitin chain formed via Lys at 48 functions mainly becomes a marker for proteolytic attack by the 26S proteasome (a eukaryotic ATP-dependent 2.5-MDa multi-subunit complex) [7,8]. In addition, the K63-linked polyubiquitylation and monoubiquitylation without the formation of an ubiquitin tree have many biological roles other than proteolysis [2,3], but the details of this type of ubiquitylation are not the focus of this review.

To date, it is known that there is a single E1 for ubiquitylation, whereas E2 enzymes consist of a family of proteins, consisting of over a dozen of species in mammals. Moreover, E3s are considered to exist as molecules with a large diversity, presumably in more than hundreds or thousands species, because E3 can trap not only target protein(s) but also ubiquitin activated by the E1–E2 coupled reaction, and thus is capable of catalyzing the successive transfer of ubiquitin to the protein (see Fig. 1). Thus, in the UPS pathway, E3 plays a critical role in the selection of target proteins for degradation, because each distinct E3 usually binds a protein substrate with a degree of selectivity for ubiquitylation in a temporally and spatially regulated fashion.

So far, E3s are classified into several groups but here we propose that they should be more appropriately categorized into four types (Table 1). One is the HECT-type E3 encompassing E3 with a domain capable of binding ubiquitin as a thioester bond, termed "HECT", which harbors 350 amino acid region of homology to the E6-AP (the first identified HECT-type E3) carboxyl terminus [9]. The major group of E3s is named RING-type E3, a general term for ubiquitin-ligases with a RING-finger motif(s) consisting of the Cys-rich consensus sequence flanked by one or two His residue(s) [10,11]. The RING-finger motif is capable of binding Zn^{2+}, and is subcategorized into typical and atypical forms. The typical RING-type E3s contain three classes with subtle differences in their structure: RING-HC (C3HC4), RING-H2 (C3H2C3), and RING-IBR-RING. The atypical RING-type E3s are structurally somewhat divergent compared with the typical types. Some of these E3s contain proteins with the PHD domain (and thus often called PHD-E3s), but this domain is not directly linked to E3 activity [12]. The third type of E3s have the U-box domain whose tertiary structure resembles the of RING-finger domain [13,14], but does not show a binding potency to Zn^{2+}, which is probably required for keeping the domain structure in RING-type E3s. Interestingly, some of the U-box E3s (e.g., Ufd2) can promote a polyubiquitin chain, provisionally termed as "E4" activity,

Table 1
Classification of E3 ubiquitin-protein ligase

HECT-type:	AIP4, Ceb1, E6-AP, Herc2, Hul4, Hul5, hHYD/EDD, Itch, Nedd4, Pub1/2, Rsp5, Smurf1/2, SU/DX, Tom1, Ufd4, WWO1, WWP2, etc.
RING-type:	
(1) RING-HC (C3HC4)-finger:	BBAP, BRCA1, (Bre1), Cbls, Chfr, DTX3 (Deltex3), Efp, Hakai, HEI10, IAPs, ICP0, IE2, LNX, Mahogunin (mahoganoid), Mdm2, Mdmx, MID1, Mind Bomb (Mib), Momo, Neuralized (Neu), Nrdp1/FLRF, RAG1, Rma1, RNF2/HIPI-3, Sakura, Siah-1, SINAT5, Staring, TRAF6, etc.
(2) RING-H2 (C3H2C3)-finger:	AO7, Apc11, ARNIP, CIP8, DTX1 (Deltex1), DTX2/Deltex2, EL5, gp78, GRAIL/GREUL1, Hrd1, kf-1, NFX-1, Pirh2, Praja1/PJA1, Rbx1, RLIM, TRC8, Tul1, Ubr1
(3) RING-IBR-RING-finger:	Dorfin, HOIL-1, Parc, Parkin, etc.
(4) Atypical RING-finger:	K3/MIR1, K5/MIR2, MEKK1, Doa10, Pib1, CNOT4, etc.
U-box type:	ARC1, CHIP, CYC4, *PRP19*, *Ufd2*, Ufd2b UIP5, etc.
Others:	ICP0 HUL-1 domain, $TAF_{II}250$, (E1+E2 activity), $(UCH\text{-}L1)_2$, p300, etc.

HECT, homologous to E6-AP carboxyl terminus; RING, really interesting new gene; HUL-1, herpes virus ubiquitin ligase-1.

in the presence of E3 [15]. The fourth group of E3 consists of very unique E3s [ICPO, $TAF_{II}250$, $(UCH\text{-}L1)^2$, and p300] that have no sequence homology to known E3 enzymes. ICPO has two catalytic sites: one RING-HC and another novel HUL-1 motif [16]. $TAF_{II}250$ has intrinsic E1 and E2 activities within a single molecule, which exhibits no homology to other E3s [17]. $(UCH\text{-}L1)_2$ is the dimeric form of UCH-L1 (functioning as a de-ubiquitylation enzyme in a monomeric form) and exhibits E3 activity (see below) [18]. p300 exhibits E4-like activity in the presence of Mdm2 E3 ligase [19]. However, whether the above E3s are truly ubiquitin-ligases remains elusive at present. It is of note that all E3s except HECT-type E3s are probably not covalently bound to ubiquitin. It is plausible that certain domains, such as RING-finger or U-box, recruit E2s to the vicinity of proteins to be ubiquitylated and thus mediate ubiquitylation by facilitating the direct transfer of ubiquitin from E2-ubiquitin to the target Lys residue.

Interestingly, eukaryotic cells contain an unexpectedly large number of deubiquitylating enzymes (DUBs), which are also called Ub-specific proteases (USPs) (see Fig. 1). They belong to a family of cysteine proteases subclassified into at least two gene families that are structurally unrelated; the UCH (ubiquitin C-terminal hydrolase) family and the UBP (ubiquitin-specific processing protease) family [20]. DUBs may contribute to the production of a functional ubiquitin moiety from its precursors as well as disassembly of degradation intermediates generated by the 26S proteasome. Indeed, ubiquitin is reutilized, but not degraded in the breakdown of ubiquitylated proteins. DUBs are also thought to catalyze the reversal of the ubiquitylation reaction for "proofreading" of incorrectly ubiquitylated proteins or "trimming" of abnormal polyubiquitin structures, which play an essential role in facilitated proteolysis mediated by the 26S proteasome [21]. Intriguingly, a loss-of-function of many DUB genes by deletion or mutation or a gain-of-function by overexpression causes the induction of abnormalities in metabolism, growth, and differentiation of cells [20]. However, the reason for the presence of so many members of DUB in cells remains to be elucidated.

Ubiquitin is encoded in two types of unique genes [1]. One is a polyubiquitin gene encoding a tandemly repeated ubiquitin structure, which belongs to "heat-shock gene". Another ubiquitin gene produces an ubiquitin fused with certain ribosomal proteins, the biological significance of which is still unknown. After synthesis, these ubiquitin precursors are converted to functional molecules, the processing reaction of which is catalyzed by a set of UCH and/or certain UBPs as mentioned above. Clearly, the presence of stress-inducible polyubiquitin gene indicates that cells need a large amount of the free ubiquitin pool to respond to various environmental stresses, which serves to prevent accumulation of abnormal proteins in cells by selective proteasomal elimination after facilitated ubiquitylation of these unnecessary proteins. Thus, it becomes clear that UPS plays a pivotal role in regulating and maintaining protein homeostasis in cells.

3. Proteasome

Most cellular proteins in eukaryotic cells are targeted for degradation by the 26S proteasome, usually after they have been covalently attached to ubiquitin in the form of a polyubiquitin chain functioning as a degradation signal. The 26S proteasome is a eukaryotic ATP-dependent protease responsible for selective degradation of the polyubiquitin-tagged proteins. It is an unusually large multisubunit proteolytic complex, consisting of a central catalytic/core particle (CP, equivalent to a 20S proteasome) and two terminal regulatory particles (RP, also termed PA700 or 19S complex), which are attached to both ends of the central portion in opposite orientations to form the enzymatically active proteasome (reviewed in Refs. [7,8] and references therein). It appears to act as a highly organized apparatus designed for efficient and exhaustive hydrolysis of proteins, and can in fact be regarded as a protein-destroying machinery. The 20S proteasome is a barrel-like particle formed by the axial stacking of four rings made up of two outer α-rings and two inner β-rings, which are each made up of seven structurally similar α- and β-subunits, respectively, being associated in the order of αββα. Three β-type subunits of each inner ring have catalytically active threonine residues at

their N-terminus (in which of β1, β2, and β3 corresponding to caspase-like, trypsin-like, and chymotrypsin-like activities, respectively), and these active sites face the interior of the cylinder and reside in a chamber formed by the centers of the abutting β rings [22]. Thus, substrates gain access to the active sites only after passing through a narrow opening corresponding to the center of the α rings and the amino-termini of the α subunits form an additional physical barrier for substrates to reach the active sites.

Interestingly, the center of the α-ring of the 20S proteasome is almost closed, preventing penetration of proteins into the inner surface of the β-ring on which the proteolytically active sites are located. The regulator, PA700, was discovered as an activator of the latent 20S proteasome. PA700 (alias RP) consists of two subcomplexes, known as "base" and "lid", which, in the 26S proteasome, correspond to the portions of PA700 proximal and distal, respectively, to the 20S proteasome [23]. The base is made up of six ATPases and two large regulatory components while the lid contains multiple non-ATPase subunits. The base complex, thought to bind ATP-dependently to the outer α-ring of the central 20S proteasome, seems to be involved in opening the gate of the α-ring for entry of the protein substrate. The metabolic energy liberated by ATP consumption is probably utilized for the assembly of the base complex with the 20S proteasome, although it may also be used for unfolding target proteins, gate opening of the 20S proteasome, and substrate translocation so that they can penetrate the channel of the α- and β-rings of the 20S proteasome [24]. On the other hand, the lid-complex is thought to be involved in the recognition of target proteins, deubiquitylation for reutilization of ubiquitin, and interactions with various proteins including proteins with an ubiquitin-like domain or certain E3(s). Interestingly, it was found that Hsp90 interacts with the 26S proteasome and plays an important role in the assembly and maintenance of the 26S proteasome [25].

4. Protein quality control and Parkinson's disease

Over 30% of the newly synthesized cellular proteins are discarded without being properly folded, i.e., misfolded, and/or unassembled, even though they are normally synthesized without mutations of their genes or errors in the translation process [26]. Such inferior quality of the protein synthesizing-machine seems surprising, because it is thought that the biological system at present has been elegantly formulated during evolution. In addition, even if proteins are synthesized and folded accurately as functional proteins with normal tertiary structures, they often undergo damages due to various stresses under poor surroundings [4]. In fact, the high density of protein molecules in the intracellular space increases the spontaneous denaturation and consequently the likelihood of partially folded or unfolded proteins to undergo off-pathway reactions, such as aggregation. In addition, environmental stresses, such as heat, oxidation (i.e., formation of free radicals), and ultraviolet, could result in the production of impaired proteins. However, it is worth emphasizing that those proteins with non-native or aberrant structures are not observed in normal cells, because they are rapidly removed inside the cells. In other words, the cell is fully equipped with a surveillance system that rapidly eliminates such abnormal proteins unwanted for their presence in the cell.

In this regard, the cellular apparatus monitoring the "normality" of proteins in the cell is usually referred to as "the protein quality control system" [4,5]. Cells are equipped with two systems that prevent accumulation of abnormal proteins formed through the protein biosynthetic pathway or postsynthesis damage. One is the molecular chaperone(s) that recognizes proteins with non-native structures to prevent them from irreversible aggregation and help their conversion to a functional conformation. The other system is UPS, which is responsible for selective destruction of misfolded/unfolded and unassembled proteins, which probably fail to refold assisted by the chaperone system. Since both the chaperones and proteasomes conceptually recognize common substrates under non-native states, these two pathways act together to prevent aggregation and accumulation of such harmful proteins, thus maintaining protein homeostasis in the cell.

This monitoring machinery is considered an integral cellular component involved in maintaining cell survival and homeostasis, because it prevents the accumulation of abnormal proteins, formation of toxic inclusion bodies, with subsequent cell death, as seen in various neurodegenerative diseases [27]. It is well known that UPS is involved in the immediate destruction of proteins impaired by environmental and/or intracellular stresses. In this regard, the importance of UPS has been recently highlighted in the non-dividing cells of the brain, since neuronal intracellular inclusions are composed of ubiquitin-positive protein aggregates that have recently been described as a common ultrastructural feature of many neurodegenerative diseases, such as PD, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) and Prion disease [27]. In fact, it is also known that proteasome inhibitors increase the frequency of ubiquitin-positive intracellular inclusions in the cells carrying many neurodegenerative disorder genes. Therefore, one could assume that a critical aspect of various neuronal degenerative diseases is failure of the UPS-protein quality control system. Indeed, ample evidence has been provided for the potential link between failure of the protein quality control and neurodegeneration. In this regard, among many neurodegenerative disorders, PD is the most interesting, because there is direct evidence showing that the cause of PD is closely linked to functional abnormality of the UPS pathway [28].

Parkinson's disease, a severe neurological disorder of movement, is the second most common neurodegenerative

disorder [29]. The prevalence of PD increases markedly with age, affecting 1–2% of the population above 65 years. PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta in the midbrain. The clinical symptoms include resting tremor, rigidity, bradykinesia, and postural instability. Sporadic PD is characterized by loss of dopaminergic neurons, i.e., selective neurodegeneration of the pigmented neurons, such as substantia nigra and locus coeruleus, in the brain stem. The pathological hallmark of PD is the presence of cytoplasmic proteinaceous inclusions known as Lewy body (LB) in surviving neurons [30]. Indeed, LB is often observed in sPD. LB displays a core and halo organization and stains strongly with anti-ubiquitin antibodies, and hence many proteins composed of LB are thought to be heavily ubiquitylated, implying that PD is associated with deviant behavior of the ubiquitin metabolism.

Approximately 5–10% of patients with PD are estimated to belong to the familial form of PD. The recent identification of several genes and gene loci linked to the familial forms of PD has significantly enhanced our understanding of the genetic mechanisms of PD [31–33]. The overlapping clinical and pathological features of idiopathic PD and familial Parkinsonism indicate a common underlying molecular pathway in all PDs. These outcomes suggest that the discovery of genes responsible for the familial forms of the disease should shed new light on the mechanism(s) leading to the selective dopaminergic cell loss in sporadic PD. Indeed, kindreds with Mendelian pattern of inheritance of PD have been known a long time ago. To date, 10 loci have been mapped in familial PD by linkage analysis and four causative genes have been cloned [31–33]. These genes of familial PD are subdivided into the autosomal dominant forms, i.e., PARK1 encoding αSynuclein (αSN) and PARK5 encoding ubiquitin C-terminal hydrolase L1 (UCH-L1), and the autosomal recessive forms, i.e., PARK2 encoding parkin and PARK7 encoding DJ-1. The biochemical features of proteins encoded by the familial PD genes are mostly unknown, but it is marvelous that parkin, a product of the causative gene PARK2 of autosomal recessive juvenile parkinsonism (ARJP), is a ubiquitin-protein ligase, indicating that ARJP is linked to impediment of proteolysis mediated by UPS. The following section will review the structure and pathophysiology of parkin.

5. Parkin

5.1. ARJP

ARJP/Park2 was first reported in 1973 by Yamamura et al. [34]. It is a levodopa-responsive parkinsonism that develops before 40 years of age (average onset around 25 years). Additional clinical festures include foot dystonia, sleep benefit, diurnal fluctuation, hyperactive tendon reflexes and less frequent resting tremor compared with sPD. Dystonic features and sleep benefit are common, particularly when the age of onset is young. In addition to these clinical features, genetic analysis of the parkin gene has expanded the clinical spectrum of ARJP. For example, Klein et al. [35] reported parkin deletions in a family with clinical features essentially identical to those of idiopathic PD and none of the family members displayed any of the clinical hallmarks described in patients with previously reported parkin mutations. The age of onset was 31 to 75 years, and showed apparently autosomal dominant inheritance. Maruyama et al. [36] also reported a Japanese family with pseudo-autosomal dominant inheritance with parkin gene mutations, although the age of onset was in the young range. These analyses broadened the clinical spectrum of ARJP, and we realize that ARJP is quite similar to adult-onset sporadic PD.

5.2. The parkin gene

The gene responsible for ARJP maps to 6q25.2–q27 chromosome, based on the indication by linkage to markers D6S305 and D6S253. The D6S305 marker was deleted in one ARJP patient [37]. By positional cloning within this microdeletion, in 1998 Kitada et al. [38] isolated a cDNA clone of 2960 bp with a 1395-bp open reading frame by positional cloning, and termed it parkin (formally PARK2). Mutations in the newly identified gene appeared to be responsible for the pathogenesis of ARJP and, therefore, the protein product was named as parkin. The *PARK2* gene is the second largest gene reported in human so far, spans over 1.4 Mb and has 12 exons. The isolated cDNA clone is 2960 bp with 1395-bp open reading frame, and encodes a protein of 465 amino acids with a molecular weight of about 52 kDa. Interestingly, the parkin gene is highly conserved across species, not only in vertebrates, such as human, rat, mouse but also in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogater* [39], suggesting that it plays a common role in various organisms. To date, various mutations such as exon deletions, exon multiplications or point mutations resulting in missense and nonsense changes of parkins have been reported in ARJP patients [40,41]. Mutations in the parkin are found in nearly 50% of patients with autosomal-recessive early-onset parkinsonism (less than 45 years of age).

5.3. The parkin protein

Parkin is characterized by the presence of an ubiquitin-like domain (UBL) at its NH_2 terminus and two RING finger motifs, flanked by one IBR (in between RING finger) motif, at its COOH-terminal region (Fig. 2). In 2000, parkin was found to be an E3 ubiquitin ligase, a critical component of the pathway that covalently attaches ubiquitin to specific proteins with a polymerization step to form a degradation signal [42–44]. Indeed, parkin catalyzes the addition of

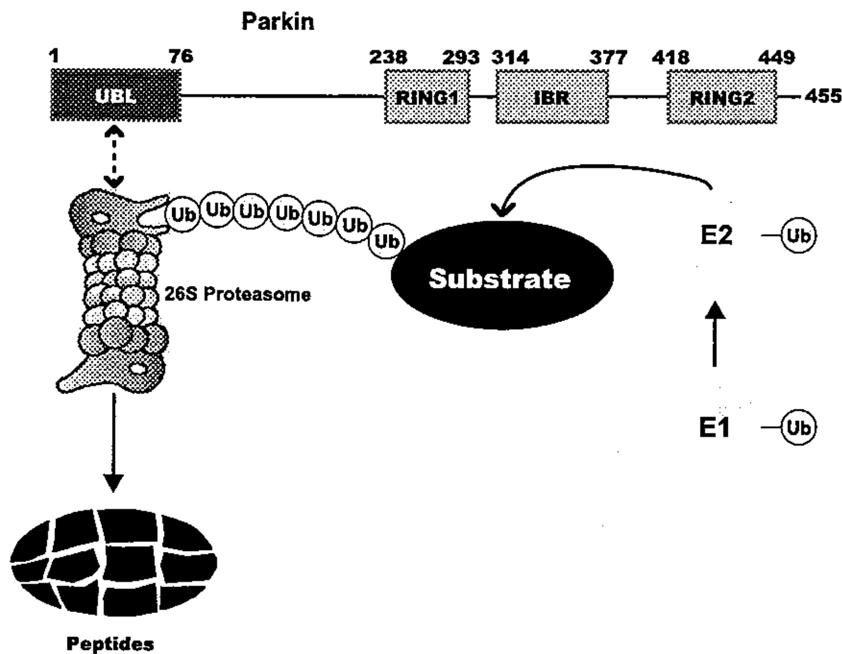

Fig. 2. Model of the parkin-directed ubiquitylation pathway. Ub, ubiquitin; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; UBL, ubiquitin-like domain. Parkin consists of two functionally distinct regions: a UBL segment responsible for interaction with the 26S proteasome and a RING-box (RING1-IBR-RING2) recruiting E2 for ubiquitylation. See text for details.

ubiquitin to target proteins prior to their destruction via the proteasome, indicating that the misregulation of proteasomal degradation of parkin substrate(s) is deleterious to dopaminergic neurons and, thus, impaired protein clearance can induce dopaminergic cell death. As mentioned, several types of mutations including deletion mutations and point mutations (both missense- and nonsense-mutations) have been detected in the parkin gene of ARJP patients. The site of these mutations spans almost all regions including the N-terminal UBL domain and the RING-IBR-RING domain, and there seems to be no difference of phenotypes among these mutation sites, indicating that the entire region of the parkin protein is essential for exerting its physiology.

As shown in Table 1, parkin belongs to a member of the RING-IBR-RING family, which is a subgroup of RING-finger type E3s. The RING-IBR-RING family is widespread in eukaryotes, with many members in animals (mammals, *Drosophila*, *Caenorhabditis*) and plants (*Arabidopsis*), although only two members exist in the budding and fission yeasts [45]. For instance, dorfin, HOIL1, and Parc are included in this family. Dorfin (double ring-finger protein) cloned from human spinal cord exhibits a RING finger-type ubiquityl ligase activity for mutant copper/zinc superoxide dismutase (SOD1) linked to ALS, but not for its wild-type [46]. Dorfin is predominantly localized in the inclusion bodies of familial ALS with SOD1 mutation as well as sporadic ALS [47]. Indeed, the cytopathological hallmark in the remaining motor neurons of ALS is the presence of ubiquitylated inclusions consisting of insoluble protein aggregates. Interestingly, dorfin is also localized with ubiquitin in LBs and colocalized in these large inclusions with ubiquitin and proteasomal components, suggesting that dorfin may be involved in LB formation and the pathogenic process of PD [48]. HOIL-1 (equivalent to XAP3/RBCK1/UIP28) resembles parkin, having the N-terminal UBL domain and C-terminal the RING-finger/IBR domain [49]. It is an E3 ligase that recognizes oxidized iron regulatory protein 2 (IRP2), a modulator of iron metabolism, suggesting that oxidation is a specific recognition signal for ubiquitylation of IRP2. Like dopamine, iron is in involved in the production of reactive oxygen species (ROS), suggesting that HOIL-1 may be responsible for neurodegenerative disorders, collaborating with or independent of parkin and/or dorfin. Parc (a parkin-like ubiquitin ligase) is identified as a cytoplasmic anchor protein in p53 (a tumor suppressor)-associated protein complexes [50]. Parc is a critical regulator of p53 subcellular localization and subsequent function. It is a large multi-domain protein containing the RING-finger/IBR domain at the C-terminal region.

The RING-IBR-RING domain is also called the TRIAD or DRILL domain, which functions by interacting with ubiquitin-conjugating enzymes (E2s), suggesting it is the catalytic site of this class of E3-enzyme family. Indeed, parkin, dorfin, and HOIL-1 bind to specific E2 species through their RING-IBR-RING motif. Intriguingly, these three RING-IBR-RING type E3s are strongly expressed in the brain.

The UBL region of parkin exhibits moderate similarity to ubiquitin, displaying approximately 20% of sequence identity. However, its role is largely unknown. The number of identified mutations of the parkin gene has recently increased in patients with early-onset parkinsonism as

described above, and a single mutation that causes Arg-Pro substitution at position 42 of the UBL domain has been identified in one family of ARJP patients [51]. This mutated parkin retains the ability to bind E2 (UbcH7), but fails to co-immunoprecipitate ubiquitylated proteins, such as *O*-glycosylated αSN [52]. Analogously, HOIL-1 interacts with oxidized IRP2 through the N-terminal UBL domain [49]. Thus, it is likely that the UBL domain contributes to the recognition of target proteins. On the other hand, accumulating evidence suggests that various proteins harboring the UBL domain, e.g., Rad23, Dsk2, and their human homologues (hHR23α/β and hPLIC1/2, respectively), provide links between the 26S proteasome and the ubiquitylation machinery [53]. In this context, it has been reported that a 50-kDa subunit Rpn10 of the human 26S proteasome, originally called S5a, could bind to polyubiquitin conjugates in vitro and, hence, could possibly function as a polyubiquitin chain-binding subunit [54]. Intriguingly, Rpn10 also binds the UBL domain of hHR23α/β and hPLIC-2 [55].

NMR studies have indicated that the three-dimensional structure of the UBL domain of parkin resembles that of ubiquitin [56]. As shown in Fig. 3, ubiquitin is a small protein with two α-helical and five β-sheet structures, which arrange in the order of ββαββαβ in the secondary structure. Overall, these structures are conserved in the UBL of parkin, indicating that both molecules appear to be structurally very similar. Intriguingly, inspection of chemical shift perturbation data revealed that UBL binds the Rpn10 subunit of the 26S proteasome with the surface area (see a model in Fig. 2). It was recently reported that one family of ARJP patients exhibited a point mutation at position 42 [51], where Arg is substituted with Pro. Intriguingly, the NMR data also indicate that Arg42 is located in the Rpn10-binding site. It is quite conceivable that this mutation induces a significant conformational change in the Rpn10-binding site of UBL, resulting in impaired proteasomal binding of parkin, which could be the structural basis of ARJP. This finding provides direct evidence that parkin is linked to cellular proteolysis, and its dysfunction presumably causes ARJP. This is of particular importance, because even if parkin is an E3 ligase, the possibility that it acts other than proteolysis could not be excluded at present.

5.4. The role of parkin in the pathogenesis of ARJP

The molecular basis of selective neuronal death in AR-JP is unknown. The biological role of parkin is also largely unknown at present. However, it is clear that a lesion in the parkin gene causes ARJP by dopaminergic neuronal loss, indicating the vital role of parkin in the survival of these neurons. Nonetheless, parkin mutations can lead to a disorder clinically similar to sporadic PD; a pathognomonic feature of ARJP is the lack of LB, which has been demonstrated in all but one reported case [57]. Why is LB missing in ARJP and how is LB formed in sporadic PD? One plausible explanation is that functional parkin plays an essential role in the formation of LBs as highly ubiquitylated inclusions.

Recent biochemical studies have clarified the type of proteins forming the LB. These include αSN, UCH-L1, parkin, synphilin-1, VPC (equivalent to p97 ATPase or CDC48), molecular chaperones, proteasome subunits [28,58,59]. The reason(s) for the formation of these proteins into LB is unknown, but they are conceptually divided into

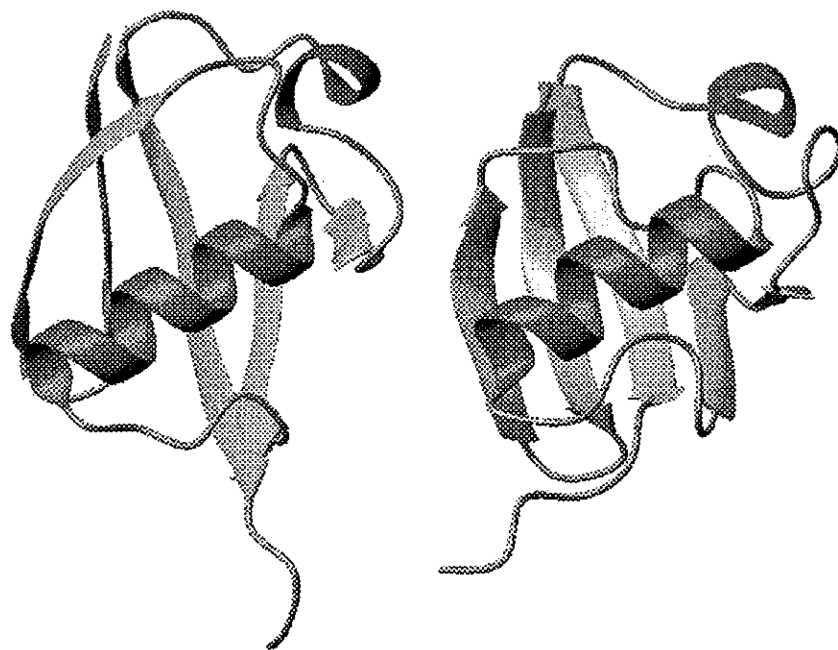

Fig. 3. The tertiary structures of ubiquitin (left) and the ubiquitin-like domain of parkin (right). α-Helices and β-sheets are shown in red and yellow, respectively.

two distinct groups based on their origin. One group belongs to specified proteins that are extraordinarily abundant in the brain and can be easily denatured and form aggregates, or other proteins associated with such proteins. The other class of proteins in the LB is involved in the cellular machinery responsible for refolding or deletion of abnormal proteins generated in cells. They include molecular chaperones and components included in UPS, respectively. Thus, the formation of LB is due to the loss of protein quality control in the cell. In other words, it is likely that certain neurons are vulnerable to the failure of UPS-mediated protein breakdown, based on the fact that multiple factors, including genetic and environmental factors, are concomitantly responsible for PD. Further studies of LB including the mechanism(s) involved in their formation and their role may shed light on the cause of PD.

LB may provide neuroprotection, because the formation of intracellular inclusions seems to be beneficial to cells. Consistently, parkin also functions in neuroprotective events (Refs. [58,59] and references therein). In considering ARJP lacking LB, parkin is thought to be an E3 ubiquitin ligase that degrades proteins with aberrant conformations [58,60]. In this context, the lack of LB in ARJP is consistent with the notion that ARJP is an early-onset disease. It is likely that failure of UPS to degrade abnormal proteins may underlie nigral degeneration and LB formation that occur in PD. Impaired protein clearance can induce dopaminergic cell death, supporting the concept that defects in the UPS may underlie nigral pathology in ARJP and perhaps sporadic forms of PD.

5.5. Parkin-interacting molecules

As mentioned, parkin interacts with E2s and Rpn10 (and hence the 26S proteasome) through the RING-IBR-RING domain and UBL domain, respectively. In addition, multiple pieces of evidence indicate that parkin interacts with various other proteins except the substrates. Of these, CHIP is an interesting molecule, because it can be regarded as "quality-control E3" that selectively ubiquitylates unfolded protein(s) by collaborating with molecular chaperones Hsp90 and Hsp70 [61]. Recently, it was reported that parkin forms a complex with CHIP, Hsp70, and Pael-R (known as parkin substrate, see below) both in vitro and in vivo [62]. The amount of CHIP in the complex is increased during stress of the endoplasmic reticulum (ER). CHIP promotes the dissociation of Hsp70 from parkin and Pael-R, thus facilitating parkin-mediated Pael-R ubiquitylation. Moreover, CHIP enhances parkin-mediated in vitro ubiquitylation of Pael-R in the absence of Hsp70. Thus, CHIP acts as a mammalian E4-like enzyme that positively regulates parkin E3 activity. On the other hand, it is also reported that parkin forms a complex with expanded poly-Gln protein, Hsp70 and the proteasome, which may be important for the elimination of the poly-Gln protein [63]. In addition, Hsp70 enhances parkin binding and ubiquitylation of poly-Gln protein, suggesting that Hsp70 may help to recruit misfolded proteins as substrates for parkin E3 ligase activity.

A recent study reported that parkin is a component of an SCF (Skp1, Cullin-1, Roc1, and F-box protein)-like ubiquitin ligase [64]. Indeed, parkin functions in a multiprotein ubiquitin ligase complex that includes the F-box/WD repeat protein hSel-10 and Cullin-1. Intriguingly, cyclin E is a substrate of this ubiquitin-ligase complex including parkin. Parkin deficiency potentiates the accumulation of cyclin E in cultured postmitotic neurons exposed to the glutamate excitotoxin kainate and promotes their apoptosis. Furthermore, overexpression of parkin attenuates the accumulation of cyclin E in toxin-treated primary neurons, including midbrain dopaminergic (DA) neurons, and protects them from apoptosis.

5.6. Putative substrates

Parkin is an E3 enzyme, hence it is conceivable that the pathogenesis of ARJP involves accumulation of unidentified neurotoxic protein(s) as a substrate of parkin. Furthermore, the abnormal accumulation of substrates caused by the loss of parkin function may be the cause of neurodegeneration in parkin-related parkinsonism. Thus, the misregulation of proteasomal degradation of parkin substrate(s) is deleterious to neurons. Therefore, accumulation of substrate(s) for ubiquitylation mediated by parkin is probably critical to our understanding of the pathogenesis of ARJP. Identification of these substrates and their role are important. Presumably, the parkin protein displays ubiquitin-ligase activity for different targets, which accumulate in the brain of ARJP patients due to parkin defect and might cause neurodegeneration. To date, accumulation of various candidate parkin-substrates has been described, including that of CDCrel-1 (a synaptic vesicle associated GTPase), Pael-R (parkin associated endothelin receptor like receptor), glycosylated αSN, synphillin-1 (an αSN interacting protein), cyclin E, α/β tubulin, and transfer RNA synthetase (p58, a key structural component of the mammalian aminoacyl-tRNA synthetase complex). Abnormal accumulation of these substrates due to loss of parkin function may be the cause of neurodegeneration in parkin-related parkinsonism, although direct links between these factors and dopaminergic cell death have not yet been established.

Pael receptor (Pael-R) identified as a protein that interacts with parkin is an attractive parkin substrate whose accumulation may account for the loss of DA neurons in ARJP [65]. Pael-R is a putative G protein-coupled transmembrane polypeptide. When overexpressed in cells, this receptor tends to become unfolded, insoluble, and ubiquitylated in vivo. The insoluble Pael-R leads to unfolded protein-induced cell death. Parkin specifically ubiquitylates this receptor in the presence of ER-resident E2s ubiquitin-conjugating enzymes and promotes the degradation of insoluble Pael-R, resulting in suppression of the cell death

induced by Pael-R overexpression. Moreover, the insoluble form of Pael-R accumulates in the brains of AR-JP patients. Accumulation of the unfolded Pael-R in the ER of dopaminergic neurons induces ER stress leading death of dopaminergic neurons in AR-JP. It was also reported in an organismal system that pan-neuronal expression of Pael-R causes age-dependent selective degeneration of *Drosophila* dopaminergic neurons [66]. Coexpression of parkin degraded Pael-R and suppressed its toxicity, whereas interfering with endogenous fly parkin function promoted Pael-R accumulation and augmented its toxicity.

Like αSN (see later), Pael-R has a propensity to misfold and aggregate. Importantly, not only the unfolded protein response (UPR) can be induced by Pael-R, but also UPR induces up-regulation of both the mRNA and protein levels of parkin. Furthermore, overexpression of parkin, but not a set of mutants without the E3 activity, specifically suppressed unfolded protein stress-induced cell death [43]. These findings suggest that parkin is involved in the ubiquitylation pathway for misfolded proteins derived from ER and contributes to protection from neurotoxicity induced by UPR. Thus, parkin functions in the ER-associated degradation (ERAD) of misfolded ER protein, and it is up-regulated by unfolded-protein stress. However, how the unfolded-protein stress response fits into a common pathway is not clear at present. In contrast, other studies indicate that parkin is not regulated by the unfolded protein response in SH-SY5Y neuroblastoma cells (Ref. [67] and references therein). Consistently, parkin levels were unaffected by thaspsiargin treatment in rat cortical neuron cultures and tunicamycin treatment in rat primary hippocampal neurons. Whether parkin truly acts as a key player in the UPR pathway awaits future studies.

αSN, consisting of 140 amino acid residues, is a highly conserved protein in vertebrates [68]. It is the primary component of the LB. It has no secondary structure and intriguingly can be degraded by the 20S proteasome as well as the 26S proteasome [69], suggesting that denaturing proteins can activate the 20S proteasome by the gate opening of the central portion of the α-ring of the 20S proteasome (see Section 3). Implicated in neurotoxicity are two αSN mutants (A53T and A30P) that cause extremely rare familial PD (PARK1). It is interesting that the *O*-glycosylated form of αSN (named αSp22) becomes a target for parkin [52]. In contrast to normal parkin, mutant parkin associated with ARJP failed to bind and ubiquitylate αSp22. Thus, αSp22 is a substrate for parkin's E3 ligase activity in normal human brain and that loss of parkin function causes pathological αSp22 accumulation. These findings demonstrate a critical biochemical reaction between the two PD-linked gene products and suggest that this reaction underlies the accumulation of ubiquitylated αSN in conventional PD. Note that nonglycosylated αSN, the major species in the brain, is not a parkin substrate in vivo or in the brain.

Overexpression of mutant αSN increases sensitivity to proteasome inhibitors by decreasing proteasome function [70]. Overexpression of parkin decreases sensitivity to proteasome inhibitors in a manner dependent on parkin's E3 ligase activity, and antisense knockdown of parkin increases sensitivity to proteasome inhibitors. Mutant αSN also causes selective toxicity to catecholaminergic neurons in primary midbrain cultures, an effect that can be mimicked by the application of proteasome inhibitors. Parkin is capable of rescuing the toxic effects of mutant αSN or proteasome inhibition in these cells. Therefore, parkin and αSN are linked by common effects on a pathway associated with selective cell death in catecholaminergic neurons.

Synphilin-1 is linked to the pathogenesis of PD since it is an αSN and parkin interacting protein. Moreover, it is a component of LB in brains of sporadic PD patients. Parkin interacts with and ubiquitylates synphilin-1 [71]. Co-expression of αSN, synphilin-1, and parkin results in the formation of LB-like ubiquitin-positive cytosolic inclusions. It was shown that familial-linked mutations in parkin disrupt the ubiquitylation of synphilin-1 and the formation of the ubiquitin-positive inclusions. These results provide a molecular basis for the ubiquitylation of LB-associated proteins and link parkin and αSN in a common pathogenic mechanism through their interaction with synphilin-1.

Several other candidate substrates for parkin E3 have been reported. For example, parkin interacts with the synaptic vesicle-associated protein, CDCrel-1, belonging to a family of spetin GTPases, through its RING-finger domains [72]. Parkin ubiquitylates and promotes the degradation of CDCrel-1, whereas its familial-linked mutations impair CDCrel-1 degradation. On the other hand, parkin is tightly bound to microtubules that are polymers of tubulin α/β heterodimers, ubiquitylates α/β tubulin and promotes their degradation [73]. Its ability to enhance the ubiquitylation and degradation of misfolded tubulins may play a significant role in protecting neurons from toxins that cause PD. Misfolded tubulin monomers are highly toxic and thus must be rapidly degraded. Parkin also interacts with, ubiquitylates and promotes the degradation of p38, a key structural component of the mammalian aminoacyl-tRNA synthetase complex [74]. Interestingly, expression of p38 in COS7 cells resulted in the formation of aggresome-like inclusions in which parkin was systematically sequestered. In the human dopaminergic neuroblastoma-derived SH-SY5Y cell line, parkin promoted the formation of ubiquitylated p38-positive inclusions. Moreover, overexpression of p38 in SH-SY5Y cells caused significant cell death against which parkin provided protection. This suggests that p38 plays a role in the pathogenesis of PD.

5.7. Physiological roles of parkin in model organisms

Several lines of evidence strongly implicate mitochondrial dysfunction as a major causative factor in PD, although the molecular mechanisms responsible for mitochondrial dysfunction are poorly understood [75]. *Drosophila* parkin null mutants exhibit reduced life span, locomotor defects,

and male sterility [76]. The tissue-specific phenotypes observed in *Drosophila* parkin mutants result from mitochondrial dysfunction and raise the possibility that similar mitochondrial impairment triggers the selective cell loss observed in ARJP. The locomotor defects are derived from apoptotic cell death of muscle subsets, whereas the male sterile phenotype is derived from a spermatid individualization defect at a late stage of spermatogenesis. Mitochondrial pathology is the earliest manifestation of muscle degeneration and a prominent characteristic of individual spermatids in parkin mutants. Thus, the tissue-specific phenotypes observed in *Drosophila* parkin mutants result from mitochondrial dysfunction and raise the possibility that similar mitochondrial impairment triggers the selective cell loss observed in AR-JP. In contrast, disruption of the parkin gene in *C. elegans* reveals no obvious phenotypes in their behaviors (Suzuki et al., unpublished results).

Inactivation of the parkin gene in mice results in motor and cognitive deficits, inhibition of amphetamine-induced dopamine release and inhibition of glutamate neurotransmission [77]. The levels of dopamine are increased in the limbic brain areas of parkin mutant mice and there is a shift towards increased metabolism of dopamine by monoamine oxidase. Although there is no evidence for a reduction of nigrostriatal dopamine neurons in the parkin mutant mice, the level of dopamine transporter protein is reduced in these animals, suggesting a decreased density of dopamine terminals or adaptive changes in the nigrostriatal dopamine system. The reduction of glutathione levels was worse in the striatum and fetal mesencephalic neurons of parkin mutant mice, suggesting that a compensatory mechanism may protect dopamine neurons from neuronal death.

6. Perspective

UPS plays a central role in the protein quality control in the cytosol as well as the ER of the cell. It is therefore conceivable that functional loss of the protein quality control system is associated with various neurodegenerative diseases. In particular, with regard to the pathogenesis of PD, parkin is an E3, indicating that the ubiquitin pathway is directly linked to the cause of ARJP [58,60]. However, loss of parkin activity due to disease-linked pathogenic mutations is not simply related to the pathogenesis of ARJP, because parkin is widely expressed in most neurons in the brain. Thus, in addition to this genetic defect, there is another factor(s) capable of inducing DA neuronal death, which may act in concert with parkin in causing the degeneration of DA neurons. Available evidence indicates that environmental factors may play a role in the diseases process. It is likely that for most cases, there is a complex interplay between these genetic and environmental influences in the causation of PD. It has been postulated that environmental conditions are closely linked to loss-of-function of certain protein(s) due to disease-linked genetic mutations. It is possible that dysfunction of the mitochondrial respiratory chain, inducing oxidative stress, could be involved in the pathogenesis of ARJP, in concert with loss of parkin activity [75]. Indeed, the mitochondrial complex I inhibitors 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone cause damage of nigral neurons by mechanisms involving oxidation. Oxidative stress seems to play a prominent role in ARJP and sporadic PD [28]. Compared with other brain regions, the substantia nigra pars compacta has a higher rate of basal protein oxidation due to the enzymatic- and auto-oxidation of dopamine. Thus, this brain region has a propensity to generate high levels of oxidatively and nitratively damaged proteins. At present, there is convincing evidence suggesting that proteolytic stress due to failure of the UPS to clear unwanted proteins is a common factor among the different familial and sporadic forms of PD [68]. In this regard, dopamine metabolism may be linked to induction of proteolytic stress through production of various molecules toxic for protein structure such as ROS. Disease mechanisms seem to converge around oxidative damage and impairment of protein catabolism. Further studies are required to clarify this issue molecularly.

It is interesting that parkin is associated with synaptic membranes, even if not conclusively. Moreover, αSN is also associated with synapses, indicating that it may play some role in the modulation of synaptic plasticity. Synphilin-1 is abundant in synaptic compartments. CDCrel-1 is associated with synapses. It is likely that the real link between these proteins is that they all regulate the signaling pathways at synapses. In addition, it is possible that failure of protein homeostasis, depending on the loss-of-function of parkin, in synaptic vesicles is linked to the cause of PD. However, it is still unknown whether parkin is directly linked to the function of synapses.

To date, hereditary factors have emerged as the focus of research in PD [31,32]. In addition to parkin (*PARK2*), three genes of familial PDs have been identified. The identification of pathogenic mutations of three genes, αSN (*PARK1*), parkin and UCH-L1 (*PARK5*), has elucidated UPS and its potential role as a casual pathway in PD. Mutations of αSN and UCH-L1 are extremely rare, while mutations of parkin are more common in early-onset cases but still in a small population of the total number of cases of PD, as discussed. UCH-L1 belongs to the family of DUBs, is expressed exclusively in neurons, and represents over 1% of total brain protein content and has tendency to aggregate, like αSN. In addition, both are major components of LB. Therefore, parkin, αSN, and UCH-L1 are on the same line of abnormality of protein homeostasis. Interestingly, UCH-L1 has a dimerization-dependent ligase activity that promotes addition of ubiquitin to preformed ubiquitin chains on proteins [18]. A polymorphic variant of UCH-L1 has reduced ligase activity but comparable hydrolase activity, relative to the wild-type enzyme. Thus, the ligase activity as well as the monomeric hydrolase activity of UCH-L1 may

play a role in proteasomal protein degradation, a critical process for neuronal health.

Recently, DJ-1 (PARK7) was identified as a novel early-onset recessive PD gene and found to encode DJ-1 protein, consisting of 189 amino acids (20 kDa) [78]. Overall, the symptoms of this disease are very similar to parkin and significantly overlap with idiopathic PD. However, it is difficult at this juncture to fully appreciate how mutations of the DJ-1 gene cause PD, since its function is basically unknown. DJ-1 is thought to participate in the oxidative stress response. This is of interest, because there is evidence that oxidative events occur during the course of PD.

To date, several new loci for the causative genes of other forms of familial PD have been documented. The identification of multiple genetic causes will provide further impetus to characterize the pathway leading to PD. New candidate genes are expressed to encode proteins either involved in UPS or sequestrated in intracytoplasmic protein aggregates. Future identification of disease genes is required for confirmation, thereby unifying the clinical and genetic heterogeneity of PD.

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Structural basis of sugar-recognizing ubiquitin ligase

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