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Role of the p62 Protein in the Formation of Neuropathological Cytoplasmic Inclusions

Doctoral dissertation

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ABSTRACT

The pathology of aging-associated neurodegenerative disorders typically involves the occurrence of proteinaceous cytoplasmic inclusions in the brain. These inclusions are seen in both neurons and glial cells, affecting disease-specific cell populations. Most types of inclusions associated with cognitive or movement dysfunction are composed of cytoplasmic aggregates of tau or α-synuclein (αS) proteins in aberrant fibrillar forms, thus defining the disease groups of "tauopathies" and "synucleinopathies".

Inclusions are usually accompanied by cell loss, suggesting that their origin is closely linked to the neuronal death and dysfunction that are responsible for the clinical symptoms. The molecular events underlying the formation of cytoplasmic inclusions are poorly understood, but it is increasingly believed that the biogenesis of different types of inclusions may share some common mechanisms. In order to elucidate the mechanisms of inclusion formation and the elicited cellular reactions, it is necessary to identify key proteins involved and investigate their roles in these events. In particular, proteins that may contribute to or regulate the aggregation process are of interest.

In the present series of studies, a gene array approach and cellular models were first utilized to identify proteins potentially involved in neurodegenerative phenomena (study **I**). Following the identification of p62, a signaling protein with several features of interest, the expression of this protein was examined in neuropathology-mimicking conditions in neuronal culture. Subsequently, the involvement of p62 was investigated in disease-associated protein aggregation in the human brain using immunohistochemistry (studies **II**–**IV**). In study **II**, brain specimens from different tauopathies (Alzheimer and Pick diseases) and synucleinopathies (Parkinson disease, dementia with Lewy bodies, and multiple system atrophy) were examined. In study **III**, the incorporation of p62 was studied in more detail with respect to the tau pathology of Alzheimer disease. In study **IV**, the morphogenesis of αS-containing inclusions characteristic of Parkinson disease was elucidated.

The main findings were: **I:** The expression of p62 transcript and p62 protein were both prominently upregulated in response to pro-apoptotic conditions and proteasomal inhibition. This upregulation might indicate the activation of survival signaling. **II:** In both tauopathies and synucleinopathies, the p62 protein was copiously present in the hallmark cytoplasmic inclusions in perikarya but was mostly absent from intraneuritic deposits. **III:** In the tau pathology of Alzheimer disease, p62 was selectively incorporated into neurofibrillary tangles and represented a relatively early constituent in these structures. **IV:** The spectrum of perikaryal αS pathology in the substantia nigra pointed to a morphogenetic sequence in which punctate αS deposits, pale bodies, and Lewy bodies arise as successive stages of a complex aggregation process. The engagement of p62 coincided with the formation of compact inclusions, possibly as a part of a cytoprotective response.

These findings are compatible with several alternative interpretations, but the pattern of p62 involvement in the various types of inclusions is viewed as evidence that p62 plays a contributory role in their formation. Since inclusions may serve as sinks for potentially noxious proteins, p62 may act to promote cell viability.

National Library of Medicine Classification: WL 359
Medical Subject Headings: Alzheimer disease; apoptosis; brain/pathology; human; immunohistochemistry; inclusion bodies; Lewy bodies; neurodegenerative diseases/pathology; neurofibrillary tangles; Parkinson disease; proteins/abnormalities; tauopathies; ubiquitin

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Kuopio, August 2004

Erkki Kuusisto

ABBREVIATIONS

αS	α-synuclein
AD	Alzheimer disease
AG(D)	argyrophilic grain (disease)
ALS-D	amyotrophic lateral sclerosis with dementia
AP-1	activator protein-1
aPKC	atypical protein kinase C
BDNF	brain-derived neurotrophic factor
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CB	coiled body
CBD	corticobasal degeneration
cdc2	cell-division-cycle 2
cdk5	cyclin-dependent kinase 5
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
cGMP	cyclic guanosine monophosphate
Chip-1	carboxyl terminus of Hsc70-interacting protein
CNS	central nervous system
COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
DLB	dementia with Lewy bodies
EGF(R)	epidermal growth factor (receptor)
ERK	extracellular-signal-regulated kinase
Ets-1	E26 transformation-specific-1
FTD	frontotemporal dementia
GABA	γ-aminobutyric acid
GAP	GTPase-activating protein
GCI	glial cytoplasmic inclusion
GFAP	glial fibrillary acidic protein
Grb14	growth factor receptor-bound protein 14
GSK-3β	glycogen synthase kinase-3β
GTPase	guanosine 5'-triphosphatase
HDAC	histone deacetylase
H&E	haematoxylin-and-eosin
HP-tau	hyperphosphorylated tau
IHB	intracytoplasmic hyaline body
IKKβ	IκB kinase β
IL-1(R)	interleukin-1 (receptor)
IRAK	IL-1 receptor-associated kinase
IUP	intrinsically unstructured protein
K_V	voltage-gated K^+ channel
LB	Lewy body
MAP(K)	mitogen-activated protein (kinase)
MarB	Marinesco body
MB	Mallory body
MEK	MAP-ERK kinase

MPTP	*N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	multiple system atrophy
MT	microtubule
MyD88	myeloid differentiation protein
MyoD	muscle-regulatory protein
NBR1	next to breast cancer 1
ND	neurodegenerative disorder
NF-κB	nuclear factor κB
NF-E2	nuclear factor-erythroid 2
NFT	neurofibrillary tangle
NGF	nerve growth factor
NP	neuritic plaque
Nrf2	NF-E2-related factor 2
NT	neurotrophin; neuropil thread
OPCA	octicosapeptide repeat / phox and cdc24p / atypical PKC-interaction
PaB	pale body
PAR-4	prostate apoptosis response-4
PB1	Phox and Bem1p domain
PBS	phosphate-buffered saline
PD	Parkinson disease
PDB	Paget's disease of bone
PDEF	prostate-derived Ets transcription factor
PEST sequences	regions rich in proline, glutamate, serine, and threonine
PHF	paired helical filament
PiB	Pick body
PiD	Pick disease
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMD	postmortem delay
PSP	progressive supranuclear palsy
RIP	receptor-interacting protein
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SF	straight filament
sGAG	sulfated glycosaminoglycan
SN	substantia nigra
Sp1	stimulating protein 1
SQSTM1	sequestosome 1
SSC	saline sodium citrate
TA	tufted astrocyte
TGF-β1	transforming growth factor-β1
TNF(R)	tumor necrosis factor (receptor)
TRADD	TNFR-associated death-domain protein
TRAF6	TNFR-associated factor 6
Trk	tyrosine receptor kinase
Ub	ubiquitin
UBA	ubiquitin-associated
ZIP	zeta-interacting protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I**–**IV**.

I Kuusisto E, Suuronen T, Salminen A (2001). Ubiquitin-binding protein p62 expression is induced during apoptosis and proteasomal inhibition in neuronal cells. *Biochemical and Biophysical Research Communications* 280, 223–228.

II Kuusisto E, Salminen A, Alafuzoff I (2001). Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. *NeuroReport* 12, 2085–2090.

III Kuusisto E, Salminen A, Alafuzoff I (2002). Early accumulation of p62 in neurofibrillary tangles in Alzheimer's disease: possible role in tangle formation. *Neuropathology and Applied Neurobiology* 28, 228–237.

IV Kuusisto E, Parkkinen L, Alafuzoff I (2003). Morphogenesis of Lewy bodies: dissimilar incorporation of α-synuclein, ubiquitin, and p62. *Journal of Neuropathology and Experimental Neurology* 62, 1241–1253.

CONTENTS

1. INTRODUCTION

As life expectancy expands, society is faced with an increasing burden of patients with dementia and movement disability due to aging-associated neurodegenerative disorders (NDs). Few of the therapeutic possibilities currently available are able even to slow, let alone halt the progression of NDs. On the other hand, there is also a growing recognition that these diverse conditions appear to share many similar pathogenetic mechanisms, giving hope that if one could untangle these mechanisms, then it might be possible to devise novel therapeutic opportunities applicable to a host of different diseases.

A conspicuous pathological feature shared by most of these disorders is the phenomenon of aberrant protein aggregation (Goedert et al. 1998). This process is manifest as the formation of inclusions, *i.e.*, focal intracellular accumulations containing fibrillar forms of cellular proteins, in the cytoplasm or nuclei of cells within the central nervous system. Various types of inclusions have achieved major importance as neuropathological markers for the classification and postmortem diagnosis of NDs (Esiri et al. 1997, Lowe et al. 1997, Lowe 1998). From the methodological standpoint, the usefulness of inclusions stems from their conspicuous nature which, together with appropriate histological staining techniques, enables their unambiguous identification and quantitative assessment in tissue sections. From the viewpoint of diagnostics, the use of inclusions for postmortem diagnosis and for disease classification is justified by the consistent associations between some clinical phenotypes and the occurrence and distribution of certain inclusion types within specific cell types and areas of the brain. Adding to the utility of inclusions is the strikingly common involvement of “inclusion pathology” within the spectrum of NDs. Most of these disorders, including the most prevalent diseases, display characteristic types of inclusions with disease-dependent intrabrain distributions (Esiri et al. 1997, Lowe et al. 1997, Lowe 1998).

In several of these diseases, the pathological aggregates are primarily composed of aberrant tau or α-synuclein proteins, which accumulate as fibrils into the cytoplasmic compartment of neurons and/or glia. These “tauopathies” (Lee et al. 2001b) and “synucleinopathies” (Jellinger 2003) account for most of the inclusion pathology associated with cognitive or movement dysfunction.

The formation of cytoplasmic inclusions is poorly understood (Alves-Rodrigues et al. 1998, Goedert et al. 1998). A closely linked and likewise unclear issue is the relation of inclusions to cell loss that typically co-occurs in affected brain areas (Terry 2000). This co-occurrence with cell loss and the association to clinical manifestations, together with the wide involvement of inclusion pathology in NDs and the fact that most types of ND-associated cytoplasmic inclusions share several common features, suggest that their formation is a fundamental process in the pathogenesis of NDs and may involve common mechanisms that are closely intertwined with the events underlying cell death.

In particular, there is accumulating evidence to suggest that in several NDs, the cellular damage associated with protein aggregation might result from toxic actions exerted by fibrillar or non-fibrillar protein assemblies that arise due to aberrant conformational changes (Taylor et al. 2002, Caughey and Lansbury 2003), whereas the sequestration of such assemblies into compact inclusions might serve to counteract their noxiousness. This sequestration may involve active cellular processes such as microtubule-dependent transport for routing aggregated proteins to proteasomal/autophagic clearance (Kopito 2000, Garcia-Mata et al. 2002), and ubiquitination that might prevent non-productive interactions (Gray 2001). Despite this emerging view of common mechanisms, wide gaps in our understanding of inclusion-related phenomena still remain, especially the chasm between *in vitro*-based models and observations made in human diseases.

In order to better understand the pathogenetic events underlying the formation of inclusions and the cellular reactions elicited by protein aggregation, it is necessary to identify proteins that might have regulatory functions in these processes. Specifically, proteins with the ability to directly interact with aggregated proteins are of interest.

The protein p62 (sequestosome 1) is a cytoplasmic protein for which physiological functions have been assigned in the context of signaling pathways activated by growth factors (Geetha and Wooten 2002). In the present series of studies, however, p62 was identified and selected for investigation as a protein displaying a number of features that suggested a possible role in neuropathological conditions. We therefore characterized the expression of p62 in neuropathology-mimicking cellular models and examined its involvement in disease-associated protein aggregation in the human brain.

2. REVIEW OF THE LITERATURE

2.1 Cytoplasmic inclusions associated with neurodegenerative disorders

The term ”inclusion” refers to abnormal accumulations of intracellular constituents, appearing as discrete bodies within the cell. Based on their primary material, location, and topography, inclusions can be broadly classified into lipid-, carbohydrate-, and protein-enriched types, into cytoplasmic and intranuclear types, and into membrane-bound types and those without delimiting membranes. Both disease-associated inclusions and those for which no overt association to pathological conditions has been found to date are known. A range of NDs are associated with various types of cytoplasmic or intranuclear inclusions that primarily contain abnormal proteins, usually lack delimiting membranes, and typically populate specific cell types and areas of the brain (Esiri et al. 1997, Lowe et al. 1997, Alves-Rodrigues et al. 1998). The remainder of this text is primarily concerned with the *cytoplasmic* subset of these disease-associated inclusions. The main emphasis will be on inclusions assumed to consist mainly of aberrant tau or α-synuclein (αS) proteins.

A growing number of cytoplasmic inclusion types have been discovered, due to increasing research interest and the improvements in the methods for their visualization in tissue sections. In early studies, inclusions could be visualized using histological stains with affinity for protein-rich material, such as silver impregnation techniques. Following the identification of the major protein constituents in the inclusions, the use of immunodetection has enabled a more sensitive and specific visualization, and facilitated the identification of novel (sub)types.

It should be noted that although the term “inclusion” is often used interchangeably with “protein accumulation” or “protein aggregation“, the latter two terms denote related but different concepts. “Protein accumulation” refers to any type of protein buildup, whether focal or diffuse, intra- or extracellular. “Protein aggregation”, in turn, denotes the direct association of normally separate proteins into aberrant multimolecular assemblies, by means of any type of biochemical interaction (*i.e.*, covalent, electrostatic, or hydrophobic).

2.1.1 Pathogenetic relevance

The role of inclusions in the pathogenesis of NDs is still poorly understood. However, several lines of evidence suggest that inclusion formation is deeply intertwined with the pathological events that account for the clinical expression of these diseases. (1) Certain types of inclusion pathology correlate well with their respective clinical phenotypes (Gibb et al. 1991, Arriagada et al. 1992, Dickson 1998). (2) Inclusions typically co-occur with cell loss in the same brain areas, pointing to close interconnections between the aberrant events underlying cell death and inclusion formation (Feany and Dickson 1996, Bobinski et al. 1997, Castellani 1998, Dickson 1998, Braak and Braak 2000). (3) The wide involvement of inclusion pathology in NDs suggests that the formation of inclusions is a central process that may reflect similar mechanisms in the pathogenesis of these conditions. (4) Despite their varied patterns of disease association, cell type specificity, regional distribution, morphology, and protein composition, most types of cytoplasmic inclusions associated with NDs share a number of common features (Table 1), supporting the hypothesis that their formation may involve common pathological processes.

Table 1. Features shared by most types of ND-associated cytoplasmic inclusions.

Occurrence	• Associated with advanced age. • Spatially selective involvement of brain regions and cell types, manifest as characteristic predilection areas and vulnerable cell populations.
Morphology	• Often voluminuous and localized adjacent to the nucleus, frequently causing an apparent displacement or indentation of the latter.
Composition	• Proteinaceous, as evident from affinity to protein-binding dyes. • Immunoreactivity for ubiquitin. May reflect a failure of the ubiquitin-proteasome system, possibly promoting the accumulation of undegraded proteins.
Ultrastructure	• Mostly composed of fibrillar material, constituted by aberrant assemblies of normal cytoplasmic proteins. • Absence of delimiting membranes.
Protein conformations	• The major protein constituents seem to have undergone aberrant conformational changes, often resulting in the enrichment of β-structure.
Effect on host cell viability	• Host cells appear to remain viable and active, as evidenced by the preservation of the cell membrane, nucleus, and metabolic markers.
Associated pathology in surrounding tissue	• Typically co-occur with cell loss. • Frequently accompanied by astrocytosis and activation of microglia. • Several inclusion types are associated with abnormal neurites.

For the above reasons, an intense research effort has been directed at elucidating the molecular nature and biogenesis of neuropathological inclusions, as well as their relation to neuronal dysfunction, cell loss, and clinical symptoms. If we could identify common degenerative processes and clarify the mechanisms relating to inclusions of a particular type, this would undoubtedly advance our understanding of the pathogenesis for the entire spectrum of "inclusion disorders" (Lee et al. 2001b).

2.1.2 Mechanistic relation to cell death and neuronal dysfunction

While abundant evidence points to a central position for inclusions in the pathogenesis of NDs, it remains a key unresolved issue how their formation is mechanistically linked to cell death and other pathological events that account for the clinical phenotypes (Castellani 1998, Komori 1999, Tran and Miller 1999). Research has been hampered by the complexity of the systems involved and the lack of good experimental models. However, a range of hypotheses have been put forward, mostly falling into four main views where inclusions are either regarded as 1) noxious to the cell, 2) an epiphenomenon, 3) structures that sequester toxic proteins, or 4) centers for the proteasomal and/or autophagic clearance of aggregated proteins. Selected evidence in support of each concept is presented below.

Inclusions have been proposed to be noxious as such, thereby promoting cell death or dysfunction (Trojanowski et al. 1998, Galvin et al. 2001). In addition to direct mechanical derangement of cellular structures by voluminous inclusions, noxiousness might result if the deposited proteins interfere with cellular processes, *e.g.* via blockage of intracellular transport (Cleveland 1996, Katsuse et al. 2003) or saturation of proteolytic capacity (Bence et al. 2001, Keck et al. 2003, Snyder et al. 2003). Detrimental effects may also be due to the entrapment of vital signaling intermediates or other cellular constituents within the inclusion (Steffan et al. 2000, Nucifora et al. 2001, Donaldson et al. 2003, Qin et al. 2004). Neuropathological evidence in support of this view encompasses the observed associations of inclusions with cell loss and clinical phenotypes, as stated above. Genetic studies have also provided important evidence for the primacy of inclusion constituents in the etiopathology of NDs, revealing that certain genes which, when mutated, underlie inheritable forms of these diseases (*i.e.*, tau, αS,

neuroserpin) encode proteins that represent the major components of the respective inclusion types (Polymeropoulos et al. 1997, Hutton et al. 1998, Davis et al. 1999). Consistent findings come from rodent models, in which the overexpression of these genes may be sufficient to cause phenotypes recapitulating many of the pathological features of the human diseases (Lewis et al. 2000, Kirik et al. 2002). Finally, numerous *in vitro* studies have shown that aggregated proteins mimicking those *in vivo* can cause a multitude of adverse effects on cellular function, such as vesicle permeabilization (Volles et al. 2001), Golgi fragmentation (Gosavi et al. 2002), microtubule disassembly (Alonso et al. 1996), neuritic degeneration (Li et al. 2001), generation of reactive oxygen species (Turnbull et al. 2001), cytotoxicity (Bodles et al. 2000, Yang et al. 2002), and apoptotic cell death (El-Agnaf et al. 1998, Kouroku et al. 2000).

According to a contrary view, inclusion formation might represent an epiphenomenon not influencing the pathological processes that cause cell death. In favor of this view, the severity of neuronal death often seems disproportionately high in comparison with the frequency of inclusions in the same brain area (Terry 2000). Vice versa, individual cases may display exceptionally high densities of inclusions in brain regions with no sign of cell loss (van Duinen et al. 1999). Similarly, in some animal models, inclusion formation is not accompanied by cell death (Auluck et al. 2002, Lo Bianco et al. 2002). Further, inclusion-bearing cells appear to remain viable, even for years (Hatanpää et al. 1996, Tompkins and Hill 1997, Morsch et al. 1999).

A view that may better explain the empirical findings is the scenario that inclusions as such may only exert a minor adverse effect on the host cell, while serving as structures which sequester aberrant proteins that otherwise would be noxious (Iqbal et al. 1998, Denk et al. 2000, Volles and Lansbury 2003). Evidence from cell-free systems, in particular, suggests that the sequestration of such proteins into inclusions may play an adaptive role, by promoting the elimination of toxic prefibrillar intermediates, “protofibrils”, favoring their conversion into a more inert, fibrillar form (Bucciantini et al. 2002, Lashuel et al. 2002a, Caughey and Lansbury 2003). Supportive evidence comes from human postmortem studies that sometimes reveal inverse correlations between inclusion abundance and cell loss (Kuemmerle et al. 1999, Takahashi et al. 2001), suggesting that cells capable of forming inclusions might be more resistant against

aberrant proteins. Consistently, some early-onset types of NDs display no inclusions characteristic of the late-onset phenotype (Mizuno et al. 2001), suggesting that the inability to form inclusions might lead to accelerated pathogenesis. Along similar lines, experiments with genetically modified mice suggest that inclusion formation renders cells less vulnerable to aggregation-prone proteins and other insults (Cummings et al. 1999, Denk et al. 2000). This conclusion is in accordance with cell culture studies showing a dissociation between the processes of inclusion formation and cell death (Lang-Rollin et al. 2003, Tanaka et al. 2004).

A question linked to the pathogenetic significance of inclusions concerns the nature of the process whereby they are formed: it is unclear to what extent inclusions emerge as a result of unregulated events, and to what extent their formation may be governed by regulatory systems (Taylor et al. 2002). The view of inclusion formation as a haphazard phenomenon, driven by aberrant intermolecular interactions, is apparently favored by the varied morphologies of different inclusions and their heterogenous compositions. In addition, some features of disease-associated inclusions, such as fibrillization, can be reproduced in cell-free conditions (Conway et al. 1998, Barghorn and Mandelkow 2002), pointing to the contribution of unregulated events. However, these findings do not preclude the involvement of regulatory mechanisms, particularly in the events whereby aberrant proteins (fibrillar or not) conjoin into larger focal assemblies. Indeed, evidence accumulating from cell culture models suggests that cytoplasmic inclusions are formed in a highly regulated manner, via the aggresomal response (Kopito 2000, Garcia-Mata et al. 2002). In this mechanism, misfolded proteins are transported along microtubuli to the pericentriolar area, where they form a condensed mass encaged by intermediate filaments (Johnston et al. 1998, Garcia-Mata et al. 1999). Aggresomes may represent a general adaptive response in conditions where misfolded or aggregation-prone proteins are produced faster than can be eliminated via refolding or degradation (Kopito and Sitia 2000, Garcia-Mata et al. 2002). This process seems to be cytoprotective when toxic proteins accumulate (Taylor et al. 2003, Tanaka et al. 2004). Since aggresomes are enriched in components of the Ub-proteasome system and heat shock proteins, they are thought to serve as sites of active proteolysis (Wigley et al. 1999). In addition, aggresomes seem to facilitate the clearance of aggregated proteins via autophagy (Ravikumar et al. 2002, Fortun et al. 2003, Taylor et al. 2003). The aggresomal response

has gained increasing recognition as a mechanism potentially underlying the formation of neuropathological cytoplasmic inclusions, but it should be noted that as yet there is little direct evidence in support of this view.

2.2 Tau-containing inclusions

Tau is a microtubule-binding protein that is abundant in the central nervous system (CNS), localizing mainly into axons where it stabilizes microtubules (MTs) and promotes their polymerization. In the CNS, tau is expressed as 6 splicing isoforms of 352–441 residues. Tau is a natively unfolded protein with little secondary structure. Functionally, the protein is divided into an N-terminal projection domain and a C-terminal MT-binding domain, the latter part containing 3 or 4 MT-binding repeats. The sequence also harbors numerous phosphorylation sites (Buee et al. 2000, Friedhoff et al. 2000, Lee et al. 2001b).

A growing number of tau-immunoreactive neuronal or glial inclusion types have been described in a variety of conditions (Feany and Dickson 1996, Esiri et al. 1997, Komori 1999). Some of the most widely recognized types, each of which is generally believed to contain the tau protein as the major constituent, are introduced below. Additional or variant types include those presenting in hereditary tau-linked degenerations (Spillantini et al. 1998a) and those incorporating tau as a minor or incidental component.

2.2.1 Neuronal types

Neurofibrillary tangles The most common type of disease-associated inclusion in the brain is the “neurofibrillary tangle” (NFT), one of the neuropathological hallmarks of AD (Esiri et al. 1997, Terry et al. 1999). Histologically, NFTs are clearly visualized by silver impregnation or amyloid stains. In AD brains, NFTs appear as flame-shaped or globoid masses of fibrous intraneuronal material, the morphology depending on the neuronal type and the developmental stage of the tangle. Ultrastructurally, NFTs mainly consist of dense bundles of paired helical filaments (PHFs) 8–20 nm across (Lee et al. 2001b). PHFs may be associated with straight filaments (SFs) and more complex types of fibrillar structures (Itoh et al. 1997b, Gomez-Ramos and Moran 1998). The principal constituent of PHFs is the tau protein in its hyperphosphorylated forms (HP-tau) (Goedert et al.

1995, Iqbal et al. 1998). Immunohistochemistry also reveals the variable incorporation of ubiquitin (Ub) (*e.g.*, Bancher et al. 1989a) and a range of other proteins into NFTs (Terry et al. 1999).

In AD brains, NFTs occur in the cerebral cortex and in several subcortical regions (Braak and Braak 1991, Terry et al. 1999). Within cortical areas, the distribution of NFTs suggests a hierarchical progression that first involves the transentorhinal cortex, from where the pathology spreads via limbic areas to the isocortex (Braak and Braak 1991). The correlation between the NFT distribution and the severity of clinical expression underlies the use of NFTs as markers in the neuropathological diagnosis of AD (NIA-RI Working Group 1997). In the neocortex, NFTs mostly appear in large pyramidal neurons within layers II–III and V, where they are usually accompanied by abnormal neuronal processes, *i.e.*, neuropil threads (NTs) and dystrophic neurites, the latter surrounding deposits of β-amyloid. Both types of aberrant neurites contain PHFs and immunolabel for HP-tau and Ub (Esiri et al. 1997). In addition to AD, NFTs occur in other tauopathies including progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), with disease-specific differences in their distribution, structure, and biochemical properties (Esiri et al. 1997, Jellinger and Bancher 1998, Vogelsberg-Ragaglia et al. 1999, Buee et al. 2000). Frequently, brains from neurologically normal elderly individuals also show a degree of NFT pathology within limbic areas (Braak and Braak 1995).

Pick bodies The “Pick body” (PiB) is characteristic of Pick disease (PiD) (Esiri et al. 1997, Dickson 1998). Silver impregnation reveals PiBs as intensely argyrophilic, rounded to irregular, well-circumscribed inclusions 10–15 μm across. Immunohistochemistry shows the presence of HP-tau, variably accompanied by Ub, phosphorylated neurofilaments, and other proteins. Ultrastructurally, PiBs contain randomly arranged SFs ~15 nm in diameter, admixed with granular constituents, twisted filaments dissimilar from AD-type PHFs, and cytoplasmic organelles. Biochemically, the filamentous components consist of HP-tau which differs from that found in AD (Buee et al. 2000). PiBs are relatively specific for PiD (Feany and Dickson 1996) and have been regarded as its defining histopathological feature (Dickson 1998). In affected brains, PiBs

are found in the cerebral cortex and in subcortical areas including many brainstem nuclei (Yoshimura 1989, Dickson 1998). In the neocortex, PiBs preferentially populate layers II and VI (Hof et al. 1994).

Argyrophilic grains Small HP-tau-immunoreactive inclusions scattered in the neuropil constitute the pathological hallmark of argyrophilic grain disease (AGD) (Jellinger 1998). Best visualized by silver impregnation, “argyrophilic grains” (AGs) appear as minute, spindle- or comma-like structures with small protrusions, mostly occurring in the hippocampus and nearby limbic areas. In addition to pure AGD, AGs frequently accompany other neurodegenerative conditions (Jellinger 1998). AGs reside within dendrites, often arranged in short rows and frequently surrounding neurons diffusely positive for HP-tau. AGs selectively incorporate the tau isoform with 4 MT-binding repeats (Togo et al. 2002). They also label variably for Ub (Ikeda et al. 1995a) and engage several tau-phosphorylating kinases (Ferrer et al. 2003). The structural elements of AGs appear as accumulations of 9–18-nm SFs or 25-nm smooth tubules (Jellinger 1998). The formation of AGs is mostly unclear, but the morphology of AG-bearing neurons is suggestive of ongoing dendritic shrinkage (Tolnay et al. 1998).

2.2.2 Glial types

Coiled bodies Several tauopathies display “coiled bodies” (CBs) in oligodendrocytes. CBs occur most numerously in CBD, but are also abundant in PSP and AGD and are less common in PiD (Komori 1999). Histologically, CBs are readily detected by Gallyas-Braak silver impregnation. They may exhibit varied morphologies including coil- or comma-like or spine-like with branches, different forms predominating in each disease. In CBD, the inclusions usually appear as a bundle of fibrils that coil around an enlarged nucleus and extend into proximal processes (Dickson et al. 2002). CBs are immunoreactive for HP-tau but generally negative for Ub (Feany et al. 1996, Komori 1999). Ultrastructurally, their constituents have been described as filamentous or tubular, straight or helical structures 9–25 nm in diameter, this variability likely reflecting disease-specific differences. CBs may populate regions of the cerebral cortex, basal ganglia, brainstem, and cerebellum, with disease-dependent but overlapping distributions. The occurrence of CBs is paralleled by that of thread-like tau-

immunoreactive structures which localize into the myelin-forming processes of oligodendrocytes (Komori 1999). The ultrastructural similarity of these thread-like structures and CBs suggests that they have an interlinked origin (Arima et al. 1997).

Astrocytic inclusions Several types of tau deposition within astrocytes occur in various conditions, most abundantly in CBD, PSP, and PiD (Chin and Goldman 1996, Komori 1999). These accumulations, named by their conspicuous features as visualized by the Gallyas-Braak technique or HP-tau immunolabeling, include tufted astrocytes, astrocytic plaques, as well as thorn-shaped and ramified astrocytes. Each type shows a different pattern of disease association(s) and disease-dependent intrabrain distribution(s), and may vary somewhat in appearance from disease to disease. This morphological diversity may reflect differing populations of affected cells and/or more subtle heterogeneity in the underlying pathological processes (Chin and Goldman 1996, Komori 1999). Compared to tau inclusions with a compact consistency (*e.g.*, NFTs), those in astrocytes generally appear to be less condense, being poorly discernible with conventional silver stains and showing few or no fibrillar constituents in ultrastructure, thus resembling the "pre-tangles" seen in AD (Ikeda et al. 1998).

"Tufted astrocytes" (TAs) are characteristic of PSP (Komori et al. 1998, Matsusaka et al. 1998). They are seen as focal aggregates of fine or thick radiating processes in a concentric, tuft-like arrangement, often obscuring the nucleus. "Astrocytic plaques" are relatively specific to CBD (Ikeda et al. 1998, Dickson et al. 2002). They are reminiscent of neuritic plaques in AD but devoid of β-amyloid, appearing as a corona-like arrangement of tau-positive, stubby distal processes around an unstained area. Both TAs and astrocytic plaques are mostly confined to the gray matter, populating regions of the cerebral cortex, basal ganglia, and brainstem. "Ramified astrocytes" are associated with PiD. They resemble TAs but appear as a more diffuse form of accumulation that occupies more of the cell body and processes (Komori 1999). The biogenesis of these disease-associated types of astroglial inclusions is poorly understood, but at least in the case of TAs, tau accumulation seems to be a degenerative rather than a gliosis-related process (Togo and Dickson 2002). Unlike the above types, "thorn-shaped astrocytes" show no

overt disease specificity. Their occurrence is mostly limited to subpial and subependymal areas and may be promoted by gliosis (Ikeda et al. 1995b).

2.2.3 Formation of AD-type neurofibrillary tangles

A variety of empirical approaches have been employed to elucidate the formation of tau-containing inclusions, utilizing either human postmortem tissues or diverse experimental models ranging from non-human primates to cell-free systems. In human tissues, the inclusion type most extensively studied is the AD-type NFT which will be the main focus here. However, much of the insight obtained from various models of human tauopathies is not specific to any particular disease but rather may apply to other types of tau inclusions besides or instead of those in AD.

Neuropathological findings While only a limited amount of mechanistic insight can be extracted from studies on human autopsy-derived brain material, it has been possible to shed light on the events underlying NFT formation by reconstructing a morphological description of the process. To this end, tau accumulations in affected brain regions of cases with various extents of AD-type pathology have been visualized using immuno-histochemical and other stains. By tentative ordering of the structures thus observed, the most likely sequence of events has been inferred, largely on morphological grounds (Bancher et al. 1989a, Braak et al. 1994, Sassin et al. 2000, Uboga and Price 2000, Lauckner et al. 2003). Additional clues into the underlying events have been obtained from human tissues by determining the identities, modifications, and ultrastructure of NFT constituents using immunohistochemistry, biochemical analyses, and electron microscopy.

The distinction of early, mature, and end stage forms of NFTs was already suggested by Alois Alzheimer (see Bancher et al. 1989a). Thereafter, the morphogenesis of NFTs has been characterized in the greatest detail in pyramidal neurons of layer Pre-α of the entorhinal/transentorhinal region (Braak et al. 1994) and in large neurons of the basal nucleus of Meynert (Sassin et al. 2000). The antibody AT8 that recognizes a phosphoepitope in early pathological tau (Su et al. 1994) and Gallyas silver impregnation were used to visualize earlier and later stages of neurofibrillary changes, respectively. The overall sequence of events was similar in the two regions, with the minor

dissimilarities likely attributable to differences in the structures of the afflicted neurons. As proposed by the authors, tau accumulation first appears as even, granular, or hatched AT8-immunoreactivity throughout the perikaryon and processes of an otherwise normal-looking neuron. The second stage is the appearance within the perikaryon of small filamentous or rod-like inclusions (early tangle) that often colocalize with lipofuscin. Concomitantly, axonal AT8-positivity is lost, and deterioration of dendrites begins, first evident in distal parts as conspicuous changes such as fragmentation, thickening, decrease of AT8-immunoreactivity, and the appearance of tortuous or rod-like portions and terminal swellings. Argyrophilia also emerges, silver impregnation revealing singular filamentous inclusions in the soma and distal neuritic fragments. The following stage represents the classic NFT, shown by both stains as a globular or flame-like mass of bundled filaments that partially displace the nucleus. Dendrites undergo further degeneration, losing much of their AT8-positivity, while tortuous fragments may remain around the soma. The final stages entail the death of the neuron as evident from the disappearance of the cell body. The NFT remains as a “ghost” tangle, which gradually loses its AT8-immunoreactivity but retains argyrophilia as a loosely packed filamentous mass (Braak et al. 1994, Sassin et al. 2000). The ghost tangles become infiltrated by processes of glial fibrillary acidic protein (GFAP)-positive astrocytes and by activated microglia (Probst et al. 1982, Cras et al. 1991, Sheng et al. 1997).

Similar morphogenetic schemes, *i.e.*, a progression from a “pre-tangle” stage (diffuse or granular cytoplasmic staining for tau/HP-tau) via classic NFT to an extracellular tangle, have been derived by other authors (Bancher et al. 1989a, Lauckner et al. 2003). The transition from the pre-tangle stage to classic NFT seems to involve a conformational change in tau (Uboga and Price 2000) and progressive phosphorylation at specific sites (Kimura et al. 1996, Augustinack et al. 2002, Lauckner et al. 2003), as shown using conformation- and phosphoepitope-dependent antibodies. Compared with tau, Ub immunoreactivity appears late in the process of NFT formation, following argyrophilia and persisting in ghost tangles (Bancher et al. 1989a, Bancher et al. 1991, Uchihara et al. 2001).

Ultrastructural studies suggest that the transition from pre-tangle to NFT is accompanied by the conversion of nonfilamentous or granular tau into fibrillar form (Bancher et al.

1989a, Weaver et al. 2000). In the pre-tangle neuron, AT8 immunoreactivity is mostly present diffusely in the cytoplasm, with focal concentrations that seem to be the initial site of fibrillogenesis. Progressive incorporation of the diffuse material into PHFs and SFs coincides with the emergence of an early NFT. Upon tangle maturation, the fibrils aggregate and align into densely packed bundles that contain PHFs peripherally and SFs in the core, finally filling most of the perikaryon as a classic NFT (Gomez-Ramos and Moran 1998).

Despite the consensus on the morphogenetic events in NFT life span, little is known on the duration of each developmental stage. However, mathematical modeling, based on quantified neuronal loss and NFT abundance in AD hippocampus, suggests that neurons might survive with NFT for 15–25 years (Morsch et al. 1999). A long lifetime for NFT-bearing neurons is also supported by stereological analyses in limbic and neocortical areas of AD brains (Bussiere et al. 2003, Hof et al. 2003). Consistent with these findings, direct measurement of neuronal activity using metabolic markers showed that NFT-bearing neurons remain active, albeit at a lower metabolic level compared to normal neurons (Hatanpää et al. 1996).

In addition to tau and Ub, an increasing number of proteins or other biomolecules have been localized into NFTs. Some of these also accumulate in pre-tangle neurons, possibly suggesting an early involvement in neurofibrillary changes. The latter constituents include the cdk5 protein kinase (Pei et al. 1998) and active forms of cdc2, MEK1/2, ERK1/2, and GSK-3β (Pei et al. 1999, Ferrer et al. 2001, Pei et al. 2002a, Pei et al. 2002b), translation initiation factor 2α (Ferrer 2002), several septins (*i.e.*, cytoskeletal GTPases) (Kinoshita et al. 1998), active calpain II (Grynspan et al. 1997), and sulphated glycosaminoglycans (sGAGs) (Goedert et al. 1996). The potential significance of these and other NFT constituents for tau fibrillization and tangle formation remains to be elucidated, but mechanistic studies suggest promoting roles for most of the above kinases as well as for calpains and sGAGs.

The total amount of tau is 4–8-fold elevated in AD, resulting from the accumulation of hyperphosphorylated tau (Khatoon et al. 1992, Khatoon et al. 1994). Besides hyper-

phosphorylation, a variety of other modifications in tau have been identified in AD, as shown by immunolocalization into NFTs and/or by biochemical analysis of brain-extracted pathological tau. These modifications include glycation, N- and O-linked glycosylation, oxidation, nitration, truncation, ubiquitination, and transglutaminase-catalyzed cross-linking (Norlund et al. 1999, Buee et al. 2000, Gamblin et al. 2003, Horiguchi et al. 2003), but quantitative data on the extents of each in different stages of tangle formation is mostly lacking. Finally, while PHFs contain the 6 isoforms of tau in proportions similar to normal tau (Goedert et al. 1995), the isoform composition and morphology of PHFs vary in different neuronal compartments (Liu et al. 1993, Kurt et al. 1997, Ishizawa et al. 2000). As in the case of the other constituents of NFTs, it is mostly unclear to what degree and how the alterations of tau structure or isoform ratios might contribute to tau aggregation into NFTs.

Mechanistic aspects Based on the morphogenetic descriptions of tau aggregation into NFTs (Braak et al. 1994, Gomez-Ramos and Moran 1998), the process can be viewed as three partially overlapping subprocesses: 1) accumulation of nonfibrillar tau throughout the cytoplasm of an initially normal neuron, 2) conversion of cytoplasmic, nonfibrillar tau into PHFs and other filamentous forms, and 3) bundling of separate fibrils into a large NFT. At the molecular level, each subprocess is poorly understood due to the scarcity of good experimental models, but studies on human material and available models have provided variable degrees of evidence for possible mechanisms.

While the changes that precede and expedite tau pathology in AD brains likely involve altered metabolism of amyloid precursor protein (Lewis et al. 2001, Delacourte et al. 2002, Hardy and Selkoe 2002), the immediate events leading to the accumulation of nonfibrillar (HP-)tau remain unclear. It is well established that AD-like hyperphosphorylation of tau may cause its detachment from MTs (Biernat et al. 1993, Bramblett et al. 1993, Friedhoff et al. 2000) and sequestration of normal tau (Alonso et al. 1994, Alonso et al. 1996, Iqbal et al. 1998), possibly underlying the somatodendritic accumulation of tau. Consistently, after genetic manipulations that increase tau phosphorylation, an elevation of unbound HP-tau is seen in cultured cells (*e.g.*, Xie et al. 1998, Hamdane et al. 2003) and somatodendritic HP-tau emerges in mice (Kins et al. 2001, Lucas et al. 2001, Liou et al. 2003). However, the dissociation of tau from MTs

may also be promoted by other factors such as sGAGs (Hasegawa et al. 1997). On the other hand, the several-fold increase of total tau in AD (Khatoon et al. 1994) implies that there is a disturbance of tau synthesis and/or degradation. Indeed, the expression of tau mRNA is increased in affected brain areas (Barton et al. 1990), possibly with a shift in the isoform ratios towards 4-repeat tau (Yasojima et al. 1999), and in mice, overexpression of normal tau leads to pre-tangle-like accumulation (Götz et al. 1995, Brion et al. 1999). Tau may also fail to be properly degraded due to modifications that render it refractory to proteolysis (Litersky and Johnson 1995, Yang and Ksiezak-Reding 1995) and/or due to a decline in proteasome activity as seen in AD (Keller et al. 2000, Keck et al. 2003). One possible cause for this decline might be the accumulation of frameshift mutant Ub (van Leeuwen et al. 1998) which inhibits the Ub-proteasome system *in vitro* (Lindsten et al. 2002, Hope et al. 2003).

Elucidation of the structural basis of tau fibrillization has been hampered, since the natively unfolded character of soluble tau has prevented its structural determination (Barghorn and Mandelkow 2002) and the fine structure of PHFs also remains debatable (Pollanen et al. 1997, Moreno-Herrero et al. 2004). In addition, attempts to produce tau fibrils in cultured cells have been frustrated by the hydrophilicity of tau (Friedhoff et al. 2000). Therefore, insight into fibril formation mostly comes from cell-free experiments, where a variety of conditions have been employed to induce fibril assembly from recombinant tau proteins (Barghorn and Mandelkow 2002). These studies show that fibrils with various morphologies, some indistinguishable from PHFs in AD, can assemble from unmodified tau isoforms over a time scale of days to weeks (Goedert et al. 1996, Perez et al. 1996, Friedhoff et al. 1998, King et al. 1999), while only the MT-binding repeat regions seem to be crucial for fibrillization (Kampers et al. 1996, Perez et al. 1996). The process is nucleation-dependent and can be greatly accelerated in the presence of cofactors such as polyanions (*i.e.*, sGAGs, RNA, and polyglutamate) (Goedert et al. 1996, Friedhoff et al. 1998) or fatty acids (Wilson and Binder 1997), which may balance the positive charges present in tau.

Findings from two well-characterized systems (Friedhoff et al. 1998, King et al. 1999) were compatible with a structural model in which the basic building block of fibril assembly is a dimer of antiparallel tau molecules that interact via their repeat regions.

Fibrils would result from stacking of such dimers, involving a conformational change and, in oxidative conditions, stabilization by disulfide bridges (Barghorn and Mandelkow 2002). Consistent with this model, PHFs in AD contain a protease-resistant core formed by the repeat regions in a cross-β arrangement (Novak et al. 1993, Barghorn et al. 2004). Although the conditions in cell-free systems differ in numerous ways from those *in vivo*, the findings suggest that also in AD, tau fibrillization might be promoted by cofactors such as RNA (Ginsberg et al. 1997) and sGAGs (Goedert et al. 1996), by oxidative stress (Markesbery 1999), or by accumulation of tau even in the absence of cofactors or modifications.

The role of phosphorylation in tau fibrillization is unclear. In the presence of polyanions, the phosphorylation of tau by several different kinases inhibited its assembly into fibrils (Schneider et al. 1999). However, in more physiological conditions utilizing no cofactors, heavy hyperphosphorylation of tau by brain extracts induced a rapid formation of PHFs and SFs which further associated into bundles (Alonso et al. 2001). This apparent disparity may suggest that the effect on fibrillization is determined by the sites and extent of phosphorylation.

Few studies have addressed the molecular events whereby separate fibrils conjoin into a large NFT. While spontaneous bundling occurs in some models (Alonso et al. 2001), this process may be promoted by glycation (Ledesma et al. 1998), which is seen in PHF bundles in AD (Ko et al. 1999). The bundling of fibrils seems to precede the emergence of Ub immunoreactivity (Iwatsubo et al. 1992), an event of unclear significance. The incorporation of Ub might reflect a malfunction of Ub-dependent proteolysis in affected neurons (Alves-Rodrigues et al. 1998), which might result from the inhibition of proteasomes by fibrillar tau (Keck et al. 2003), causing the general buildup of Ub-conjugated proteins. Alternatively, the late-emerging Ub-positivity in NFTs might arise from the ubiquitination of tangle constituents as part of an adaptive cellular response. For example, it might indicate an attempt at proteasomal degradation (Layfield et al. 2003), as evidenced by the presence of some proteasomal subunits in NFTs (Fergusson et al. 1996). However, it should be noted that most of the Ub conjugated to PHFs seems to occur in the monomeric form (Morishima-Kawashima et al. 1993), a poor signal for

efficient proteasomal targeting (Thrower et al. 2000), while *in vitro*, tau can undergo proteasomal degradation without any requirement for ubiquitination (David et al. 2002). Therefore, pathways for Ub conjugation might also be defective in tangle-bearing neurons. Another suggestion, based on the localization of the Ub-conjugation sites in the MT-binding region of tau, is that the core of PHFs could be highly refractory to proteasomal digestion (Morishima-Kawashima et al. 1993). One further possibility, proposed by Gray (2001), is that the (mono)ubiquitination of PHFs might serve a non-proteolytic role as a "nonstick coating" that would prevent the further growth of the aggregates and limit detrimental interactions with normal cellular constituents. However, there is currently little direct evidence in support of any of the above hypotheses.

2.3 α-Synuclein-containing inclusions

α-Synuclein is an abundant presynaptic protein that in the brain is expressed mostly as an isoform of 140 residues (Goedert 2001, Lotharius and Brundin 2002, Dev et al. 2003). It is located mainly in nerve terminals, where it is found in close proximity to synaptic vesicles, both membrane-associated and free in the cytosol. The functions of αS are not well established, but its key function is thought to be in the regulation of synaptic vesicular transport, including that of dopamine, possibly via inhibition of phospholipase D2. Other aspects of dopamine metabolism may also be regulated by αS. Similar to tau, αS is a natively unfolded protein. It comprises an N-terminal domain harboring 11-amino acid repeats that bind to membranes by assuming an α-helical structure, a hydrophobic intermediate region, and a C-terminal acidic region with chaperone-like activity. A number of proteins and other ligands including fatty acids interact directly with αS (Goedert 2001, Lotharius and Brundin 2002, Dev et al. 2003).

Several pathological conditions are associated with the formation of cytoplasmic inclusions that assumably contain αS as their main protein component (Galvin et al. 2001). The most common types of these are described below.

2.3.1 Neuronal types

Lewy bodies The most common type of αS-containing inclusion is the "Lewy body" (LB), which are usually classified into brainstem and cortical types (Gibb et al. 1991,

Forno 1996, Lowe et al. 1997). In the brainstem, “classic” LBs appear within neuronal perikarya as eosinophilic, conspicuously spherical inclusions with a concentric lamination (core, body, and halo), although not all layers are usually discernible. Ultrastructurally, the core is densely granular, while the outer layers contain haphazardly to radially arranged filaments. Cortical LBs usually display a less rounded and more uniform morphology. Both brainstem and cortical LBs are assumed to be mainly composed of aberrant αS fibrils, 5–10 nm in diameter (Baba et al. 1998, Spillantini et al. 1998b, Goedert 2001). Immunohistochemistry shows a less consistent incorporation of numerous other proteins (Dev et al. 2003) including polyubiquitinated species (Iwatsubo et al. 1996) and phosphorylated neurofilaments (Bancher et al. 1989b).

LBs may occur in various predilection sites of the brain as well as in sympathetic ganglia (Pollanen et al. 1993), showing variable distributions which often associate with clinical states that reflect the pattern of involvement (Lowe et al. 1997). In particular, the presence of LBs in the substantia nigra (SN) and other pigmented brainstem nuclei is a diagnostic hallmark of PD (Forno 1996), whereas their occurrence in the cerebral cortex is used as a criterion for the diagnosis of dementia with LBs (DLB) (McKeith et al. 1996). However, “incidental” LBs also occur in neurologically normal aged individuals. Perikaryal LBs are usually accompanied by thread-like or swollen αS-immunoreactive neurites, as well as by globular αS-immunoreactive inclusions within neuronal processes or apparently free-lying in the neuropil (Spillantini et al. 1998b). These extrasomal inclusions are also commonly referred to as LBs, despite their somewhat differing morphology as compared to the perikaryal ones.

Pale bodies Another type of PD-associated inclusion is the “pale body” (PaB). These are weakly or non-eosinophilic, globular or irregular bodies of homogenous or uniformly granular material that seems to displace neuromelanin but fails to stain with many common histological stains or silver impregnation (Roy and Wolman 1969, Pappolla et al. 1988, Gibb et al. 1991). However, they are slightly reactive with the Ninhydrin-periodic acid-Schiff method, indicating a low protein content (Pappolla et al. 1988). Ultrastructurally, PaBs consist of disorganized, αS-immunoreactive fibrils interspersed with vacuoles and granular matter (Gibb et al. 1991, Arima et al. 1998b, Wakabayashi et

al. 1998a). Besides moderate to strong immunoreactivity for αS, most PaBs stain variably for Ub, a minority also for phosphorylated neurofilaments (Dale et al. 1992). PaBs have been found in neurons of the SN and locus coeruleus, but not in several other predilection areas for LB pathology (Gibb et al. 1991). The pathogenetic relation of PaBs and LBs remains unclear. Although PaBs seem to occur in the SN of all PD patients (Pappolla et al. 1988), they are generally not used for diagnosis.

2.3.2 Glial types

Glial cytoplasmic inclusions The defining feature of multiple system atrophy (MSA) is the presence of "glial cytoplasmic inclusions" (GCIs) within oligodendrocytes (Castellani 1998, Gilman et al. 1999). GCIs are faintly eosinophilic, sickle- or flame-shaped or ovoid inclusions that seem to displace the nucleus. They can be visualized with the Gallyas silver technique but are mostly negative for other histological stains. Similar to LBs, GCIs immunostain strongly for αS (*e.g.*, Wakabayashi et al. 1998b). Ultrastructurally, GCIs contain loosely aggregated αS-immunoreactive tubules or filaments, 20–40 nm across, associated with granular material and organelles (Arima et al. 1998a, Wakabayashi et al. 1998a, Burn and Jaros 2001). A detailed study on immunoisolated GCIs revealed bundles of amorphous material-coated filaments, the core of which consisted of paired strings of 3–6 nm particles, possibly representing αS oligomers (Gai et al. 2003). Biochemical analysis identified Ub, αB crystallin, and tubulins as additional constituents (Gai et al. 1999), while other proteins were variably present as determined by immunolabeling (Castellani 1998, Burn and Jaros 2001).

In MSA brains, GCIs are widely distributed (Papp and Lantos 1994, Inoue et al. 1997) and represent the predominant inclusion type. They may be accompanied by inclusions in neurons and in glial and neuronal nuclei, as well as by neuropil threads (Lowe et al. 1997). Apart from glial nuclear inclusions, each structure immunolabels positively for αS (Wakabayashi et al. 1998a). The mechanisms underlying the formation of GCIs as well as their pathogenetic significance remain mostly unknown. However, biochemical analyses of MSA brain specimens indicate that the conversion of αS to poorly soluble forms may contribute to GCI formation (Tu et al. 1998, Dickson et al. 1999, Duda et al. 2000, Campbell et al. 2001), while immunohistochemical evidence points to the

involvement of αS phosphorylation (Fujiwara et al. 2002) and nitration (Giasson et al. 2000) in the pathogenesis of synucleinopathies including MSA.

2.3.3 Formation of brainstem-type Lewy bodies

Compared to AD-type NFTs, the formation of LBs is less well characterized and understood since their assumed main component, αS, was not identified until 1997 (Polymeropoulos et al. 1997, Spillantini et al. 1997). On the other hand, a wider range of options are available for studying αS aggregation in animal models, where it is possible to induce a PD-like phenotype not only by αS overexpression but also pharmacologically by several types of compounds. Below, the focus will be on brainstem-type LBs, which have been more extensively characterized than the cortical type.

Neuropathological findings Before the introduction of αS immunolabeling, studies were biased towards conspicuous inclusions visualizable using nonspecific stains, therefore missing earlier types of pathological change. Among the initial observations on nigral inclusions was the distinction of PaBs from LBs by their histological staining properties (Redlich 1930) and ultrastructure (Roy and Wolman 1969). These authors' interpretation was that PaBs are an early change in LB development. However, in subsequent studies utilizing histological stains, electron microscopy, and immunolabeling for neurofilaments, PaBs and LBs were deemed to be not directly related on the basis of their dissimilar staining and ultrastructural profiles (Pappolla et al. 1988, Gibb et al. 1991). The lack of intermediate forms between PaBs and LBs was also pointed out, whereas a more likely precursor of LBs seemed to be a third type of fibrillar accumulation, termed as "Lewy-body-like matter" (Gibb et al. 1991). However, this latter type has not been described in other studies.

On the other hand, the above studies also revealed that PaBs are always accompanied by LBs in the SN (Pappolla et al. 1988) and co-occur in the same neuron more often than expected by chance (Gibb et al. 1991). These findings implied that the formation of LBs and PaBs is closely related, with the possibility that they might be formed as successive stages of the same process. Indeed, immunolabeling for Ub had revealed inclusions interpreted as intermediary forms of PaBs and LBs (Leigh et al. 1989). Dale et al. (1992)

found that PaBs and LBs correlate in abundance, and that PaBs seemed to emerge before LBs, but the issue on separate processes vs. successive stages remained unsettled. Subsequently, transitional forms between PaBs and LBs were also illustrated ultrastructurally in the locus coeruleus (Takahashi et al. 1994), with the suggestion that PaBs would give rise to LBs, although only one atypical non-parkinsonian case was examined.

The introduction of αS immunostaining enabled the detection of less conspicuous types of αS accumulation, appearing as "diffuse" (Wakabayashi et al. 1998a, Saito et al. 2003) or "cloud-like" (Gomez-Tortosa et al. 2000) cytoplasmic αS-positivity in otherwise normal-looking neurons, and interpreted as early changes, giving rise to the larger inclusions in the SN. In pigmented neurons of lower brain stem nuclei, another subtle change was found, *i.e.*, "dust-like" αS particles, but their presence in the SN was not examined (Braak et al. 2001). These studies also revealed the αS-positivity of PaBs. Ultrastructurally, the αS-positive material in PaBs and LBs appeared to be similar, consisting of (granulo-)filamentous components, more densely packed in LBs (Arima et al. 1998b, Wakabayashi et al. 1998a), lending support to the view that PaBs are precursors to LBs. Thus, Wakabayashi et al. (1998a) suggested a model for the formation of nigral LBs, in which αS is initially seen as weak, diffuse cytoplasmic staining, followed by the appearance of irregularly shaped accumulations that evolve into clearly demarcated inclusions (PaBs) and further to typical LBs. This process seemed to be accompanied by progressive loss of neuromelanin and the increase of αS immunoreactivity. A consistent scheme was proposed by Gomez-Tortosa et al. (2000), with the note that Ub-positivity seemed to be engaged upon the appearance of more compact inclusions. Results obtained by immunolabeling for Ser-129-phosphorylated αS also conformed to the above model (Saito et al. 2003). Taken together, the consensus view is that LB formation begins from a dispersed cytoplasmic accumulation of αS (diffuse, cloud-like, or dust-like), from which PaBs and LBs evolve sequentially, but the fine structure of the initial change as well as the sequence of intermediate events remain to be defined.

In addition to the above findings on events confined to neuronal perikarya, studies on the overall progression of αS pathology in PD suggest that the emergence of αS-positive neurites precedes that of perikaryal inclusions in brainstem nuclei including the SN (Del Tredici et al. 2002, Braak et al. 2003). The neurites were presumed to be mostly axonal (Braak and Braak 2000). However, it has not been determined how this intraneuritic accumulation of αS relates to that within perikarya.

The pathological αS that accumulates in LB-containing tissues differs from normal αS in several ways, as indicated by biochemical and immunohistochemical analyses (Dev et al. 2003). It is less soluble (Campbell et al. 2000, Kahle et al. 2001), contains partially truncated as well as higher molecular weight species of αS (Baba et al. 1998, Tofaris et al. 2003), and is mostly phosphorylated at Ser-129 (Fujiwara et al. 2002). The pathological αS is also partially conjugated to Ub (with mono- to polyubiquitinated species) (Hasegawa et al. 2002, Sampathu et al. 2003) and contains a modified ~23-kD form of αS (Tofaris et al. 2003). In addition, pathological αS is nitrated at tyrosine residues (Giasson et al. 2000) and is enriched in transglutaminase-catalyzed crosslinks (Junn et al. 2003, Andringa et al. 2004). Epitopes for advanced glycation endproducts also appear in nigral LBs (Munch et al. 2000). In addition to insoluble αS, PD and DLB brains show elevated levels of soluble αS oligomers (Sharon et al. 2003), which seem to form in the presence of polyunsaturated fatty acids (Perrin et al. 2001) and were suggested by Sharon et al. to precede the aggregation of αS into insoluble forms. The significance of these disease-associated alterations in αS for LB formation remains unclear. However, *in vitro*, several of them affect the propensity of αS to undergo aggregation (p. 41).

In addition to αS and Ub, numerous other protein species, as well as lipids, accumulate into LBs (*e.g.*, Pollanen et al. 1993, Trimmer et al. 2004). While the protein constituents include signaling and cytoskeletal proteins and those involved in cell stress and protein degradation and might thus serve causal, structural, or adaptive functions in LB formation or only be passively entrapped into the LB (Pollanen et al. 1993), there is currently little evidence to assign specific functional roles for most of the identified components. In contrast to LBs, few proteins have been reported to localize into PaBs, in

accordance with their histological staining properties that suggest a low protein content (Pappolla et al. 1988).

Mechanistic aspects Analogous with the case of NFTs and tau, the events underlying LB formation constitute three overlapping subprocesses: 1) accumulation of αS within perikarya and neurites, 2) fibrillogenesis from normal αS, and 3) segregation of pathological αS into discrete inclusions. Some of the relevant findings providing mechanistic insight into these phenomena are compiled below. It should be noted that much of the observations from cell culture and cell-free studies may be relevant to αS-containing inclusions regardless of their type.

It is unclear whether the perikaryal accumulation of αS results from increased expression, impaired degradation, or from a redistribution of αS. Consistent with the notion that an elevation of αS mRNA level may promote αS accumulation, a triplication of the αS locus was identified in a family with autosomal dominant PD (Singleton et al. 2003). Likewise, in several mice and primate models, overexpression of normal human αS causes diffuse perikaryal accumulation of αS (Kahle et al. 2000, Matsuoka et al. 2001, Richfield et al. 2002, Kirik et al. 2003). However, in a sample of 9 PD cases, a decrease of αS mRNA expression was found in the SN (Neystat et al. 1999), whereas in neocortical areas of DLB brains, no change (Wirdefeldt et al. 2001) or an increase (Rockenstein et al. 2001) in the αS mRNA level was observed. Although the latter three studies reveal no consistent relation to LB pathology, it should be noted that αS mRNA levels in tissues with advanced pathology may not reflect the events at earlier stages.

The accumulation of αS may also stem from impaired proteolysis (McNaught and Olanow 2003). Both PD and DLB brains show a loss of proteasomal subunits and activity in the SN (McNaught and Jenner 2001, McNaught et al. 2003, Tofaris et al. 2003), and mutations in two enzymes of the Ub-proteasome pathway cosegregate with early-onset PD (Kitada et al. 1998, Leroy et al. 1998). In addition, fibrillization renders αS more resistant towards proteinase K digestion (Giasson et al. 2001, Neumann et al. 2002), while both monomeric and aggregated αS inhibit proteasomal activity (Snyder et al. 2003, Lindersson et al. 2004). Although the mechanism of αS degradation is unclear,

at least some of the normal αS may be degraded via the proteasome (Bennett et al. 1999, Webb et al. 2003), albeit in a Ub-independent manner (Tofaris et al. 2001). Thus, a self-maintaining cycle may ensue in which proteasomal dysfunction promotes the accumulation of αS and vice versa, leading to a buildup of proteasome-dependent substrates. Accordingly, drug-induced inhibition of proteasomes in cell culture (Rideout et al. 2001, Rideout and Stefanis 2002) and in rat brain (McNaught et al. 2002a, Fornai et al. 2003) results in the formation of inclusions, many of which contain αS. On the other hand, the data on the proteasomal degradation of αS have been disputed (Ancolio et al. 2000, Biasini et al. 2004), with other studies pointing to autophagic (Webb et al. 2003) or lysosomal (Paxinou et al. 2001, Lee et al. 2004) pathways. Further, the accumulation of αS in PD seems to precede that of Ub which is only present in a subset of LBs (Dale et al. 1992), a finding corroborated by results from transgenic models (Dev et al. 2003). These findings might suggest instead that proteasomal dysfunction may not contribute to the early stages of αS accumulation, but becomes manifest later as the incorporation of Ub into LBs due to the overload of Ub-dependent degradation pathways (Layfield et al. 2003).

The perikaryal accumulation of αS might also be contributed to by a redistribution of the normally presynaptic αS into the neuronal bodies, as seemed to occur in an MPTP-induced primate model (Kowall et al. 2000). In PD, this kind of redistribution might ensue from a retardation in axonal transport, as observed for αS in aging mice, also providing a possible explanation for the occurrence of the first αS aggregates within neurites (Li et al. 2004). Evidence for defective axonal transport was also provided by the finding that axonally transported proteins seem to progressively accumulate into developing LBs (Katsuse et al. 2003).

Oxidative stress and mitochondrial defects are implicated in PD pathogenesis (Schapira 1996, Jenner 2003) and could contribute to the abnormal buildup of αS. Indeed, mitochondrial toxins (*i.e.*, MPTP, rotenone, paraquat) caused αS accumulation within the perikarya of nigral neurons in both mice (Betarbet et al. 2000, Vila et al. 2000, Manning-Bog et al. 2002) and the baboon (Kowall et al. 2000), an effect that may be mediated by retarded degradation of αS (Sherer et al. 2002) and/or upregulation of αS expression

(Vila et al. 2000, Gomez-Santos et al. 2002). Further, spontaneous formation of αS-containing inclusions that replicated many of the antigenic and structural features of LBs occurred in a long-term culture of neuroblastoma cells expressing mitochondrial genes from PD patients (Trimmer et al. 2004). Although the underlying events in these models are unclear, complex self-maintaining mechanisms may be involved, based on the multiple interconnections between oxidative stress, mitochondrial and proteolytic dysfunction, and accumulation/aggregation of αS (Lotharius and Brundin 2002, Jenner 2003).

As regards the structural basis of αS fibrillization, it may be relevant that αS (Weinreb et al. 1996) as well as tau (Schweers et al. 1994) belong to the class of intrinsically unstructured (natively unfolded) proteins (IUPs), characterized by extended, flexible, random-coil-like conformations with little secondary structure (Tompa 2002). Notably, the structural plasticity and other characteristic features of the IUPs that are essential for their functions might also favor the formation of pathological fibrils (Uversky and Fink 2004). In particular, many IUPs, including αS and tau (Syme et al. 2002), show a preference for the type II polyproline helical conformation that, while providing flexibility for molecular recognition, may also confer a propensity for the conversion to β-sheet structure (Tompa 2002).

Nevertheless, it is poorly understood how the natively unfolded and soluble αS becomes arranged into insoluble, ordered fibrils. In concentrated solutions, αS monomers spontaneously aggregate within days to months into fibrils similar to those in LBs (*e.g.*, Conway et al. 1998, Giasson et al. 1999), although the morphology of the fibrils is highly dependent on the assembly conditions (Hoyer et al. 2002). Fibrillogenesis appears to occur via oligomeric, metastable intermediates, “protofibrils”, the formation of which involves a change from random coil to β-sheet conformation (Conway et al. 2000b, Ding et al. 2002, Lashuel et al. 2002b). The protofibrils are heterogenous, including spherical, chain-like, and annular types (Volles and Lansbury 2003), and are converted to stable fibrils with an amyloid-like cross-β structure (Conway et al. 2000a, Serpell et al. 2000). Fibrillization requires the central portion of αS which forms the protease-resistant core of the fibrils (Miake et al. 2002, Der-Sarkissian et al. 2003). The process is nucleation-

dependent (Wood et al. 1999), seeming to require oxidative dimerization by dityrosine cross-linking as a rate-limiting step (Krishnan et al. 2003).

Fibril formation *in vitro* is accelerated by diverse factors such as oxidative stress (Hashimoto et al. 1999), pesticides (Uversky et al. 2001b), polyvalent metal ions (Uversky et al. 2001a), crowding by inert macromolecules (Uversky et al. 2002), tubulins (Alim et al. 2002), sGAGs (Cohlberg et al. 2002), phosphorylation at Ser-129 (Fujiwara et al. 2002), and truncation of either terminus (Kessler et al. 2003, Murray et al. 2003), whereas the formation of soluble oligomers is enhanced by polyunsaturated fatty acids (Perrin et al. 2001). Fibrillogenesis is inhibited by β- and γ-synuclein (Uversky et al. 2002b), as well as by nitration (Yamin et al. 2003) and dopamine (Conway et al. 2001) which stabilize oligomeric αS species. Although other lines of evidence also link most of the above factors with LB pathology, it is unclear to what degree they might influence αS fibrillization *in vivo*.

In cell culture, the formation of αS-containing inclusions can be induced and/or promoted by a variety of treatments including overexpression (McLean et al. 2000), proteasomal inhibition (Rideout and Stefanis 2002), mitochondrial toxins (Lee et al. 2002), nitrative insult (Paxinou et al. 2001), and transglutaminase cross-linking (Junn et al. 2003), but in most studies the sequence of events has not been characterized. However, overexpression or rotenone treatment (Lee and Lee 2002, Lee et al. 2002) gave rise to small spherical aggregates of nonfibrillar αS throughout the cytoplasm. These aggregates, suggested to represent protofibrils, were transported to the juxtanuclear region where they coalesced forming a large, fibrillar and stable inclusion enriched in mitochondria and lysosomes (Lee and Lee 2002, Lee et al. 2002). This process was consistent with an aggresomal response (Johnston et al. 1998), in line with the findings that some aggresomal markers colocalize with LBs in PD and DLB brains (McNaught et al. 2002b). Nevertheless, a marked disparity remains between the morphologies of authentic LBs and aggresomes seen *in vitro* (Lee et al. 2002), suggesting that LB formation involves mechanisms poorly mirrored by the cellular models.

2.4 Physiology of p62

The p62 protein is a cytosolic, conserved protein with an apparent molecular weight of ~62 kD and bearing no overall homology to other proteins. It was first identified in studies of mitogenic signaling as a ligand of the tyrosine kinase $p56^{lck}$ (Park et al. 1995). Thereafter, in studies on other aspects of cellular physiology, p62 has been identified in several different fields where regulatory proteins have been searched for signaling proteins, ion channels, and transcription factors. Most studies to date implicate p62 in the regulation of atypical protein kinase C (aPKC), but many poorly understood aspects remain about its physiological role.

For historical reasons, the p62 gene/transcript/protein is referred to with several different names. While the approved gene name is “sequestosome 1” (SQSTM1), alternative names in use include “aPKC-interacting protein p62”, “ubiquitin-binding protein p62”, “zeta-interacting protein” (ZIP), and “A170”. The latter two names also refer to the rat and mouse counterparts, respectively. In the remainder of this text, “p62” will be used to denote the gene/transcript/protein irrespective of species. It should be noted that in other fields, the labels “p62” and “ZIP” are being used in reference to proteins unrelated to SQSTM1.

2.4.1 Structure

In the human genome, the p62 mRNA is transcribed from a single gene (GeneID no. 8878. Entrez. The Life Sciences Search Engine 2004) comprising 8 exons that encode a protein of 440 amino acids (Joung et al. 1996, Vadlamudi and Shin 1998). The sequence contains a number of regions suggestive of functional motifs or domains, including a PB1 domain that embeds an OPCA motif, a ZZ zinc finger, two PEST sequences, and a UBA domain (Fig. 1a). PB1 domains are present in many cytoplasmic signaling proteins and mediate the formation of heterodimeric complexes between PB1 domain-containing proteins (Ito et al. 2001, Ponting et al. 2002). The majority of PB1 domains also contain an OPCA motif, an acidic region necessary for heterodimerization (Nakamura et al. 1998, Ito et al. 2001). The ZZ domain encodes a putative zinc finger that is thought to mediate protein-protein interactions (Ponting et al. 1996). PEST sequences are characteristic of rapidly degraded proteins, many of which serve important regulatory

functions (Rechsteiner 1988). The UBA domain is found in diverse enzymes involved in the ubiquitination pathway and other systems. It mediates noncovalent binding to mono- and poly-Ub but can also bind other proteins and even mediate dimerization (Hofmann and Bucher 1996, Bertolaet et al. 2001, Buchberger 2002). The sequence of p62 also has many predicted phosphorylation sites for serine/threonine kinases (Fig. 1a) (Ishii et al. 1996, Joung et al. 1996), but it is unclear whether the regulation of p62 involves phosphorylation. At least in some cell types, p62 seems to be phosphorylated, likely by casein kinase II (Yanagawa et al. 1997).

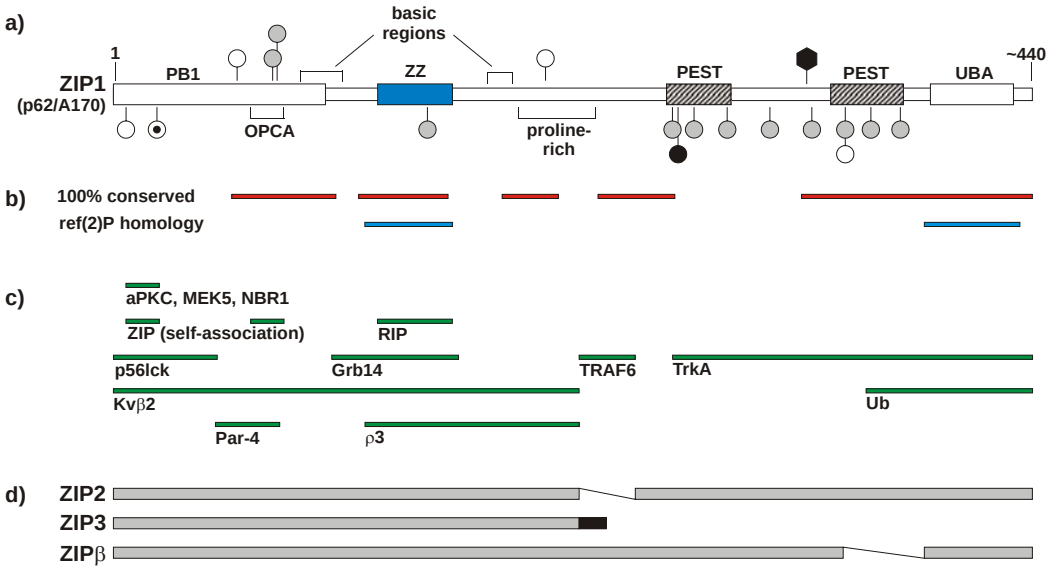

Figure 1. Primary structure, interaction sites, and isoforms of p62. **a)** Predicted functional domains and motifs (adapted from Okazaki et al. 1999). Phosphorylation sites are indicated by spheres (*white* = PKC; *grey* = casein kinase II; *black* = MAP kinase; *dotted* = cAMP/cGMP-dependent protein kinase). The possible N-linked glycosylation site is indicated by a hexagon. **b)** *Top:* Regions 100 % identical in human, rat, and mouse homologues. *Below:* Areas with high similarity to the *Drosophila* counterpart ref(2)P. **c)** Partially defined sequence stretches encompassing critical residues for protein bindings. **d)** Alternatively spliced isoforms. (From Joung et al. 1996, Vadlamudi et al. 1996, Gong et al. 1999, Sanz et al. 2000, Wooten et al. 2001, Avila et al. 2002, Cariou et al. 2002, Chang et al. 2002, Ciani et al. 2003, Croci et al. 2003, Lamark et al. 2003, Noda et al. 2003, Wilson et al. 2003).

The human amino acid sequence is 91 % identical with the rat and mouse counterparts. Notably, the sequence contains several stretches that in the three species are 100 % identical, suggestive of critical functional roles (Fig. 1b). These areas encompass the

OPCA motif, most of the ZZ domain, the C-terminal PEST sequence, and the UBA domain. The ZZ and UBA domains also show relatively high similarity to those of ref(2)P, the *Drosophila* homologue of p62 (Fig. 1b) (Okazaki et al. 1999, Avila et al. 2002).

Proteins interacting with p62 The p62 protein is capable of a large number of noncovalent interactions, as demonstrated using various affinity assays. Its binding partners include cell surface receptors (TrkA–C), receptor-associated proteins (RIP, TRAF6, $p56^{lck}$, Grb14), other signaling proteins (PKC-ζ, PKC-λ/ι, PAR-4, MEK5, $p38^{MAPK}$, p120 ras-GAP, NBR1), a transcription factor (COUP-TFII), ion channel proteins (Kvβ2, ρ3), Ub, and p62 itself (Park et al. 1995, Marcus et al. 1996, Vadlamudi et al. 1996, Puls et al. 1997, Sanchez et al. 1998, Gong et al. 1999, Sanz et al. 1999, Sanz et al. 2000, Sudo et al. 2000, Wooten et al. 2001, Cariou et al. 2002, Chang et al. 2002, Croci et al. 2003, Geetha and Wooten 2003, Lamark et al. 2003, Noda et al. 2003). For most of the interactions, some information on the respective binding sites in p62 has been obtained by using mutated constructs (Fig. 1c). Nonetheless, the structural basis underlying these interactions is poorly understood, since no crystal/solution structure has been published for the entire p62 or many of its binding partners. However, the structure of the p62 UBA domain has been determined (Ciani et al. 2003) and can explain the noncovalent p62–Ub binding in terms of hydrophobic interaction between Ub and the UBA domain (Mueller and Feigon 2002). Some of the remaining interactions can be understood based on structural studies on PB1 domain-containing proteins. PB1 domains can heterodimerize in a front-to-back arrangement, promoting the interaction of p62 with aPKC, MEK5, and NBR1 (Noda et al. 2003, Wilson et al. 2003). Notably, p62 also displayed an exceptional propensity for strong PB1-mediated self-association (Lamark et al. 2003, Wilson et al. 2003).

2.4.2 Expression

Tissue distribution In human tissues, p62 mRNA is ubiquitously expressed (Joung et al. 1996), consistent with findings from rodents (Joung et al. 1996, Puls et al. 1997, Gong et al. 1999, Okazaki et al. 1999, Croci et al. 2003). The distribution of p62 protein has not been studied in human tissues, but in the rat, the protein was detected in all tissues tested

(Gong et al. 1999). Within rat brain, p62 was mostly expressed in neurons, especially in pyramidal cells of the cerebrum and cerebellar Purkinje cells (Nakaso et al. 1999). Four alternatively spliced isoforms of p62 have been identified from rat tissues (Fig. 1a, d). In comparison to the full-length (ZIP1) isoform, ZIP2 (Gong et al. 1999) and ZIPβ (Cariou et al. 2002) lack regions containing the TRAF6 binding site and a PEST sequence, respectively, while ZIP3 has a truncated, variant C-terminus (Croci et al. 2003). The protein seems to be mostly expressed as the longest isoform (Ishii et al. 1996, Joung et al. 1996, Puls et al. 1997, Sanchez et al. 1998, Chang et al. 2002).

Subcellular localization In several cell types, p62 displays a punctate cytoplasmic pattern that colocalizes with late endosomes (Sanz et al. 2000, Aono et al. 2003, Geetha and Wooten 2003) as well as with the distribution of aPKC (Sanchez et al. 1998, Samuels et al. 2001). Since p62 lacks membrane-spanning domains, it probably resides on the cytosolic side of the vesicles. The molecular basis underlying the association of p62 with late endosomes is not understood. However, studies on PB1 domain-containing proteins have suggested that the punctate distribution of p62 requires its oligomerization, while aPKCs appear to be anchored into the punctate structures by p62 (Lamark et al. 2003). On the other hand, stimulation of cells by growth factors (NGF, EGF, IL-1) caused the internalization of the respective receptors (TrkA, EGFR) and receptor-associated proteins (IRAK, TRAF6) within endocytic vesicles, resulting in their partial colocalization with the vesicular pattern of p62 (Sanchez et al. 1998, Sanz et al. 2000, Geetha and Wooten 2003, Lamark et al. 2003). This localization of p62 was therefore speculated to reflect a function in the endocytic trafficking of receptors.

Regulation The expression of p62 mRNA and/or protein is induced by diverse physiological and exogenous stimuli, including the mitogenic or differentiation-inducing factors IL-3, NGF, TGF-β1, serum, PMA, and Ca^{2+} ionomycin (Lee et al. 1998, Gong et al. 1999, Okazaki et al. 1999), proteasome inhibitors (Ishii et al. 1997), oxidative stress-generating agents (Ishii et al. 1996, Ishii et al. 2000), and glycol ethers (Syed and Hecht 1998). The rapid induction of p62 expression by some of these stimuli justify the classification of p62 as an immediate early response gene (Lee et al. 1998). The regulatory regions of the human p62 gene harbor CpG islands (Vadlamudi and Shin

1998), characteristic of highly regulated genes essential for cell vitality (Bird 1986). Accordingly, the 5' regions flanking the transcribed sequences in human and mouse genes contain putative binding sites for transcription factors including AP-1, Sp1, NF-κB, c-myc, Ets-1 family, NF-E2, MyoD, and C/EBP, possibly accounting for the responsivity of p62 expression to the above-mentioned stimuli (Vadlamudi and Shin 1998, Okazaki et al. 1999). However, the regulation of the p62 transcript level also seems to involve mRNA stabilization (Lee et al. 1998).

2.4.3 Functions

Currently, the best-characterized aspects of p62 function are found in the context of aPKC regulation, where p62 has been implicated in the engagement of aPKC in response to cell-surface receptor stimulation and in the aPKC-mediated modulation of ion channels. However, other activities apparently unrelated to aPKC have also been described for p62. Knowledge on its physiological role is mostly based on *in vitro* studies, since mice ablated for the p62 gene were only described recently (Duran et al. 2004).

2.4.3.1 Regulation of atypical protein kinase C

Atypical PKCs (PKC-ζ and PKC-λ/ι) play critical roles in the control of cell growth, differentiation, survival, and polarity, as well as glucose transport (Wooten 1999, Moscat and Diaz-Meco 2000, Farese 2002, Etienne-Manneville and Hall 2003). These effects are partly mediated by aPKC-dependent regulation of gene expression via key signaling pathways (Hirai and Chida 2003, Suzuki et al. 2003). In particular, aPKC plays an important role in the activation of NF-κB (Diaz-Meco et al. 1993, Leitges et al. 2001), a transcription factor involved in a range of cellular functions including inflammatory and immune responses and regulation of cell survival (Karin and Lin 2002). This effect of aPKC is assumably exerted via the activation of IKKβ (Lallena et al. 1999). The activity of aPKCs is regulated by extracellular stimuli (Farese 2002, Hirai and Chida 2003, Suzuki et al. 2003), but the mechanisms determining the target specificity of aPKCs are largely unclear. However, many protein kinases are regulated by adapter, anchoring, and scaffold proteins, which confer specificity to the kinase by determining its site of action or by bridging it to target proteins (Pawson and Scott 1997, Mochly-Rosen and Gordon

1998, Garrington and Johnson 1999). Several studies suggest that p62 may serve as an adapter or scaffold for aPKC.

NF-κB activation The observations that p62 could bind aPKC (Puls et al. 1997) but appeared to be neither its substrate nor a regulator as such (Sanchez et al. 1998) lead to several studies which demonstrated a critical role for p62 in signal chains that activate NF-κB via aPKC in response to growth factors. First, p62 was implicated in the activation of NF-κB by TNFα, known to require aPKC. Upon stimulation by TNFα, p62 was found to bridge aPKC to RIP, one of the proteins recruited to the TNFR1 receptor (Sanz et al. 1999). Thereby, a novel signal chain from TNFα to NF-κB activation was established, with p62 as an essential intermediary. Collectively, the findings suggested a model in which TNFα stimulation induces the assembly of a receptor-signaling complex, involving the interactions TNFR1–TRADD–RIP–p62–aPKC, resulting in the aPKC-mediated activation of downstream events towards NF-κB activation (Sanz et al. 1999).

Subsequent studies revealed that p62 plays an analogous role in the activation of NF-κB in response to IL-1 and NGF. Thus, upon stimulation by IL-1 (Sanz et al. 2000) or NGF (Wooten et al. 2001, Mamidipudi et al. 2002), p62 associated with and linked aPKC to the respective receptor-signaling complexes and was necessary for NF-κB activation. In the case of IL-1, p62 interacted with the receptor-associated protein TRAF6. The findings suggested that IL-1 induces the formation of a receptor-signaling complex in which the signal is transduced from TRAF6 via p62 to aPKC (Sanz et al. 2000). Upon NGF stimulation, p62 interacted simultaneously with TRAF6 and one of the receptor proteins (TrkA), but otherwise appeared to serve a similar function in the aPKC-mediated activation of NF-κB (Wooten et al. 2001, Mamidipudi et al. 2002). Recently, p62 was further shown to be recruited to the TrkB and TrkC receptors (Geetha and Wooten 2003), potentially increasing the number of p62-involving signal chains activated by extracellular ligands.

Collectively, the above studies indicate that p62 connects aPKCs to signal pathways initiated by several growth factors, by binding to components of the receptor-signaling complexes. The association of p62 and aPKC with these complexes occurs in a ligand-

induced/enhanced manner, consistent with the colocalization of p62 with internalized receptors or their associated proteins upon stimulation by growth factors. A common pathway initiated by these events leads to the activation of NF-κB and requires both p62 and aPKC (Sanz et al. 1999, Sanz et al. 2000, Samuels et al. 2001, Wooten et al. 2001, Mamidipudi et al. 2002, Geetha and Wooten 2003). This mechanism appears to be highly conserved, since the *Drosophila* counterparts of p62, aPKC, and NF-κB serve analogous roles (Avila et al. 2002). Precisely how p62 promotes aPKC activation remains unclear, but several studies suggest that the activation of aPKC requires the simultaneous binding of p62 to upstream proteins such as TRAF6 (Sanchez et al. 1998, Wooten et al. 2001, Cariou et al. 2002, Chang et al. 2002). In this sense, the role of p62 is that of an adapter protein (Sanz et al. 2000).

Consistent with the above scheme, p62 was found to antagonize the effects of PAR-4, an inducible inhibitor of aPKC (Chang et al. 2002). By inhibiting aPKC, PAR-4 can suppress NF-κB activation, thereby sensitizing cells to apoptosis (Diaz-Meco et al. 1999, El-Guendy and Rangnekar 2003). However, both effects were counteracted by coexpressed p62. Since PAR-4 and p62 assembled into a ternary complex with PKC-ζ, they seem to constitute an antagonistic pair of inducible regulators for PKC-ζ (Chang et al. 2002).

ERK5 pathway In addition to its role in NF-κB activation, p62 seems to be pivotal for other cellular events, including the NGF-induced internalization of TrkA and activation of the ERK5 pathway (Geetha and Wooten 2003). The mechanisms underlying these phenomena are unclear. However, the requirement of p62 was also demonstrated for ERK5 activation upon EGF stimulation (Lamark et al. 2003), suggesting that the ERK5 pathway might constitute a signal chain activated by several p62-recruiting receptors. ERK5 is activated by MEK5 (Kamakura et al. 1999, Kato et al. 2000), promoting neuronal survival in the CNS (Watson et al. 2001). There is evidence that MEK5 can be activated directly by p62 (Lamark et al. 2003, Noda et al. 2003).

Channel proteins In addition to linking aPKCs to upstream signaling complexes, p62 appears to bridge PKC-ζ to some of its phosphorylation targets, including two channel

proteins. First, p62 and PKC-ζ were implicated in the regulation of voltage-gated K^+ channel (K_V) activity (Gong et al. 1999). In excitable cells, K_V channels are responsible for establishing the resting membrane potential and for the modulation of action potentials. K_V channels consist of pore-forming α subunits, associated with β subunits serving modulatory and structural functions (Martens et al. 1999). In rat brain, two isoforms of p62 were found to bind to K_Vβ2 (Gong et al. 1999), the most abundant of the β subunits. Further investigations demonstrated the assembly of a ternary complex where p62 seems to target the phosphorylation activity of PKC-ζ to K_Vβ2 (Gong et al. 1999). Similarly, p62 and PKC-ζ were implicated in the modulation of $GABA_C$ receptor function (Croci et al. 2003). $GABA_C$ receptors are ligand-gated Cl^- channels with an unclear functional role (Bormann 2000, Zhang et al. 2001). The ρ3 subunit of $GABA_C$ receptors was found to bind the ZIP3 isoform of p62. Analogous to the case of K_Vβ2, the study provided evidence for the formation of a ternary complex containing p62, PKC-ζ, and the channel protein, ρ3 (Croci et al. 2003).

The above findings from the context of channel proteins are compatible with the view of p62 as a scaffold protein (Croci et al. 2003) that directly bridges a protein kinase (aPKC) to its targets. At present it is unclear whether the “scaffold” roles of p62 (*i.e.*, interaction with aPKC targets) are linked to its “adapter” functions in receptor-mediated signaling.

2.4.3.2 Noncovalent Ub-binding and “sequestosomes”

An interesting feature of p62 is its ability to bind noncovalently to Ub via the UBA domain (Vadlamudi et al. 1996). Although the highly conserved sequence of the UBA domain points to a critical function (Fig. 1b), its physiological role remains unknown. Of potential relevance, treatment of HeLa cells with proteasomal inhibitors resulted in the enlargement of p62-containing punctate structures into amorphous, membrane-free masses that became enriched in Ub-conjugated proteins. These structures were suggested to represent an entity that sequesters Ub-conjugated proteins from the cytoplasm, hence the term ”sequestosome”, with the proposal that p62 regulates their formation (Shin 1998). However, scant evidence was provided in support of these claims. Since ubiquitination is also involved in ligand-stimulated internalization of cell-surface receptors (Hicke 1999), p62 might serve a role in connecting receptor-mediated signaling

events to Ub-dependent regulatory mechanisms (Geetha and Wooten 2002). On the other hand, the targets of UBA domains may also be proteins other than Ub (Buchberger 2002).

2.4.4 Disease associations

Hepatocytic inclusions The first direct indication of a role for p62 in disease came in the context of liver cancer, where the p62 protein was identified as a major constituent of intracytoplasmic hyaline bodies (IHBs) in hepatocellular carcinoma (Stumptner et al. 1999). IHBs are globular, eosinophilic inclusions composed of granulo-filamentous material (MacDonald and Bedard 1990). Apart from p62, no other protein species has been reported to be consistently found in IHBs. Very little is known about the pathogenesis of IHBs and the cause of p62 accumulation therein. However, in IHBs, p62 was hyperphosphorylated at epitopes similar to those of tau and neurofilaments in AD (Stumptner et al. 1999). By analogy with NFTs, this suggests that aberrant phosphorylation might contribute to IHB formation.

In studies on another hepatocytic inclusion type, the Mallory body (MB), p62 was also identified as a major constituent (Zatloukal et al. 2002). MBs are associated with a variety of chronic liver disorders. They are irregular, variously sized cytoplasmic inclusions with a mostly filamentous ultrastructure. MBs are primarily composed of misfolded, abnormally phosphorylated and crosslinked cytokeratins, while also incorporating stress-inducible proteins including Ub. Many aspects of their pathogenesis are poorly understood (Denk et al. 2000, Denk et al. 2001, Stumptner et al. 2001). Interestingly, in a mouse model of drug-induced MB formation, p62 was rapidly induced and accumulated in hepatocytes before the appearance of any cytokeratin aggregates. The growing MBs seemed to incorporate hyperphosphorylated p62 as an early component that was as abundant as cytokeratins (Stumptner et al. 2002, Zatloukal et al. 2002). Further experiments indicated that p62 associates with aggregated cytokeratins likely via binding to Ub chains in the aggregates. Based on these findings it was speculated that p62 might participate in a defensive response against misfolded proteins (Zatloukal et al. 2002).

Paget's disease of bone (PDB) Mutations in the p62 gene have been implicated in the etiology of PDB (OMIM entry no. 602080. Online Mendelian Inheritance in Man 2003), a skeletal disorder that affects up to 3 % of individuals above 55 (Cooper et al. 1999). The disease is characterized by focal increases of bone turnover due to activated osteoclasts, causing bone pain, enlargement, and deformities. The etiology is heterogenous, involving several genetic loci with likely contributions from environmental factors (Hamdy 1995). A missense mutation in p62 was found to account for a high proportion of autosomal dominant PDB cases (Laurin et al. 2002). Subsequently, five other PDB-causing mutations in p62 were identified, predicted to result in amino acid substitution or C-terminal truncation (Hocking et al. 2002, Johnson-Pais et al. 2003). The mechanisms by which these mutations lead to the disease are not known. However, findings on the genetics and pathophysiology of PDB suggest that the mutations may result in aberrant activation of NF-κB (Hocking et al. 2002, Laurin et al. 2002), supported by recent findings from p62 gene-ablated mice (Duran et al. 2004). Although all 6 mutations affect the Ub-binding region of p62, the disease-causing effect did not appear to stem from a loss of Ub binding (Ciani et al. 2003), suggesting that the C-terminal region of p62 may have some as yet unknown functions which can influence NF-κB activation.

Pathological associations based on disease models Work on animal and cellular models has indicated other pathological conditions possibly involving p62. First, oxidative stress-generating agents were found to upregulate p62 *in vitro* at both the mRNA and protein levels (Ishii et al. 1996, Ishii et al. 1997). Since oxidative stress may contribute to excitotoxic injury (Michaelis 1998), the expression of p62 was also examined in a rat model of excitotoxicity. In several brain regions, the levels of p62 mRNA and protein were mildly to moderately elevated following the excitotoxic insult (Nakaso et al. 1999). This increase of p62 levels appeared to be less marked in neurons most vulnerable to excitotoxicity, prompting the speculation that p62 might be playing a protective role. Thirdly, in studies addressing the molecular basis of the reproductive toxicity of 2-methoxyethanol and methoxyacetic acid, these glycol ethers were found to rapidly induce p62 mRNA expression in Sertoli cells (Syed and Hecht 1998).

At present it is difficult to reconcile the pathological roles of p62 with the currently available knowledge of its physiology. However, one obvious common feature in the findings from hepatocytic inclusions, neoplasias, and the disease models is the elevation of p62 expression levels. In this respect, p62 displays a similarity to stress proteins. Collectively, the studies on the normal function and pathological associations of p62 are compatible with the view that p62 is a stress-responsive protein which, via the modulation of aPKC function, normally regulates downstream effectors including NF-κB, in a manner that may involve Ub-binding as an important feature. In stressful conditions, p62 might thereby potentiate receptor-stimulated adaptive processes involving aPKC and NF-κB. However, the diversity of its interacting proteins suggests that p62 may also possess additional functions.

3. AIMS OF THE STUDY

A characteristic feature of most neurodegenerative disorders is the occurrence of proteinaceous inclusions, abnormal accumulations of cellular constituents, within neurons and/or glia in the central nervous system. The inclusions are thought to originate in close association with the pathological events that lead to neuronal loss or dysfunction in these disorders. In order to elucidate the pathogenetic mechanisms underlying inclusion formation, it is necessary to identify the key proteins involved and to investigate their structure, function, and regulation. In this study, a gene array approach and cellular models were first utilized to identify a candidate protein (p62) possibly involved in neurodegenerative phenomena, and subsequent experiments proceeded to examine the involvement of p62 in disease-associated protein aggregation in the human brain.

The specific aims were as follows:

1) To identify genes potentially involved in neuronal apoptosis by looking for gene expression changes in a cellular model. Subsequently, to investigate how the expression of p62 responds to neuropathology-mimicking conditions, by studying the effects of pro-apoptotic treatments and proteasomal inhibition on the levels of p62 mRNA and protein (**Study I**). The hypothesis regarding p62 expression was that an upregulation occurs during both apoptosis and the accumulation of ubiquitinated proteins.

2) To study the involvement of p62 in human tauopathies (Alzheimer and Pick diseases) and synucleinopathies (Parkinson disease, dementia with Lewy bodies, and multiple system atrophy), focusing on their characteristic cytoplasmic inclusions (**Study II**). The hypothesis was that p62 is enriched in these inclusions.

3) To examine in detail the extent of p62 involvement in the various types of neuronal pathology typical of Alzheimer disease (*i.e.*, neurofibrillary tangles, neuritic plaques, and neuropil threads) (**Study III**). The hypothesis was that p62 is predominantly incorporated into perikaryal as opposed to intraneuritic protein aggregates.

4) To elucidate the morphogenesis of Lewy bodies and their relation to pale bodies and other types of α-synuclein-containing accumulations characteristic of Parkinson disease, using α-synuclein, p62 and ubiquitin as windows into the aggregation process (**Study IV**). The hypothesis was that different forms of perikaryal α-synuclein-containing deposits in the substantia nigra evolve sequentially and show differential incorporation of p62.

4. MATERIALS AND METHODS

4.1 Neuronal culture (I)

Mouse neuroblastoma Neuro-2a cells (CCL 131) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO, USA) containing 1000 mg/l D-glucose and supplemented with 10 % fetal calf serum (Life Technologies, Rockville, MD, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Rat hippocampal neurons were prepared from 17-day old Wistar embryos and cultured in B27-supplemented Neurobasal medium (Life Technologies) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mM L-glutamine, as described by Brewer et al. (1993).

Approx. 24 h after plating, apoptosis was induced in exponentially growing Neuro-2a cells either by withdrawal of serum or by treatment with okadaic acid (10–30 nM), etoposide (8 µM), or trichostatin A (2 µM). Proteasomal inhibitors MG-132, lactacystin, and proteasome inhibitor I (PSI) were used at concentrations of 2–5 µM. Rat hippocampal neurons were treated with okadaic acid (10 nM) or lactacystin (5 µM) after 7 days *in vitro*. All drugs were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Cells were analyzed for nuclear morphology, caspase-3 activity, and DNA integrity as previously described (Solovyan et al. 1999).

4.2 Gene expression analysis using cDNA arrays (I)

Atlas mouse cDNA expression arrays (Clontech, Palo Alto, CA, USA), containing 588 cDNA fragments from genes belonging to various functional classes of proteins, were used for the identification of genes upregulated during apoptosis. Briefly, Neuro-2a cells were cultured in the absence or presence of serum, to obtain apoptotic and control cultures, respectively, and total RNA was extracted from the cells after 24 h using TRIzol reagent (Life Technologies). ^{32}P-labelled cDNA probes were prepared from deoxyribonuclease-treated samples of apoptotic and control RNA and hybridized to a pair of arrays according to the manufacturer's protocol. The ^{32}P signals were detected using a Storm PhosphorImager and spot intensities quantified using the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA, USA). For each detectable spot in the

array pair, signal intensity ratios (*i.e.* apoptotic : control) were calculated and compared to the signal ratios of control genes represented in the array.

4.3 Quantitation of p62 mRNA level (I)

Total RNA was extracted using TRIzol from cultured Neuro-2a cells and rat hippocampal neurons. Equal amounts of RNA (10–15 µg) were denatured and resolved in an agarose/formaldehyde gel, transferred in 10× saline sodium citrate (SSC) onto a Magnacharge positively charged nylon membrane (Osmonics, Westborough, MA, USA), and fixed by ultraviolet crosslinking at 120 mJ/cm^2 (Stratalinker 2400; Stratagene, La Jolla, CA, USA). As a control to ensure equal loading and transfer efficiency, the blots were stained with methylene blue to display ribosomal RNA bands (Sambrook et al. 1989). A ^{32}P-labeled cDNA probe specific for p62 mRNA was prepared from the expressed sequence tag clone #576791 (RZPD, Berlin, Germany) using High Prime labelling system (Roche Molecular Biochemicals, Indianapolis, IN, USA). The blots were prehybridized for 2 h and hybridized overnight at 65 °C in a solution containing 0.5 M (with respect to Na^+) sodium phosphate, 7 % sodium dodecyl sulfate (SDS), 1 % bovine serum albumin, and 1 mM ethylenediaminetetraacetic acid. Thereafter, the membranes were washed 2 × 15 min in 2× SSC / 0.1 % SDS and 2 × 15 min in 0.1× SSC / 0.1 % SDS at room temperature. The ^{32}P signals were detected and quantified using the Storm PhosphorImager and ImageQuaNT program.

4.4 Immunoblot analysis of p62 and Ub-conjugated proteins (I)

Neuro-2a cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in radioimmunoprecipitation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100, 0.5 % sodium deoxycholate) supplemented with 1× Complete protease inhibitor cocktail (Roche). The lysates were stored on ice for 30 min and centrifuged at 10 000 g for 10 min. The supernatant was used for immunoblotting. Rat hippocampal neurons were washed twice with PBS, collected in PBS using a rubber policeman, and centrifuged at 15 000 g for 20 s. The cell pellet was lysed in PBS containing 1 % Triton X-100 and 1× Complete protease inhibitor cocktail. The lysates

were stored on ice for 30 min, centrifuged at 12 000 g for 20 min, and the supernatant was used for immunoblotting.

Equal amounts of proteins (10–20 µg) were analyzed in 10 % polyacrylamide-sodium dodecyl sulfate gels according to the standard protocol (Sambrook et al. 1989) and transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a Trans-Blot SD semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The blots were blocked in Tris-buffered saline containing 3 % nonfat dried milk and 0.05 % Tween-20, and incubated with primary and secondary antibodies according to standard protocols (Sambrook et al. 1989). For labeling of p62, rat polyclonal antiserum against the mouse homologue A170 (gift from Dr. T. Ishii, Univ. Tsukuba, Japan) was used at a dilution of 1:2500. Ubiquitin-conjugated proteins were labeled using a rabbit polyclonal antibody (Z0458; DAKO, Carpinteria, CA, USA) diluted 1:500. As secondary antibodies, anti-mouse or anti-rabbit $F(ab')_2$ immunoglobulin fragments conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) were employed. The protein bands were visualized using a chemiluminescent detection system (SuperSignal; Pierce, Rockford, IL, USA) onto Hyperfilm ECL (Amersham Pharmacia Biotech).

4.5 Human subjects (II–IV)

Postmortem adult human brain specimens were obtained from the Kuopio Brain Bank. The brain material used for studies **II**–**IV** represented a total of 45 subjects autopsied during 1991–2000 at the Department of Pathology in the Kuopio University Hospital. The subjects and brain areas examined are summarized in Table 2. The studies were carried out following the guidelines and with the approval of the National Board for Medico-legal Affairs and the Research Ethics Committee of the Kuopio University Hospital and the University of Kuopio.

For study **II**, specimens were obtained from 4 clinicopathologically diagnosed subjects representing tauopathies (definite AD and PiD), and from 7 cases representing synucleinopathies (PD, DLB, and MSA). The brain areas selected for analysis were those

that typically show high densities of pathological inclusions in these diseases (Table 2). The cases are described in more detail in publication **II**.

Table 2. Summary of human postmortem material.

Study	Group	N	Description	Age (yr)	Brain weight (g)	Postmortem delay (h)	Brain areas examined
II	AD	3	definite Alzheimer disease	83.3 (78–86)	1067 (965–1200)	80 (48–96)	entorhinal cortex and hippocampus
	PiD	1	Pick disease	82	820	10	hippocampus
	PD	2	Parkinson disease	73 (68–78)	1303 (1290–1315)	72 (48–96)	midbrain incl. substantia nigra
	DLB	3	dementia with Lewy bodies	65 (52–79)	1222 (1020–1350)	48 (48–48)	cingulate gyrus
	MSA	2	multiple system atrophy	51.5 (49–54)	1348 (1295–1400)	48 (48–48)	basal forebrain and striatum
III	AD	15	demented; abundant neuritic plaques and neurofibrillary tangles in neocortex	84.9 (81–97)	1008 (740–1400)	13.6 (2–104)	parietal cortex (Brodmann's area 39)
	high-plaque non-demented	9	no dementia; abundant neuritic plaques but no tangles in neocortex	84.8 (82–92)	1277 (1085–1410)	79 (5–216)	
IV	PaB/LB	7	abundant PaBs and LBs in substantia nigra	69 (57–80)	1480 (1150–1640)	91.9 (24–206)	midbrain incl. substantia nigra
	control	3	no pathology in substantia nigra	69 (57–80)	1355 (1280–1495)	72 (72–72)	

For study **III**, subjects from the Brain Bank were initially screened using the following criteria: 1) age at death > 80 years and 2) abundant neocortical neuritic plaques in silver staining. Into the first group ("AD cases"), 15 subjects were selected that in addition to criteria 1–2 showed both abundant neocortical NFTs and clinical signs of dementia (Table 2). Of the AD cases, 14 met the clinicopathological criteria of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD; Mirra et al. 1991) for "definite AD" and 1 for "probable AD", while Braak stages were 5–6. To examine the possible

involvement of p62 in Ub-positive, tau-negative neocortical neuritic plaque pathology, 9 age-matched subjects were selected that met the above criteria 1–2 but were devoid of neocortical NFTs and showed no clinical signs of dementia. These "(high-plaque) nondemented cases" met the CERAD criteria for "possible AD", and Braak stages varied from 1 to 4. All cases, as well as methods for neurofibrillary staging, CERAD classification, and scoring of neocortical NPs and NFTs are described in detail in publication **III**.

For study **IV**, cases from the Brain Bank were prescreened for the extent of nigral LB and PaB pathology in midbrain sections stained with haematoxylin-and-eosin (H&E). PaBs were identified as globular, weakly eosinophilic cytoplasmic areas that lacked a halo and seemed to displace neuromelanin. Of cases showing abundant LBs and PaBs, seven were selected for this study (Table 2). In the SN, these cases displayed abundant αS pathology (Parkkinen et al. 2003) but no appreciable signs of pathology in tau immunolabeling (see below). In addition, three age-matched control subjects, showing no nigral inclusions in H&E stain or tau immunodetection, were examined for the extent of αS immunoreactivity in histologically normal SN. The cases are described in more detail in publication **IV**.

4.6 Histological techniques (II–IV)

Seven-µm-thick serial sections cut from formalin-fixed, paraffin-embedded brain specimens were used for all stainings. The antibodies, dilutions, and epitope unmasking treatments for immunohistochemistry are described in Table 3.

Single immunodetection and common stains. Adjacent sections from brain areas of interest were rehydrated, subjected to epitope unmasking treatments (for p62 and αS), and stained with antibodies specific for p62, Ub, αS, and HP-tau. For the purpose of confirming and/or comparing the specificities of the antibodies, immunostainings were performed using using 2–4 different antibodies for p62 (**II**–**IV**), Ub (**III**, **IV**), and αS (**IV**). For detection, the enzyme-conjugated streptavidin-biotin method (Histostain-Plus / Histostain-SAP; Zymed) was used, employing either horseradish peroxidase (**II**–**IV**) or alkaline phosphatase (**II**) as the enzyme. The chromogens used were 3,3'-

diaminobenzidine (Zymed; **II**, **III**), Vector Red (Vector Laboratories, Burlingame, CA, USA; **II**), or Romulin 3-amino-9-ethylcarbazole (Biocare Medical, Walnut Creek, CA, USA; **IV**).

Table 3. Description of antibodies (FA = formic acid, mAb = monoclonal antibody, pAb = polyclonal antibody).

Name, type	[1]Epitope (immunogen)	[2]Pretreatment	Dilution	Source
NCL-ASYN, mouse mAb	αS (human αS_{1-140})	autoclave + 80 % FA, 5 min	1:1000	Novocastra (Burlingame, CA, USA)
Synuclein-1, mouse mAb	αS_{91-99}	80 % FA, 60 min	1:1000	Transduction Laboratories (Lexington, KY, USA)
LB509, mouse mAb	$\alpha S_{115-122}$	80 % FA, 60 min	1:100	Zymed (San Francisco, CA, USA)
AT8, mouse mAb	tau phosphorylated at Ser-202/Thr-205	–	1:500	Innogenetics (Ghent, Belgium)
p62-N, guinea pig pAb	p62, N-terminal (human peptide)	autoclave	1:500	Progen (Heidelberg, Germany)
p62-C, guinea pig pAb	p62, C-terminal (human peptide)	autoclave	1:500	Progen
ZIP P-15, goat pAb	p62, C-terminal (human peptide)	autoclave	1:500	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
p62 lck ligand, mouse mAb	p62 (human $p62_{257-437}$)	autoclave	1:1000	Transduction Laboratories
Z0458, rabbit pAb	free + poly-Ub (bovine Ub)	–	1:350	DAKO (Carpinteria, CA, USA)
NCL-UBIQ, rabbit pAb	Ub (human Ub)	–	1:1000	Novocastra
mNCL-UBIQ, mouse mAb	Ub (human Ub)	–	1:20	Novocastra

[1]For unmapped epitopes, immunogen is given in parentheses.

[2]Autoclaving conditions: 120 °C for 10 min in 10 mM sodium citrate (pH 6.0).

To preclude possible nonspecific staining by the detection reagents, adjacent control sections were processed with omission of the primary antibody. These controls were invariably blank. After immunostaining, sections were counterstained with Harris' haematoxylin to reveal nuclei. For comparison, adjacent sections were stained with Gallyas or Bielschowsky silver impregnation techniques (**II**, **III**) or with H&E (**II**–**IV**). All sections were mounted in DePeX (BDH Laboratory Supplies, Poole, UK). Variations in postmortem delay or age of death had no obvious effect on any of the staining patterns.

*Double immunodetection (**III**).* To visualize p62 in combination with either HP-tau or Ub, double immunostaining was performed using the Histostain-DS kit (Zymed), employing 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to label p62 and 3-amino-9-ethylcarbazole to display HP-tau- or Ub-positive structures. As the primary antibodies, p62-N, AT8, and NCL-UBIQ were used, respectively. To preclude cross-reactions, the manufacturer's protocol for the second staining was modified by using a peroxidase-conjugated secondary antibody against mouse or rabbit IgGs (Pierce) for detection of HP-tau or Ub, respectively. Control sections with omission of both primary antibodies were processed to ensure that no nonspecific labeling resulted from any of the detection reagents. To check that the reagents of the second staining neither recognized nor were masked by any of the reagents of the first staining, additional control slides were included with the omission of either of the primary antibodies. As the dark blue precipitate from the first staining might have concealed any cross-reactivity between reagents of the first and second stainings, further slides were processed with omission of both the first chromogen and the second primary antibody. Neither nonspecific staining nor interference between stainings was observed. Nuclei were counterstained with haematoxylin. The sections were embedded in Clearmount (Zymed) and coverslipped with DePeX.

*Reversible immunodetection and H&E staining (**IV**).* A reversible chromogenic staining technique was utilized to enable the comparative visualization of two overlapping antigens (p62 and αS) and tissue morphology as revealed by H&E. Briefly, sections from representative subjects were immunostained using the p62-N antibody and temporarily mounted in DePeX. Photomicrographs were obtained from structures of interest,

whereafter the coverslips were unmounted in xylene and the dye was removed in 70 % ethanol. The sections were then treated with formic acid to unmask αS epitopes, and immunostained using the NCL-ASYN antibody. To preclude cross-reactions, the latter antibody was detected using a peroxidase-conjugated antibody against mouse IgGs (Pierce), instead of the biotin-amplified system. After counterstaining and mounting, the sections were re-imaged, using cellular landmarks to locate the structures imaged for p62. After re-imaging, the sections were again unmounted and destained, followed by staining with H&E, mounting and imaging for the third time. The controls employed to exclude possible artifacts introduced by this staining protocol are detailed in publication **IV**.

4.7 Microscopic analysis and quantitation (II–IV)

The stained sections were analyzed using a Nikon Optiphot-2 microscope. For quantitation of the immunopositive structures (**III**), sections were first scanned at 40× magnification to locate the area of gray matter showing the highest involvement. From this area, the abundances of NFTs, NPs, and NTs were then scored by counting or visual estimation of 3 or 10 adjacent microscopic fields at 200×–40× objective, depending on the frequency of labeled structures, as specified in publication **III**.

4.8 Photomicrography (II–IV)

Digital photomicrographs were obtained using a microscope-attached Nikon Coolpix 990 camera. Image brightness, contrast, and color levels were moderately adjusted using the Adobe Photoshop 5.5 program to restore the perceived quality of the microscopic view. In study **II**, identification and comparison of the same cellular structures visualized using different stains in adjacent sections were accomplished by alignment of photomicrographs taken from the successive sections. In publications **II**–**IV**, photomicrographs with representative staining patterns are shown for each stain and group of subjects.

4.9 Statistical analysis (I, III)

Data was analyzed using SPSS for Windows (release 7.5.1). Changes in p62 mRNA level at different timepoints were analyzed using one-way ANOVA followed by Bonferroni's post-hoc multiple group comparison (**I**). Intra-group comparison of means for NFT and NP scores was performed using the Wilcoxon signed-rank (2-tailed) test (**III**). Statistical associations between all variables were measured using the 2-tailed Spearman rank correlation test (**III**). To facilitate viewing of data, some of the continuous variables were recoded into 3-step-scales (+...+++), defined by the 20^{th} and 80^{th} percentiles (**III**).

5. RESULTS

5.1 Neuronal apoptosis and p62 expression (I)

5.1.1 Identification of p62 as a candidate apoptosis-involved gene

In order to identify genes potentially involved in neuronal apoptosis, Neuro-2a cells were subjected to serum deprivation, and changes in mRNA expression were detected using Atlas mouse cDNA arrays at 24 h. One of the mRNAs that was upregulated at least ~50 % in apoptotic cultures corresponded to oxidative stress-induced protein A170 (**I**: Fig. 1), the mouse counterpart of human p62 (GenBank accession nos. U40930 and NM_003900, respectively).

5.1.2 Expression of p62 is upregulated at the mRNA and protein levels by pro-apoptotic treatments

To investigate whether the upregulation of p62 is a consistent feature of neuronal apoptosis regardless of the type of apoptotic inducer, the expression of p62 mRNA was monitored following a range of treatments known to induce apoptosis in Neuro-2a cells, *i.e.*, serum deprivation, okadaic acid, etoposide, and trichostatin A (Salminen et al. 1998, Solovyan et al. 1999). All treatments increased the level of p62 mRNA markedly during 12–48 h, with varying time courses (**I**: Fig. 2A).

To examine whether a similar induction of p62 expression occurs during apoptosis in primary cultured cells, rat hippocampal neurons were treated with okadaic acid. A marked increase was seen in p62 mRNA level at 12–24 h (**I**: Fig. 2B).

The response of p62 protein expression to certain apoptotic inducers was analyzed by immunoblotting to determine whether the upregulation of p62 mRNA was reflected at the protein level. In lysates of Neuro-2a cells, the A170 antiserum recognized a 62-kD protein, the level of which was increased by serum deprivation or treatment with okadaic acid at 12–36 h (**I**: Fig. 3A, B). In rat hippocampal neurons, okadaic acid caused a similar increase in the level of the 62-kD protein at 24 h (**I**: Fig. 3C).

5.2 Proteasomal function and p62 expression in neuronal cells (I)

5.2.1 Inhibition of proteasomal activity upregulates p62 mRNA and protein

The presence of a Ub-binding area in the C-terminal region of p62 and its ability to bind Ub noncovalently (Vadlamudi et al. 1996) suggested that p62 might have a regulatory function connected to the Ub-proteasomal system. To study whether the accumulation of ubiquitinated proteins could affect the expression of p62, Neuro-2a cells were treated with proteasomal inhibitors, and the levels of p62 mRNA and protein were monitored.

The level of p62 mRNA was upregulated at least 2-fold by all three inhibitors tested (**I**: Fig. 4A). In the case of MG-132, p62 mRNA reached a steady state as early as 6 h, whereas lactacystin increased p62 mRNA continuously during 0–24 h. The highest upregulation, over 4-fold, was obtained with PSI. In rat hippocampal neurons, lactacystin upregulated p62 mRNA by a comparable magnitude at 12–24 h (**I**: Fig. 4D, upper panels).

At the protein level, p62 expression was similarly increased in Neuro-2a cells by all three proteasomal inhibitors, with a maximum at 24–36 h (**I**: Fig. 4B). Comparable results were obtained in rat hippocampal neurons (**I**: Fig. 4D, lower panel). In the case of PSI, a higher M_w band appeared at ~70 kD (**I**: Fig. 4B, bottom panel), possibly representing p62 conjugated to monoubiquitin.

5.2.2 Upregulation of p62 vs. increase of Ub-protein conjugates

To examine the relationship between p62 upregulation and the amount of Ub-protein conjugates, the latter were visualized by immunoblotting. In Neuro-2a cells subjected to proteasomal inhibition, a pronounced increase in the level of Ub-immunoreactive proteins during 12–36 h was evoked by PSI (**I**: Fig. 4C), as well as by MG-132 and lactacystin. Similarly, in rat hippocampal neurons, proteasomal inhibitors potently increased the amount of Ub-immunoreactive proteins, peaking at 12 h (**I**: Fig. 4E).

5.3 Incorporation of p62 into disease-associated inclusions in human brain (II)

The involvement of p62 in cytoplasmic protein aggregates characteristic of two tauopathies (AD and PiD) and three synucleinopathies (PD, DLB, and MSA) was studied in comparison to HP-tau, αS, and Ub.

5.3.1 Tauopathies

In the AD cases, immunostaining for HP-tau, as expected, revealed numerous NFTs and dystrophic neurites in the entorhinal cortex and the CA1 region of the hippocampus (**II**: Fig. 1c). A similar pattern was seen for Ub (**II**: Fig. 1d). The NFTs were also strongly immunoreactive for both p62 antibodies, showing a morphology very similar to HP-tau and Ub (**II**: Fig. 1e, f). In contrast, few neurites were labeled for p62. The number of HP-tau-positive NFTs was somewhat higher compared to p62 or Ub (**II**: Table 2). No immunoreactivity for p62 was detected in deposits of β-amyloid.

In the hippocampus of the PiD case, labeling for HP-tau visualized strongly immunoreactive, globular PiBs in granule cells of the dentate gyrus and some pyramidal cells in the CA1–CA3 regions (**II**: Fig. 1i). A subset of PiBs also stained for Ub, albeit more weakly (**II**: Fig. 1j). In addition, the Ub antibody displayed diffuse perikaryal staining in several neurons and distinct granular immunoreactivity in occasional pyramidal cells. The p62 antibodies showed moderate labeling of a subset of PiBs, mostly with a fuzzy appearance, and weaker diffuse staining in many neurons (**II**: Fig. 1k, l; Table 2).

5.3.2 Synucleinopathies

In the cingulate gyrus of the DLB cases, immunodetection of α-synuclein (αS) and Ub revealed scattered LBs in deeper cortical layers (**II**: Fig. 2c, d). The LBs were also intensely immunopositive for both p62 antibodies (**II**: Fig. 2e, f), with a staining pattern similar to those of αS and Ub.

In the substantia nigra of the PD cases, αS immunoreactivity was present as robustly positive LBs, diffuse cytoplasmic staining, and αS-positive neurites. While Ub

immunostaining displayed both LBs and some neurites, immunoreactivity for p62 was only observed in LBs (not shown).

In the MSA cases, αS immunostaining of basal forebrain and striatum showed in white matter abundant cells containing glial cytoplasmic inclusions (GCIs), exhibiting a variable degree of αS-positivity, and a typically caplike shape (**II**: Fig. 2i). Staining for Ub showed a similar staining pattern (**II**: Fig. 2j). Immunodetection of p62-C showed more distinct labeling of the GCIs in comparison to αS and Ub, while the p62-N antibody displayed fewer inclusions and with lower intensity (**II**: Fig. 2 k, l; Table 2).

5.3.3 Comparison of HP-tau, αS, Ub, and p62 incorporation

Overall, the pattern of p62 immunoreactivity associated with the five types of inclusions correlated most closely with that of Ub, while differing somewhat from HP-tau and αS (**II**: Table 2). The notable differences between HP-tau and p62/Ub patterns were the higher frequency of HP-tau-reactive NFTs in AD and the presence of diffuse p62/Ub-positivity within hippocampal neurons in PiD. Compared to αS, the pattern of p62/Ub differed in PD by showing no diffuse cytoplasmic staining within nigral neurons.

In contrast to the similarity between p62 and Ub staining patterns in perikaryal accumulations, abnormal neurites were generally immunonegative for p62, although Ub immunodetecion revealed dystrophic neurites and neuropil threads in AD and αS-positive neurites in PD. Unlike Ub, immunoreactivity for p62 was also absent from aging-related intracellular accumulations not associated with disease, *i.e.* neuropil grains and corpora amylacea.

The results obtained with both antibodies for p62 were similar apart from three differences noted: 1) In AD, p62-N and -C were slightly selective for NFTs located in deeper and upper cortical layers, respectively. 2) Both PiBs and nigral LBs stained somewhat more numerously for p62-N. 3) In MSA, p62-N displayed markedly fewer GCIs and weaker labeling than p62-C. No immunoreactivity for either antibody was detected in tissue areas devoid of disease-associated accumulations.

5.4 Accumulation of p62 into AD-type neurofibrillary pathology (III)

A quantitative study was conducted to analyze the extent of p62 incorporation in neurofibrillary changes characteristic of AD and in neocortical neuritic plaque pathology not presenting with cognitive impairment.

5.4.1 AD cases

In parietal cortical sections of the AD cases, immunoreactivity for p62-N and p62-C was predominantly observed in NFTs (**III**: Fig. 1a). Both antibodies displayed the classical morphology of NFTs as perikaryal masses from which thin endings sometimes extended into proximal neurites. The NFTs also labeled robustly for HP-tau and Ub (**III**: Fig. 1b, c). Similar results were obtained using other antibodies for Ub and p62. Stainings for HP-tau and p62 displayed similar numbers of NFTs (**III**: Fig. 2a), whereas the number of Ub-positive (Ub^+) NFTs was 61 % and 53 % lower compared to HP-tau and p62, respectively ($p < 0.001$ for both). Despite the fewer Ub-staining NFTs compared to HP-tau or p62, the abundance of $p62^+$ NFTs correlated better with Ub^+ NFTs ($r_s = 0.713$, $p < 0.01$) than with HP-tau^+ NFTs ($r_s = 0.579$, $p < 0.05$; **III**: Fig. 2c).

In contrast to HP-tau and Ub which revealed dense meshworks of neuropil threads (NTs), antibodies for p62 did not exhibit any appreciable labeling of NTs (**III**: Fig. 1a–c). However, in 6 cases, a faint network of very thin NTs positive for p62-N was discernible at high magnification in laminae II–III and V, but even this was usually not detected for p62-C (**III**: Table 2). No association was found between the presence of $p62^+$ NTs and any of the clinical or neuropathological variables, although there was a significant correlation between the abundances of HP-tau^+ and Ub^+ NTs ($r_s = 0.674$, $p < 0.01$).

Similar to NTs, neuritic plaques (NPs) were generally unreactive for p62, while 7 cases displayed at least one $p62^+$ NP as a cluster of short $p62^+$ neurites (**III**: Table 2; Fig. 1a, inset). However, the mean number of $p62^+$ NPs was only ~1 % compared to HP-tau^+ or Ub^+ NPs (**III**: Figs. 1b,c and 2b). The abundance of $p62^+$ NPs did not correlate significantly with any of the clinical or neuropathological variables. Deposits of β-amyloid displayed no immunoreactivity for p62.

Double immunostainings verified that p62 was almost invariably present in HP-tau-staining NFTs, and confirmed the general lack of p62 immunoreactivity in NTs and NPs (**III**: Fig. 3a, b). As a whole, the data from the AD cases revealed a large variability in the relative amounts of NFTs, NTs, and NPs immunopositive for a given antigen (**III**: Table 2).

5.4.2 Nondemented cases

This group consisted of 9 age-matched cases with abundant neocortical NPs but no neocortical silver-stainable NFTs nor signs of cognitive impairment. These cases thus met the CERAD criteria for possible AD, while Braak stages varied from 1 to 4 (**III**: Table 1). Although the group had shown no neocortical NFTs with silver stain, 6 of the cases displayed in the parietal cortical sections early neurofibrillary pathology, *i.e.*, occasional HP-tau$^+$ NFTs or NPs (**III**: Table 3; Fig. 1e). No NTs immunoreactive for HP-tau, Ub, or p62 were seen in any of the cases.

The nondemented cases were mostly negative for p62, while occasional p62$^+$ NFTs or NPs were detected in 3 cases, all of which were within the HP-tau$^+$ subgroup (**III**: Table 3; Fig. 1d). The numbers of p62$^+$ NFTs were similar to those reactive for HP-tau, but no Ub$^+$ NFTs were seen (**III**: Table 3). The case with the highest numbers of p62$^+$ NFTs and NPs also showed exceptionally many HP-tau$^+$ NPs (**III**: Table 3, case 17). Immunolabeling for Ub displayed abundant NPs (**III**: Fig. 1f, i; Table 3), which were 4–50-fold more frequent than the occasional HP-tau$^+$ ones. The numbers of Ub$^+$ NPs did not correlate with either HP-tau$^+$ or p62$^+$ NPs or NFTs.

Since p62$^+$ and HP-tau$^+$ NFTs were found in similar numbers in two HP-tau$^+$ cases, possibly representing preclinical AD, the findings suggested that p62 is incorporated early during NFT formation. To test this possibility, an additional nondemented subject with Braak stage I was analyzed, displaying occasional NFTs in the transentorhinal cortex. Also in this brain area, p62 and HP-tau immunostainings revealed similar numbers of NFTs (not shown).

5.5 Formation of αS-containing inclusions associated with PD (IV)

To elucidate the morphogenesis of Lewy bodies and their relationship to other types of αS^+ accumulations associated with PD, nigral specimens from cases with abundant αS pathology were analyzed using immunohistochemistry for αS, p62 and Ub.

5.5.1 Perikaryal types of αS accumulation

All cases with abundant nigral pathology showed moderate to severe loss of pigmented neurons, neuronal fragmentation, and αS accumulations (NCL-ASYN antibody) throughout the SN. Nonstaining neurons were present in numbers similar to those with perikaryal αS accumulations (**IV**: Fig. 1B–V). Two main types of perikaryal accumulation were distinguished, referred to below as punctate αS (**IV**: Fig. 1B, C) and compact inclusions. The latter could be divided into spherically symmetric, intensely immunoreactive types (**IV**: Fig. 1S–V), and less rounded or irregular, somewhat less immunoreactive forms (**IV**: Fig. 1G–L). As the spherical types were mostly consistent with classical LBs, they will be referred to as "LBs". Most of the non-symmetrical inclusions conformed to the morphological criteria of PaBs (Pappolla et al. 1989, Gibb et al. 1991). Although many did not fully meet these criteria, all non-symmetrical forms showed a similar level of immunoreactivity for αS, as well as for p62 and Ub, and will be referred to as "PaBs".

Punctate αS Punctate perikaryal immunoreactivity consisted of intensely αS^+ granules (dia. 0.5–1 µm) of varying density (**IV**: Fig. 1B, C). The granules were generally dispersed throughout the perikaryon, sometimes including proximal dendrites, but usually lay more sparsely in pigmented areas. Punctate αS was often accompanied by faint diffuse labeling, but the latter was not seen without punctate αS. Perikarya displaying only punctate αS but no compact inclusions typically accounted for one-third of αS^+ somata. Punctate αS was also seen in most somata harboring compact inclusions (**IV**: Fig. 1G–V).

In neurons containing punctate αS but no typical PaBs or LBs, the area with punctate αS occasionally encompassed a small, irregular body (**IV**: Fig. 1D–F). These appeared as

"aggregation centers" of the αS^+ granules, possibly representing a transition from punctate to compact accumulation. These structures were distinct from LBs, whereas the larger ones resembled small PaBs (**IV**: compare Figs. 1F, G).

Compact inclusions LB-type inclusions usually displayed a concentric laminar morphology 4 to ~20 µm in diameter (**IV**: Fig. 1S–V). Of all compact perikaryal inclusions, LBs represented about one-third, while the majority belonged to the diverse PaBs. These were generally larger, globular to irregular, with granular to homogeneous texture and clearly demarcated to ill-defined outlines (**IV**: Fig. 1G–L). Recurring patterns could be identified, including "uniformly granular" and "arch-like" forms (**IV**: Fig. 1J, K), and a roundish type with strongly staining, unbroken periphery (**IV**: Fig. 1L). The appearances of the latter two types suggested that their peripheral regions were undergoing progressive condensation.

LBs and PaBs often coexisted in the same perikaryon. In addition, a variety of morphologies displayed LB- and PaB-type features within a contiguous mass, giving the impression of LB formation within or from a PaB (**IV**: Fig. 1M–R). Frequently, the periphery of a PaB contained small LBs or less discrete spherical foci of pronounced αS-positivity (**IV**: Fig. M–P). Sometimes the part of the PaB between the foci was only weakly αS^+ (**IV**: Fig. 1R), suggestive of focal compaction of the PaB into several LBs, with the disappearance of the intervening material (**IV**: compare Figs. 1O, R, T).

5.5.2 Extrasomal types of αS accumulation

Numerous αS-immunoreactive inclusions also lay outside neuronal bodies, displaying globular, fusiform, or drop-like shapes, with near-homogenous to granular texture, distinct to fuzzy outlines, and uniform to uneven labeling (**IV**: Fig. 1W). Since they were not surrounded by glia and were clearly αS^+, these inclusions probably lay within neuronal processes (Iseki et al. 2000). Punctate αS, in contrast, was never seen outside perikarya and proximal dendrites. Immunoreactivity for αS was further present as mostly thin αS^+ neurites (**IV**: Fig. 1X) and in many neuronophagic events (**IV**: Fig. 1Y). Similar results were obtained using the Synuclein-1 antibody. The LB509 antibody was exceptional in that it displayed no punctate αS.

5.5.3 Selective incorporation of p62 into compact somal inclusions

In compact perikaryal inclusions, the immunoreactivity for the p62-N antibody was very similar to that of αS, revealing variform PaBs, PaB/LB intermediates, and LBs (**IV**: Fig. 2B–K). In contrast, neither punctate accumulation nor extrasomal inclusions were detected, and only occasional short neurites were labeled. Similar results were obtained using three other antibodies for p62, with the exception that one (p62 lck ligand) labeled somewhat more neurites.

Unexpectedly, p62 antibodies displayed the most robust labeling in small inclusions within neuronal nuclei (**IV**: Fig. 2L). Based on their size, shape, and immunoprofile ($\alpha S^{-}/Ub^{+}$), these inclusions could be identified as Marinesco bodies (MarBs) (Bancher et al. 1989b, Fujigasaki et al. 2001). Some nuclei with MarBs also exhibited diffuse staining (**IV**: Fig. 2L).

Sequential staining of p62 and αS verified that compact perikaryal inclusions were invariably p62-immunoreactive, with a close similarity between αS and p62 staining patterns (**IV**: Fig. 3E–K). Areas of punctate αS generally did not label for p62 (**IV**: Fig. 3B), but focal p62-positivity was seen at "aggregation centers" of punctate αS (**IV**: Fig. 3C, D). All extrasomal inclusions and most αS^{+} neurites were devoid of p62 (**IV**: Fig. 3K, F). MarBs were αS-negative (**IV**: Fig. 3L).

5.5.4 Accumulation of Ub is less selective compared to p62

In perikaryal inclusions, the patterns of Ub immunoreactivity resembled those of p62, with the exception that the inclusions appeared more fuzzy, and in LBs, the labeling was more centrally distributed (**IV**: Fig. 4B–K). The overall sensitivity of Ub immunodetection, however, was lower in comparison to p62. In contrast to p62, extrasomal inclusions labeled robustly (**IV**: Fig. 4L). Neurites were stained in numbers intermediate between αS and p62. The immunoprofiles for the different αS^{+} deposits are summarized in Table 3 of publication **IV**. Ub immunolabeling also revealed MarBs but in fewer numbers than seen for p62.

5.5.5 Comparison of αS immunodetection and H&E staining

Compared to αS immunolabeling, much fewer compact inclusions were positively identified using the H&E stain, and neither punctate deposition nor abnormal neurites were revealed. Direct comparison of αS and H&E phenotypes by means of sequential staining confirmed that only a minority of LBs and PaBs were unambiguously identified using H&E (**IV**: Fig. 5A, C), whereas the majority were poorly defined and/or overlapped with neuromelanin (**IV**: Fig. 5B, D). Most PaBs that were clearly delineated by neuromelanin in H&E were uniformly granular and globular (**IV**: Fig. 5C). Poorly delineated PaBs usually corresponded to irregular or non-uniform morphologies for αS, often different from the H&E phenotype (**IV**: Fig. 5D, E). Punctate αS without compact inclusions often occupied slightly eosinophilic areas with little or no neuromelanin (**IV**: Fig. 5F).

5.5.6 Control cases

The three control cases showed no obvious signs of neuronal loss in the SN. Two cases were completely αS-negative, whereas one showed punctate αS within two neurons, indicative of very early αS pathology. Neither p62 nor Ub immunoreactivity was detected within nigral neurons, apart from MarBs.

6. DISCUSSION

In the present series of studies, p62 (sequestosome 1) was identified and selected for investigation since this protein possesses several features indicating that it may have a possible role in neuropathological conditions. The expression of p62 was first examined in neuropathology-mimicking cellular models (study **I**). The involvement of p62 was then characterized in disease-associated protein aggregation in the human brain, focusing on cytoplasmic inclusions occurring in tauopathies and synucleinopathies (studies **II**–**IV**).

6.1 Methodological considerations

Cellular models The cell types used in study **I** have been widely employed for *in vitro* modeling of diverse neuropathological phenomena, including apoptosis (Krohn et al. 1998, Kim et al. 2000) and proteasomal impairment (Fenteany and Schreiber 1996, Keller and Markesbery 2000). To preclude possible cell type-specific effects or artefacts arising from the use of immortalized (Neuro-2a) cells, the main findings were confirmed in primary cultured (rat hippocampal) neurons. However, it is obvious that both the neuropathology-mimicking treatments as well as the cellular responses in these models are likely to differ in several ways from their counterpart events *in vivo*, for instance by being more acute and pronounced. Nonetheless, the models serve as useful indicators for novel phenomena with potential relevance for human disease.

Neuropathological studies Data obtained from postmortem tissues may be influenced by artefacts due to agonal and postmortem events and variations in tissue processing. The epitopes and pathological structures probed by the antibodies in studies **II**–**IV** seemed to be relatively insensitive to the length of the postmortem delay (PMD). In study **III**, none of the immunodetection-based variables correlated with PMD, and in study **IV**, variations in PMD had no obvious qualitative effect on the labeling patterns. Any effects arising from agonal events or tissue processing also seemed to be negligible in the cases examined, since no anomalies were seen in the staining results for any of the antibodies.

The specificity of immunodetection was ensured by employing well-characterized and/or widely used antibodies (*e.g.*, AT8 and LB509) and by using multiple antibodies per antigen, in particular for p62 in each study and for αS in study **IV**. The use of multiple antibodies per antigen also served to eliminate the possibility of false negative results that might be caused by masked or modified epitopes. In double (**III**) and sequential (**IV**) immunolabeling, a number of controls were utilized to exclude possible cross-reactivity between the staining reagents for different epitopes.

Immunohistochemistry is inherently limited by being only semi-quantitative, with a low dynamic range. In studies **II**–**IV**, the sensitivity of immunodetection was therefore adjusted for optimal visualization of pathological changes of interest (*i.e.*, inclusions), whereas the normal expression of the proteins remained below the detection level, as is evident from the virtual absence of background staining. Consequently, the pathological accumulation visualized is considered to represent greatly elevated focal densities of the proteins, as compared to their physiological distribution. On the other hand, it is possible that less conspicuous types of protein buildup may have gone unnoticed.

In study **IV**, an attempt was made to reconstruct the morphological sequence of events in LB formation by performing a tentative ordering of immunostained structures. An essentially similar approach has been employed in the case of NFTs (*e.g.*, Bancher et al. 1989a). Although a range of apparently intermediary forms were observed that suggested a particular sequence, the ordering nevertheless remains subjective and relies on several assumptions about the nature of the process (*e.g.*, unidirectional and stereotypical). More direct tracing of the events will require the development of novel experimental models, since those currently available do not duplicate the morphology of PD-associated inclusions to a satisfactory degree.

Obviously, neuropathological studies do not allow the elucidation of the underlying molecular mechanisms. However, while descriptive in character, the data provided will be instrumental for the testing of current hypotheses and for the delineation of novel concepts.

6.2 Expression of p62 in neuropathology-mimicking models (I)

6.2.1 Neuronal apoptosis and p62 expression

The initial cDNA array observation of an increase in p62 mRNA upon serum deprivation of Neuro-2a cells was confirmed and extended by the findings that several different apoptosis-inducing treatments (*i.e.*, serum deprivation, okadaic acid, etoposide, and trichostatin A) cause a prominent upregulation of p62 at both the transcript and the protein levels. Since consistent findings were obtained in rat hippocampal neurons, the findings suggested that the induction of p62 expression might be a common event in neuronal apoptosis also *in vivo*. These were the first reported data linking neuronal apoptosis to changes in p62 expression.

As such, the present findings, associating the upregulation of p62 with apoptosis, neither reveal the mechanisms accounting for this increase nor indicate directly whether the upregulation of p62 is functionally involved in, or is unrelated to the apoptotic response. While each of the 4 treatments used induces apoptosis (Salminen et al. 1998, Solovyan et al. 1999), it could be argued that they might upregulate p62 expression via separate pathways not necessarily linked with apoptotic signaling. However, in that scenario, a consistent upregulation of p62 by the different pro-apoptotic conditions, each of which elicits distinct primary events, would be unlikely, and thus this favors the involvement of common events behind the upregulation of p62. Furthermore, these putative common events need not be specific to apoptosis. Indeed, while no other work addressing p62 expression in apoptosis has been reported to date, an elevation of p62 level was observed in several models of pathological conditions, including exposure *in vitro* to oxidative stress-generating agents (Ishii et al. 1996, Ishii et al. 1997) or glycol ethers (Syed and Hecht 1998) and *in vivo* to excitotoxic insult (Nakaso et al. 1999) or the MB-inducing drug 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Stumptner et al. 2002). Since it is upregulated upon oxidative stress, p62 was referred to as a “stress protein” by Ishii et al. (1997), although it is clearly structurally distinct from the established types of stress proteins such as the heat shock proteins. Nevertheless, the responsiveness of p62 to diverse stimuli that impose stress upon the cell is consistent with the possibility that the upregulation of p62 may manifest a stress-related response.

It is tempting to speculate that a common mediator for the upregulation of p62 might be oxidative stress, since reactive oxygen species (ROS) are generated in each of the pathological p62-inducing conditions (Michaelis 1998, Syed and Hecht 1998, Denk et al. 2000), including apoptosis (Sastry and Rao 2000). In line with this, the induction of p62 expression in the present study clearly preceded the activation of caspase-3 in these models (Salminen et al. 1998, Solovyan et al. 1999) and thus paralleled more upstream events that involve ROS generation (Greenlund et al. 1995, Schulz et al. 1997).

Oxidative stress may induce p62 expression via redox-sensitive transcription factors that have binding sites in the p62 promoter region (Vadlamudi and Shin 1998, Okazaki et al. 1999) and/or via the stabilization of p62 mRNA (Lee et al. 1998). One of the critical factors appears to be Nrf2, since ROS-generating agents increase its activity and since the ablation of the Nrf2 gene largely cancels ROS-induced upregulation of p62 (Ishii et al. 2000). Nrf2 is a subunit of the NF-E2 transcription factor that has binding sites in the regulatory regions of the p62 gene. Interestingly, Nrf2 is essential for the coordinated transcriptional activation of detoxication enzymes that contribute to defense mechanisms against oxidative stress (Itoh et al. 1997a, Nguyen et al. 2003b). The increase of p62 expression may also receive contributions from other redox-sensitive factors such as NF-κB (Ishii et al. 2000), likewise activated by ROS (Kaltschmidt et al. 1999), although their roles in the regulation of p62 have not been addressed. It is noteworthy that NF-κB not only seems to regulate transcription from the p62 gene, but is also itself activated in a p62 protein-dependent manner (Sanz et al. 1999), suggesting the presence of a feedback mechanism that controls the parallel activation of p62 and NF-κB. However, in addition to the ROS-mediated pathways outlined above, the pro-apoptotic treaments used in the present study may also influence the expression of p62 via stimulus-specific pathways, possibly explaining the varying time courses of p62 upregulation.

The implication of p62 in aPKC-mediated activation of NF-κB, currently the best-characterized aspect of p62 function, provides one attractive framework for interpreting the significance of the present findings. It would be expected that conditions which elevate the level of p62 should also result in the enhancement of NF-κB activation and other signaling downstream from p62, such as ERK5 activation. Indeed, upon oxidative

stress, both NF-κB (Li and Karin 1999) and ERK5 (Abe et al. 1996) are activated. Since both NF-κB (Wooten 1999, Foehr et al. 2000) and ERK5 (Watson et al. 2001, Liu et al. 2003) act as survival factors in neurons, another predicted consequence would be the enhancement of neuronal survival. Accordingly, three *in vitro* studies suggest that the upregulation of p62 has survival-promoting effects. In HEK and PC12 cells, overexpression of p62 potently enhanced NF-κB activation and antagonized cell death, while the opposite was seen with antisense p62 (Wooten et al. 2001). Overexpressed p62 also antagonized PAR-4-mediated inactivation of NF-κB and apoptosis (Chang et al. 2002). In addition, overexpression of p62 in PC12 cells enhanced the activation of ERK5 (Geetha and Wooten 2003). *In vivo*, indirect evidence for a protective role for p62 against cell death was obtained in a rat model of excitotoxicity, where an inverse correlation was seen between the degree of p62 upregulation and the vulnerability to the excitotoxic insult (Nakaso et al. 1999).

Taken together, the above considerations suggest a scheme in which various conditions that involve the generation of ROS may, possibly via the activation of redox-sensitive factors such as Nrf2, induce the expression of p62, which in turn potentiates pro-survival signaling via pathways including the activation of NF-κB and ERK5. Viewed in this context, the present findings suggest that the upregulation of p62 is a common event in neuronal death-promoting conditions and is involved in the activation of protective signaling.

6.2.2 Proteasomal function and p62 expression

As the reported ability of p62 to bind noncovalently to Ub (Vadlamudi et al. 1996) pointed to a functional role in ubiquitination-mediated processes, it was of interest to see how the expression of p62 responds to conditions in which Ub-conjugated proteins accumulate. The proteasomal inhibitors MG-132, lactacystin, and PSI prominently increased the expression of p62 at both mRNA and protein levels in Neuro-2a cells. Similar results were obtained in hippocampal neurons, suggesting that the upregulation of p62 is a common event in neurons subjected to proteasomal inhibition. These were the first data showing a response in p62 mRNA expression to proteasomal inhibitors. Previously, Ishii et al. (1997), using 5–20-fold higher concentrations of inhibitors, had

reported an induction of p62 protein, to a degree comparable to that in our study. However, since any protein normally degraded via the proteasome is likely to accumulate upon proteasomal inhibition, analysis of protein levels alone is not very informative. In contrast, the observed increase at the transcript level of p62 might signify a functional response specifically involving p62. Our findings on both mRNA and protein levels are consistent with those more recently obtained in normal and transformed breast epithelial cell lines (Thompson et al. 2003).

As expected, the proteasomal inhibitors also caused a marked accumulation of Ub-conjugated proteins in both types of neuronal cells. These findings raised the possibility that the upregulation of p62 could represent a response to the concomitant increase in the level of ubiquitinated proteins, as would be expected if the function of p62 involves interactions with Ub. The latter assumption is currently not substantiated in the light of the work that has established a physiological role for p62 in aPKC-mediated signal transduction, yielding functional models that assign no significance for the Ub-binding ability of p62 (Sanz et al. 1999, Sanz et al. 2000, Wooten et al. 2001, Mamidipudi et al. 2002). However, it should be noted that the findings from the context of PDB (Ciani et al. 2003) as well as the highly conserved sequence of the UBA domain of p62 (Avila et al. 2002) point to the importance of the UBA region for the normal functioning of p62. Accordingly, it has been suggested that an interaction of p62 with ubiquitinated receptor-associated proteins might play an important role in receptor-mediated signaling (Geetha and Wooten 2002, Mamidipudi and Wooten 2002), but such putative interactions remain to be characterized.

It is likewise unclear what mechanisms underlie the observed elevation of p62 level upon proteasomal inhibition. It is not known if a sensor mechanism exists that would monitor the level of Ub-conjugated proteins and, upon their accumulation, would induce adaptive changes in gene expression. However, when the expression of p62 protein was compared to the level of ubiquitinated proteins in normal and cancerous breast specimens, no correlation was found (Thompson et al. 2003), suggesting that the amount of Ub conjugates is not a major determinant for p62 expression, although the result may have been confounded by the inclusion of neoplastic tissues in the analysis. In any case, proteasomal inhibition results in other events that may account for the upregulation of

p62. For example, it causes the buildup of transcription factors that are normally degraded via the proteasome (Hochstrasser and Kornitzer 1998). Interestingly, as noted by Aono et al. (2003), proteasomal inhibitors mimick other stress agents by inducing the accumulation of Nrf2, with the consequent induction of Nrf2-driven genes (Nguyen et al. 2003a, Stewart et al. 2003). A similar mechanism may apply for other transcription factors such as AP-1 (Wu et al. 2002) and C/EBP (Hungness et al. 2002) that are activated upon proteasomal inhibition and target the p62 promoter. In addition, proteasomal inhibition leads to the production of reactive oxygen and nitrogen species (Lee et al. 2001a, Hungness et al. 2002) and might thereby activate pathways similar to those initiated by oxidative stress. On the other hand, in a promoter activity assay, proteasomal inhibitors did not affect the basal transcription activity of p62 despite increasing its mRNA level, suggesting that the increase occurs via mRNA stabilization (Thompson et al. 2003). However, the latter results may not be conclusive, since a tumorigenic cell line was used and the analysis was confined to a 1.8-kb region in the promoter, possibly lacking the elements mediating the response of p62 to proteasomal inhibitors.

Whether or not the upregulation of p62 reflects functional links with Ub, it might also possess an adaptive role in the aPKC context, as suggested to be the case in the condition of pro-apoptotic stimuli. However, any adaptive role that p62 might serve upon proteasomal inhibition should differ from that in the case of pro-apoptotic stresses, since NF-κB activation is generally blocked by proteasomal inhibitors (Traenckner et al. 1994, Maggirwar et al. 1995). It could also be argued that the processes of apoptosis and production of ROS differ fundamentally from the condition of proteasomal impairment in the sense that the former are part of normal tissue development and cell metabolism and therefore associated with devoted and adaptive cellular responses, whereas the same may not be true for overt proteasomal dysfunction, a pathological state lacking counterparts in normal physiology. Therefore, the observed upregulation of p62 in this experimentally induced condition might also represent a fortuitous event. However, while further studies should resolve the functional significance of p62 upregulation upon proteasomal inhibition, the findings predict that p62 may similarly accumulate *in vivo* upon conditions involving decreased proteasomal activity.

Collectively, the results from study **I** demonstrated that p62 expression in neurons shows a marked responsiveness to two types of pathological conditions relevant to ND disorders, *i.e.*, apoptosis-promoting stresses and proteasomal impairment. Together with previous work implicating p62 in protein aggregation in liver pathology (Stumptner et al. 1999), these findings raised the question as to whether p62 might be similarly involved in neurodegenerative events in the human brain.

6.3 Incorporation of p62 into tau- and αS-containing inclusions (II)

This study was carried out to examine the immunoreactivity of p62 in relation to that of Ub, HP-tau, and αS in affected brain areas of human tauopathies (AD, PiD) and synucleinopathies (PD, DLB, MSA). The unexpected result of the study was the selective accumulation of p62 into each of the cytoplasmic inclusion types characteristic of these diseases, *i.e.*, NFTs, PiBs, brainstem and cortical LBs, and GCIs, respectively. Since the immunoreactivity for p62 was confined to inclusion-harboring cells in the staining conditions employed, the accumulations detected likely represent greatly elevated focal amounts of p62 compared to its physiological levels. These findings suggested that the pathological aggregation of HP-tau and α-synuclein might share common mechanisms involving p62.

In comparison to previously reported constituents of the above inclusion types, p62 was exceptional by showing copious incorporation into both tau- and αS-containing perikaryal inclusions yet was generally absent from deposits within neurites (*i.e.*, neuropil threads and dystrophic neurites in AD and αS^{+} neurites in PD). Furthermore, p62 immunoreactivity was absent from amyloid plaques as well as from aging-related Ub-positive structures not associated with disease (*i.e.*, neuropil grains and corpora amylacea). These findings rule out the possibility that p62 would be nonspecifically incorporated into all types of protein aggregates or Ub-containing structures and suggest that p62 accumulates selectively into disease-associated perikaryal inclusions.

Little is known about the mechanisms underlying the enrichment of p62 into cytoplasmic inclusions. However, when the staining pattern of p62 was compared with those seen for Ub and HP-tau or αS in inclusion-containing perikarya (**II**: Table 2), it appeared that for

each inclusion type, the staining pattern of p62 was similar to that of Ub but it exhibited some differences in comparison to HP-tau and αS. For example, in the dentate gyrus of the PiD case, the robust and globular HP-tau-immunoreactivity of PiBs contrasted with the weaker and more diffuse labeling for Ub and p62, whereas in the SN of the PD cases, diffuse cytoplasmic immunoreactivity was detected for αS but not for Ub or p62. Apart from the fact that abnormal Ub-positive neurites were generally immunonegative for p62, the similarity between the staining patterns for p62 and Ub in disease-associated deposits, together with the Ub-binding ability of p62 (Vadlamudi et al. 1996), suggested that the accumulation of p62 and Ub might be interconnected.

Based on the findings that p62 proteins can self-associate *in vitro* with strong affinity (Puls et al. 1997), likely causing its oligomerization *in vivo* (Gong et al. 1999) and the formation of large aggregates upon ectopic expression in several cell types (Puls et al. 1997), we proposed a mechanism whereby dimeric p62 might coaggregate with Ub-conjugated proteins. As the regions of p62 necessary for self-association and Ub-binding lie near the opposite termini of its sequence, we surmised that a p62 dimer would be capable of binding two Ub chains simultaneously. Thereby, p62 dimers and Ub-conjugated proteins might be linked into a net-like arrangement, possibly favoring their coaggregation (**II**: Fig. 3). In this manner, p62 might be sequestered into pathological inclusions via binding to ubiquitinated tau/αS or other Ub-conjugated species therein. Alternatively or in parallel, Ub conjugates might be redistributed and enriched into structures containing p62, as observed in cells exposed to proteasomal inhibitors (Shin 1998).

Our assumption that the incorporation of p62 into inclusions reflects binding to Ub was corroborated by a study employing a cellular model of intranuclear polyglutamine aggregates. These aggregates were shown to recruit normal cellular Ub-binding proteins (p62 and ataxin-3), in a manner that required the presence of functional Ub-binding motifs (Donaldson et al. 2003). However, it is not known whether the same mechanism might apply to inclusions that form in the cytoplasm. Further, although an intact UBA domain was shown to be necessary for the recruitment of p62 (Donaldson et al. 2003), it should be noted that UBA domains may also bind proteins other than Ub (Buchberger

2002, Mueller et al. 2004). Findings from a cell culture model of MB formation were also interepreted to suggest Ub-dependent sequestration of p62 (Zatloukal et al. 2002), although also these results left open other possibilities, *e.g.* the direct interaction between p62 and misfolded cytokeratins.

The model based on p62 self-association was also strengthened, albeit with slight modification, by structural studies on PB1 domain-containing proteins, showing that PB1 domains can heterodimerize in a front-to-back arrangement via electrostatic interaction (Noda et al. 2003, Wilson et al. 2003). Interestingly, among 8 different proteins studied, p62 was exceptional by displaying strong PB1-mediated self-association (Lamark et al. 2003), resulting in high-molecular weight aggregates *in vitro* (Wilson et al. 2003). Thus, unlike the back-to-back dimers assumed in our model (**II**: Fig. 3), the front-to-back arrangement demonstrated by these studies also seems to enable the chain-like oligomerization of p62, while possibly allowing simultaneous interactions with Ub (Lamark et al. 2003, Wilson et al. 2003).

It is conceivable that proteasomal dysfunction, as occurs in AD (Keller et al. 2000) and PD (McNaught and Jenner 2001) as well as *in vitro* upon protein aggregation (Bence et al. 2001), might be an underlying factor that contributes to concomitant accumulation and aggregation of p62 and Ub. In affected cells, a decline in proteasomal activity might be triggered and/or exacerbated by PHF-tau (Keck et al. 2003) or αS (Snyder et al. 2003), resulting in the accumulation of both p62 and Ub-conjugated proteins, as observed in cultured neurons treated with proteasomal inhibitors (study **I**). The co-accumulation of p62 with ubiquitinated proteins would be expected to favor the PB1-mediated self-association of p62 as well as its interactions with Ub-conjugated proteins including tau and αS. In addition to proteasomal dysfunction, the accumulation of p62 might be promoted by oxidative stress, which has been implicated in the pathogenesis of both neurodegenerative diseases and liver inclusions (Zatloukal et al. 2002).

Studies on other diseases have revealed further types of p62-immunoreactive inclusions (Table 4). In hepatocellular carcinoma, p62 was identified as a major constituent of IHBs (Stumptner et al. 1999). After our study, Zatloukal et al. (2002) confirmed the presence

of p62 in NFTs and LBs and demonstrated its abundance also in MBs in liver diseases and its presence in GFAP-containing Rosenthal fibers in astrocytoma. More recently, p62 was shown to accumulate into Ub-positive, tau-negative (Ub^+/tau^-) intraneuronal inclusions seen in frontotemporal dementia (FTD) (Arai et al. 2003). In this disease, also Ub^+ neurites labeled for p62. Similarly, in amyotrophic lateral sclerosis with dementia (ALS-D), at least a subset of the Ub^+tau^- neuronal inclusions in the dentate gyrus and neocortical regions were immunoreactive for p62 (Arai et al. 2003, Nakano et al. 2004). Notably, in a case with ALS-D, Arai et al. also found $p62^+$ oligodendrocytic and astroglial inclusions negative for both Ub and tau. Further, p62 was present in tau^+ glial inclusions characteristic of PSP and CBD (Arai et al. 2003).

Based on the above findings, the engagement of p62 appears to be a general feature of proteinaceous cytoplasmic inclusions that form within the cytosol. In published work, no contrary example has been reported to date. As a rule, p62 also seems to be incorporated into most inclusions of each type (Table 4). The only contrasting result was the detection by Nakano et al. (2004) of p62 in less than 50 % of the Ub^+ neuronal inclusions in ALS-D. However, the authors used a p62 antibody not recommended for paraffin-embedded sections. When the same inclusion type was examined in FTD using two other antibodies for p62 and a different epitope unmasking method, similar numbers of $p62^+$ and Ub^+ inclusions were observed (Arai et al. 2003). It is also noteworthy that p62 serves currently as a candidate primary constituent for the Ub^+ neuronal and Ub^- glial inclusions in ALS-D, in addition to the hepatocytic IHBs.

On the other hand, at least immunohistochemically, the incorporation of p62 seems to be more copious and frequent than that of Ub in several inclusion types (Table 4). These types comprise the Ub^- oligodendrocytic and astroglial inclusions in ALS-D, only revealed by p62 immunostaining (Arai et al. 2003), as well as the IHBs which are mostly Ub^- (Stumptner et al. 1999). Likewise, tau^+ glial inclusions labeled positively for p62 while displaying variable to no immunoreactivity for Ub (Ikeda et al. 1998, Komori 1999). PaBs and MarBs also showed preferential labeling for p62 in comparison to Ub (study **IV**). Further, the incorporation of p62 seemed to precede that of Ub in NFTs (study **III**) and in MBs (Zatloukal et al. 2002). These observations suggest that p62 may

accumulate into inclusions in an Ub-independent manner, the findings being apparently contradictory with the coaggregation model presented above.

The possibility remains that the “Ub-negative” inclusions might contain Ub-conjugated proteins in amounts too low to be revealed by conventional immunodetection yet sufficient for the recruitment of p62. If this is not the case, it is noteworthy that p62 seems to be incorporated into inclusions in a manner that requires neither Ub nor any particular one of their main constituent species (Table 4). The question therefore arises as to the molecular determinant(s) that would account for the incorporation of p62 into the manifold inclusions with tau, αS, cytokeratins, GFAP, p62 itself, or unknown proteins as their assumed primary constituents. Conceivably, such molecular determinants that are common to the misfolded, aggregated proteins in these diseases might be constituted by aberrant structural features (*e.g.*, hydrophobic surfaces, oxidized residues, or β-sheet conformation), to which p62 might bind either directly or indirectly. The possibility of direct binding is intriguing in light of the identification of antibodies that recognize common epitopes present in amyloid fibrils or soluble oligomers derived from structurally unrelated proteins (O'Nuallain and Wetzel 2002, Kayed et al. 2003). Alternatively, p62 might associate with aggregates indirectly, via binding to a chaperone protein, for example, since various types of chaperones are recruited to misfolded proteins (Richter-Landsberg and Goldbaum 2003).

Thus, findings to date provide evidence for both Ub-dependent and -independent mechanisms to explain the deposition of p62 into cytoplasmic inclusions. It is possible that both mechanisms might be involved. Be that as it may, it is important to point out that if p62 binds as oligomers to its target proteins in the aggregate, this would be expected to result in strong, multivalent interaction of p62 arrays with the target proteins, by analogy with high-avidity bindings between multivalent antigens and antibodies (Alberts et al. 1994). Such incorporation of oligomeric p62 might therefore promote the compaction of misfolded and/or ubiquitinated proteins into dense inclusions. However, the common engagement of p62 into diverse inclusions might also reflect some other, as yet unidentified functional role(s), possibly as part of more complex molecular interplay (pp. 95–98).

Collectively, study **II** demonstrated the presence of p62 in the hallmark tau and αS inclusions of AD, PiD, DLB, PD, and MSA, showing selective accumulation of p62 into perikaryal deposits. The observations also suggested that p62 might coaggregate with ubiquitinated proteins. These findings spurred more detailed investigations into the relation between p62 and other inclusion constituents in the intraneuronal protein aggregates characteristic of AD and PD.

6.4 Involvement of p62 in AD-type neurofibrillary pathology (III)

In this study the extent of p62 involvement in intraneuronal AD-type pathology was examined in the neocortex of demented and cognitively unimpaired aged individuals. The first main finding was that within the spectrum of AD-type neuronal lesions, the immunoreactivity for p62 appears to be tightly linked to neurofibrillary pathology but occurs independently of NP-associated, Ub-positive and AT8-negative ($Ub^+/AT8^-$) neuritic dystrophy. Thus, in the AD cases, p62 was almost invariably present in NFTs, while the nondemented cases were virtually or completely devoid of p62 staining, though rich in $Ub^+/AT8^-$ NPs. The second observation of major interest was that in the AD cases, p62 immunoreactivity was almost exclusively confined to perikaryal HP-tau aggregates, *i.e.* NFTs, as opposed to NTs or dystrophic neurites of NPs. This was in clear contrast to the staining profiles of HP-tau (AT8) and Ub. These findings strengthened the view from study **II** that p62 accumulates selectively into disease-associated perikaryal inclusions, rather than simply associating with any type of focal buildup of Ub.

It is presently unclear why p62 localizes preferentially in NFTs, as opposed to intraneuritic deposits of HP-tau. A similar predilection of p62 for perikaryal vs. intraneuritic aggregates was observed in αS pathology (**IV**). One possibility is that this pattern might reflect the normal intraneuronal distribution of p62. However, at least in neuronal culture (*i.e.*, NGF-differentiated PC12 cells), endogenous p62 was found in both perikarya and neurites (Geetha and Wooten 2003), and it is likely that the same is true for neurites in the brain. Further, in FTD, p62 was shown to accumulate into Ub^+/tau^- neuritic sturctures (Arai et al. 2003), previously localized to distal dendrites (Iseki et al. 1998). These findings may suggest that either the normal intraneuronal distribution of p62 varies by cell type or some other factors determine whether or not p62 is

incorporated into intraneuritic deposits. One underlying factor might be compartment-dependent heterogeneity in the structure and composition of the aggregates, as observed for pathological tau (Liu et al. 1993, Kurt et al. 1997, Ishizawa et al. 2000). Nonetheless, it should be noted that if p62 promotes inclusion formation (**II**), the degree of p62 incorporation might, conversely, be one factor determining the intracellular site where aggregated proteins pack into voluminous masses.

In the life span of NFTs, the incorporation of p62 appears to be a relatively early event, since $p62^+$ neocortical NFTs were also found in two nondemented cases with occasional NFTs in AT8 labeling, possibly representing preclinical AD. Consistently, NFTs immunoreactive for p62 were also detected in the transentorhinal cortex of a nondemented subject displaying only a few $AT8^+$ NFTs in layer Pre-α cells (Braak stage I). Importantly, several observations suggested that p62 accumulates into NFTs before Ub, thereby calling for a re-evaluation of the initial model (study **I**) in which p62 and Ub-conjugated proteins would co-accumulate into inclusions due to direct p62–Ub binding. First, in the nondemented cases, NFTs seemed to incorporate p62 before Ub (**III**: Table 3), the immunoreactivity for which appears late in tangle formation (Bancher et al. 1989a). Secondly, in the AD cases, significantly fewer NFTs labeled for Ub than for p62 or AT8. Third, correlation data from the AD cases agreed with the interpretation that the p62 is incorporated into NFTs before Ub, since the regression line for Ub^+ NFTs intersected the p62 NFT axis at a positive value (**III**: Fig. 2c). The opposite was seen for $AT8^+$ NFTs, as expected if the aggregation of HP-tau occurs before the incorporation of p62. Collectively, the present findings therefore lend support to the view that p62 may also be engaged into inclusions in an Ub-independent manner. Nevertheless, the data are compatible with the possibility that upon its incorporation, p62 might promote the coaggregation of Ub-conjugated proteins. This would be consistent with the marked correlation between the numbers of $p62^+$ and Ub^+ NFTs (**III**: Fig. 2c).

In summary, study **III** showed that in AD pathology, the immunoreactivity for p62 is predominantly detected in perikaryal HP-tau aggregates, *i.e.* NFTs, while being mostly absent from intraneuritic HP-tau deposits. The findings also suggested that in NFTs, p62 is a relatively early constituent, preceding the incorporation of Ub.

6.5 Formation of αS-containing inclusions associated with PD (IV)

This study elucidated the morphogenesis of nigral LBs and their relation to PaBs in cases with abundant inclusions in the SN, also examining the role of p62 in these pathological structures. The two main findings were: 1) Wide morphological heterogeneity of perikaryal αS-containing aggregates, ranging from punctate αS to variform compact inclusions (PaB- to LB-type). 2) Selective and consistent incorporation of p62 into compact perikaryal inclusions, combined with its absence from punctate and intraneuritic αS accumulation. Although several types of nigral αS accumulation have been described previously (Baba et al. 1998, Spillantini et al. 1998b, Wakabayashi et al. 1998a, Gomez-Tortosa et al. 2000), the diversity observed in this study encompassed novel types and intermediate forms, enabling the reconstruction of a model for the formation of perikaryal αS-containing inclusions.

The most inconspicuous type of αS accumulation consisted of cytoplasmic areas of punctate immunoreactivity, often with faint diffuse staining but lacking reactivity for p62 or Ub, suggesting that this pattern represents the earliest immunohistochemically observable stage of pathological αS accumulation. Consistent with this was the occurrence in one of the control cases of two solitary αS^+ neurons showing only punctate αS. To our knowledge, this type of αS-immunoreactivity has not been described previously in the SN. Although the αS^+ granules were somewhat reminiscent of the "dust-like particles" detected in lower brain stem nuclei in PD (Braak et al. 2001), the latter were solely seen within pigment deposits, unlike the granules in our study. However, the diffuse αS component observed in our study might correspond to the "diffuse cytoplasmic" αS staining detected by Wakabayashi et al. (1998a), while the "cloud-like αS staining" illustrated by Gomez-Tortosa et al. (2000) appears as a more compact type of aggregation. Interestingly, punctate αS similar to that in our study accumulated in cultured cells subjected to mitochondrial inhibitors or αS overexpression (Lee and Lee 2002, Lee et al. 2002).

Notably, the commonly used antibody LB509 showed no punctate staining yet clearly labeled compact inclusions, providing a clue into the early events of αS accumulation.

Since this antibody recognizes a minimum epitope at residues 115–122 of αS (Jakes et al. 1999), the punctate staining might represent αS modified within or close to this epitope, *e.g.* via phosphorylation at Tyr-125 or Ser-129 (Ellis et al. 2001, Fujiwara et al. 2002). Alternatively, the punctate αS might lack the above epitope, since partially truncated αS was shown to be a component of LBs in DLB brains (Baba et al. 1998), and since C-terminal truncation enhanced the fibrillization of αS (Crowther et al. 1998).

As regards the origins of LBs, their conspicuously spherical and concentric morphology would suggest two essentially opposite schemes for their formation. In the first, they might form by expansive, radially symmetrical growth, resulting from the deposition of αS and/or other proteins onto a smaller precursor body, originating from a putative nucleation center. However, this "growth model" would predict the existence of minuscule "early LBs", but such structures were never detected in this study. Instead, there seemed to be a minimum diameter (~4 µm) for αS-stained LBs. Therefore, LBs are more likely to arise in the opposite direction, *i.e.* in a compaction-like manner, from a more voluminous yet looser precursor structure. Our data suggest that it is the PaB which serves as the matrix for LB formation, since a continuum of intermediate morphologies ranging from typical PaBs to classical LBs were found. Most importantly, peripheral regions of PaBs frequently contained smallish but unmistakable LBs or less distinct spherical masses of heightened immunoreactivity, with the appearance that focal compaction occurs at these sites. Similar transitional forms were ultrastructurally described in the locus coeruleus (Takahashi et al. 1994). In other neurons, classical LBs lay abutting onto what appeared to be the remains of a PaB, or as clusters with no intervening αS-positivity.

The interpretation that LBs arise from PaBs is concordant with previous immunohistochemical studies in the SN (Wakabayashi et al. 1998a, Gomez-Tortosa et al. 2000). Admittedly, the possibility cannot be ruled out that PaBs and LBs could reflect separate yet parallel aggregation processes, as interpreted by some authors before the αS era, based on ultrastructural and staining differences between PaBs and LBs (Pappolla et al. 1988, Gibb et al. 1991). However, such differences would be expected if these entities represent consecutive stages of a complex aggregation process. On the other hand, in our

study, both PaBs and LBs contained not only three common protein components (αS, p62, and Ub) but also in similar relative abundances, an unlikely result if PaBs and LBs were to originate via separate processes.

The “compaction vs. growth” reasoning can also be applied to the origin of PaBs. However, their usually larger size makes it improbable that PaBs would be formed via compaction of a more voluminous precursor. Instead, they probably arise by growing, via the gradual incorporation of new material, and/or via the coalescence of already formed bodies into a larger mass. Therefore, irrespective of its molecular composition, the precursor material of PaBs should appear with diffuse or particulate morphology, or both. We demonstrated abundant neurons containing punctate/diffuse αS reactivity, suggesting that either of these components may represent the source material of PaBs. This model would imply that “early PaBs” should be detectable as αS^{+} accumulations which are smaller than typical PaBs yet morphologically distinct from LBs. Accordingly, such entities were detected in perikarya showing punctate αS, in the form of variously sized clump-like masses, possibly serving as “aggregation centers” that grow into typical PaBs. Notably, while punctate αS was never associated with p62-positivity, the latter was invariably present in PaBs, and was also seen in the aggregation centers. Thus, p62 seems to be incorporated upon PaB formation.

Bringing together the above considerations, a pathogenetic sequence can be envisaged, assuming that the morphological spectrum of perikaryal αS-containing structures reflects a stereotypic and unidirectional aggregation process. In this scheme, punctate αS-positivity appears in perikaryal areas of sparse neuromelanin as the earliest histologically observable sign of protein accumulation in the SN, consistent with the abundant evidence pointing to an early role for αS in the process (Lotharius and Brundin 2002). The next step is the appearance of a larger body within the αS-containing granules, representing their coalescence into a clump-like mass, whereby p62 is incorporated. These masses enlarge by assimilating the less aggregated αS, giving rise to what appears as a PaB. Concomitantly, immunoreactivity for Ub increases, likely due to the sequestration of Ub-conjugated proteins into the growing PaB. Neuromelanin is gradually displaced by the PaB, which may adopt varied morphologies, possibly reflecting differences in the

intraneuronal environment or maturation stage. Starting peripherally, the PaB undergoes progressive condensation, leading to the emergence of one or more spherical foci. By further compaction and incorporation of ambient αS^+ material, these foci develop into classical LBs, while the remains of the PaB eventually disappear.

The selective and consistent incorporation of p62 into PaBs and LBs is suggestive of an important role for p62 in their formation, supported by the presence of p62 in the aggregation centers. Unlike p62, Ub shows less consistent and less selective accumulation (Bancher et al. 1989b, Dale et al. 1992, Wakabayashi et al. 1998a), being variably incorporated into αS deposits in both perikarya and neurites, possibly reflecting a generalized buildup of Ub-conjugated proteins due to proteasomal dysfunction (McNaught and Jenner 2001). These findings also lend support to the view that the recruitment of p62 into inclusions is not dependent on Ub.

Combined with findings from other contexts about cytoplasmic protein aggregation, the present observations hopefully shed more light on the significance and mechanisms of PaB and LB formation. It is increasingly believed that the deposition as inert inclusions serves as a ”third line of defence” against misfolded proteins (Zatloukal et al. 2002). Important supportive evidence was obtained from transgenic studies on MB formation, which was shown to protect hepatocytes in conditions where incorrectly assembled cytokeratins accumulate (Denk et al. 2000, Zatloukal et al. 2000). Since p62 is an early and abundant component of MBs (Stumptner et al. 2002, Zatloukal et al. 2002), its similar engagement in PaBs and LBs provides further evidence that also these inclusions confer cytoprotection against aberrant proteins.

Consistent with the above, LBs are immunoreactive for several markers of aggresomes (McNaught et al. 2002b), pericentriolar deposits that form in response to cytoplasmic accumulation of misfolded proteins (Johnston et al. 1998, Garcia-Mata et al. 1999, Wigley et al. 1999). McNaught et al. therefore proposed that LB formation may represent an “aggresome-like response” to excess levels of unwanted proteins. However, LBs exhibit some notable differences compared to typical aggresomes. Most importantly, multiple LBs often occupy the same neuron, whereas the aggresome is, by definition, a

singular structure, residing in the pericentriolar area. Neither is an aggresomal response suggested by the highly symmetrical, spherical morphology of classical LBs. On the other hand, in our data, PaBs appeared to arise from punctate αS via gradually growing "aggregation centers" in a manner that was morphologically similar to the sequence of events in the prototypic aggresomal response seen *in vitro* (Johnston et al. 1998, Garcia-Mata et al. 1999). Further, PaBs were usually present singly per cell. These considerations suggest that PaBs might be more closely related to aggresomes, whereas the formation of the spherical bodies (LBs) likely involves a different, yet possibly related, mechanism.

In comparison to perikaryal accumulation of αS, intraneuritic deposits exhibited two marked differences. Unlike inclusions in perikarya, intraneuritic deposits were consistently immunonegative for p62, and far fewer abnormal neurites were stained than for αS or Ub. Interestingly, this predilection of p62 for perikaryal aggregates duplicated the analogous findings on AD-type tau pathology (**III**). There was a further difference to perikaryal accumulation; no punctate αS was detected at extrasomal sites. These findings may suggest that intraneuritic αS inclusions form via a mechanism fundamentally different from perikaryal inclusions.

An unexpected finding in study **IV** was the copious presence of p62 in MarBs. Although their origin and pathological significance remain obscure (Beach et al. 2004), nigral MarBs were shown to be increased after severe agonal psychophysiological stress, suggesting that MarBs may result from stress at the neuronal level, such as energy crisis or excitotoxicity (Quan et al. 2001a, Quan et al. 2001b). This possibility is consistent with the engagement of p62, the expression of which is upregulated upon stressful conditions (p. 76). Of possible relevance, activators of the stress-related protein kinase p38 were reported to induce the nuclear translocation of p62 (Sudo et al. 2000). The presence of p62 in MarBs could also indicate that the recruitment of p62 is not confined to cytoplasmic inclusions but rather may represent a more general event, triggered by protein aggregates whether in the cytosol or the topologically equivalent nucleoplasm. This finding therefore predicts that one would find the incorporation of p62 also in the

morphologically similar intranuclear inclusions seen in polyglutamine expansion diseases.

Taken together, the results of study **IV** suggest that in the SN, punctate perikaryal αS, PaBs, and LBs originate as successive stages of a complex aggregation process. The early and consistent involvement of p62 in this process supports the view that the formation of PaBs and LBs represents a cytoprotective response against the accumulation of noxious proteins.

Table 4. Relation of p62 to primary constituents and Ub in pathological inclusions.

Inclusion type	Assumed primary constituents (candidates)	p62 vs. primary constituent	Ub vs. primary constituent	p62 vs. Ub	Reference
neurofibrillary tangle (AD)	tau	**mostly p62+**	Ub late component	**p62 precedes & outnumbers Ub**	Study III
Pick body	tau	p62 weaker	Ub weaker	similar	Study II
tau+ glial inclusions (PSP, CBD)	tau	n/a	Ub weaker / absent	p62 stronger?	Arai et al. 2003
glial cytoplasmic inclusion (MSA)	α-synuclein	similar	similar	similar	Study II
pale body	α-synuclein	**p62 already present in the earliest PaBs**	variable	p62 stronger	Study IV
Lewy body (brainstem-type)	α-synuclein	**~identical numbers**	mostly Ub+	similar	Study IV
Ub+ neuronal inclusions (FTD, ALS-D)	? (p62)	?	?	similar / [2]**Ub outnumbers p62**	Arai et al. 2003, Nakano et al. 2004
Ub– glial inclusions (ALS-D)	? (p62)	?	all Ub–	only p62 present	Arai et al. 2003
Rosenthal fiber	GFAP	mostly p62+	mostly Ub+	n/a	Zatloukal et al. 2002
intracytoplasmic hyaline body	? (p62)	p62 = the primary constituent?	mostly Ub–	p62 much stronger & outnumbers Ub	Stumptner et al. 1999
Mallory body	cytokeratins and p62	**p62 already present in the earliest MBs**	mostly Ub+	**p62 precedes & outnumbers Ub**	Stumptner et al. 2002, Zatloukal et al. 2002
[1]Marinesco body	? (ataxin-3, p62)	?	?	p62 outnumbers Ub	Study IV

n/a = not analyzed. Bold type indicates results based on quantitative or temporal analyses.
[1]No overt disease association to date. [2]The result may be affected by suboptimal detection of p62 (see p. 84).

6.6 Possible roles of p62 in protein aggregation

The role of p62 in cytoplasmic inclusions is enigmatic. Based on the findings to date, the presence of p62 appears to be a general feature in pathological proteinaceous inclusions lacking delimiting membranes (Table 4). This generality suggests that the involvement of p62 in inclusions might indicate a functional role that provides adaptive value and could thereby have been moulded by evolution. Such a functional involvement might not be restricted to disease-associated protein deposition but could also be of importance in non-pathological conditions where proteins may accumulate, for example during cellular differentiation.

Although several studies have indicated a role for p62 in the regulation of aPKC, this does not preclude additional functions for p62. In fact, there is a growing list of well-characterized proteins performing two or more different functions. These “moonlighting proteins” are involved in diverse cellular systems and include receptor, chaperone, proteasomal, and regulatory proteins (Jeffery 1999, Jeffery 2003). While some of them seem to provide a means for coordinating related cellular activities, in other cases there is no overt connection between their different functions. Thus, the recruitment to inclusions might reflect a second function for p62, possibly unrelated to the aPKC context. Interestingly, the mechanisms whereby a multifunctional protein can switch between functions include complex formation and oligomerization (Jeffery 1999, Jeffery 2003).

Alternatively, the involvement of p62 might represent an epiphenomenon in the evolutionary sense, *i.e.*, an event that is dictated by the structural and functional properties of p62 yet has not been shaped by evolutionary selection. While this issue has both practical and theoretical implications, at present too little data is available for judging between these possibilities. Be the evolutionary aspect as it may, the key questions from the pathogenetic standpoint remain the same and concern the mechanisms underlying, and the significance of p62 involvement in cytoplasmic inclusions. The findings accrued to date are compatible with several possible scenarios, where the effect of p62 on inclusion formation ranges from promoting through neutral to antagonizing. Conceivable mechanisms comprise both “p62-autonomous” ones (*i.e.*, not prescribing the

engagement of additional proteins) as well as scenarios involving p62 as part of more complex, organized responses.

As discussed above (p. 85), currently available evidence suggests that the apparent affinity of p62 for protein aggregates (whether resulting from its binding to Ub or to other molecules therein) may act in concert with the oligomerization of p62 to favor the compaction of aggregated proteins into dense inclusions. Although this view may be intuitively appealing, it should be noted that at present there is no direct evidence for the presumed promoting effect of p62. Resolving this issue will require work on experimental models of inclusion formation, where the consequences of interfering with p62 function can be directly assayed.

It is also possible that the incorporation of p62 might have essentially no effect on the process of inclusion formation. Nevertheless, even if such “passive entrapment” might be a mere epiphenomenon for inclusion formation, it might interfere with the function of p62 as an adaptor in receptor-activated signaling and as a scaffold for aPKC. In polyglutamine expansion diseases, the depletion of vital signaling intermediates by sequestration into intranuclear inclusions appears to contribute to the pathogenesis (Nucifora et al. 2001, Chai et al. 2002, Qin et al. 2004). At least in a model of (intranuclear) polyglutamine aggregates, p62 was redistributed from its normal cytosolic locale to the inclusions (Donaldson et al. 2003). If cytoplasmic inclusions cause a similar depletion of p62, this might disrupt pro-survival signaling (*e.g.*, via NF-κB and ERK5), since p62 is thought to play an important role in mediating the responsivity to neurotrophic factors such as NGF, NT-3, NT-4/5, and BDNF (Samuels et al. 2001, Wooten et al. 2001, Mamidipudi et al. 2002, Geetha and Wooten 2003). On the other hand, it is unclear if the effect would be deleterious to the cell, since p62-deficient mice were reportedly born “grossly normal” without any overt anatomic abnormalities (Duran et al. 2004). However, p62 might figure more prominently in pathological conditions.

It has also been speculated that p62 could play a role in masking hydrophobic surfaces on aggregated proteins (Zatloukal et al. 2002), thereby preventing detrimental interactions with other cellular constituents. In this role, p62 would thus function in a chaperone-like manner. Moreover, p62 might act in a way that would promote the disaggregation of

inclusion components, as observed for molecular chaperones in experimental models (*e.g.*, Koyama and Goldman 1999, Cummings et al. 2001, Abu-Baker et al. 2003). Here, p62 might even aid in the targeting of aggregated proteins for proteolysis, since in the receptor-signaling context, the p62 ligand TRAF6 has a Ub ligase activity (Deng et al. 2000). Thus, the role of p62 could be analogous to that of heat shock protein 70, which recognizes misfolded proteins and enables their ubiquitination by the co-chaperone Chip-1, predisposing the tagged proteins for degradation (Meacham et al. 2001, Chavez Zobel et al. 2003).

The p62 protein might also be part of a more complex mechanism promoting aggregation. In view of its presence in diverse inclusions, the possible involvement of p62 in the aggresomal response is an intriguing possibility. Several aspects of the aggresomal mechanism are unclear (Garcia-Mata et al. 2002), pointing to the involvement of as yet unidentified factors. For example, it is poorly understood how abnormal proteins are recognized and taken up for delivery into the aggresome (Johnston et al. 2002). In view of its Ub-binding ability, p62 has been put forward to be a strong candidate for the binding of Ub-conjugated proteins to MTs and recruiting them to the pericentriolar area (Wilkinson 2000). However, such a function was recently identified for another protein, histone deacetylase 6 (HDAC6), which was shown to link polyubiquitinated misfolded proteins to dynein motors (Kawaguchi et al. 2003). On the other hand, HDAC6 only appeared to be crucial for the formation of polyubiquitin-enriched aggresomes, while neither the formation of aggresomes *in vitro* (Garcia-Mata et al. 1999, Johnston et al. 2000) nor the incorporation of p62 into inclusions seems to require polyubiquitin. Therefore, p62 might play an analogous role in the recognition of misfolded proteins in an Ub-independent manner.

Regardless of the specific function that p62 might serve in the aggresomal response, the involvement of p62 is suggested by the finding that it accumulates early and abundantly into MBs (Stumptner et al. 2002, Zatloukal et al. 2002) which display some aggresomal features (Riley et al. 2002, Riley et al. 2003a, Riley et al. 2003b). However, it should be noted that MBs differ from typical aggresomes by being irregularly shaped and often present as multiple bodies per cell (Denk et al. 2001). Although this disparity might be

due to the disruption of the MT array by the aggregating cytokeratins (Nakamichi et al. 2002), the relation of MBs to aggresomes remains to be defined.

In the case of neuropathological cytoplasmic inclusions, most of which also seem to incorporate p62 (Table 4), it is likewise unclear to what extent an aggresomal mechanism might contribute to their formation. The only type for which there is published evidence from human postmortem studies for aggresomal involvement is the LB (McNaught et al. 2002b) which, nevertheless, exhibits clearcut differences from aggresomes formed *in vitro* by diverse aggregation-prone proteins (pp. 91–92). Similarly, most types of tau-enriched inclusions display morphologies seemingly disparate with *in vitro* aggresomes. This is not surprising, since tau pathology is accompanied by a breakdown of the MT network, at least in AD (Iqbal et al. 1998), thus disrupting the MT-dependent transport necessary for aggresome formation. However, tau inclusions, as well as MBs, might represent an aggresomal response gone awry.

Our data suggested at least a morphological similarity between the formation of PaBs and aggresomes (**IV**). The recruitment of p62 upon the emergence of "aggregation centers" would thus agree with a possible role in aggresome formation. On the other hand, the presence of p62 also in the intranuclear MarBs (**IV**) would argue against a specific role in the aggresomal response, rather suggesting that whatever mechanistic role p62 has in inclusion formation, it is likely to affect protein aggregation whether in the cytosol or nucleoplasm.

While the above scenarios are based on the known structural and functional properties of p62, it is also possible that the incorporation of p62 into inclusions involves a major structural change in p62, resulting in its conversion to β-sheet-enriched fibrils as is the case with tau and αS. There is increasing evidence suggesting that the ability to form β-sheet-rich fibrils is a generic property of proteins (Dobson 2001), including globular ones mostly composed of α helices (Fändrich et al. 2001). The putative fibrils of p62 might represent a minor or major constituent of the aggregate, depending on the inclusion type. This possibility is intriguing in view of the partially filamentous ultrastructure of IHBs (MacDonald and Bedard 1990), where p62 is the only abundant constituent identified to date. Further, since structurally unrelated proteins such as tau and αS may synergistically

promote mutual fibrillization (Giasson et al. 2003), it is conceivable that misfolded, fibrillar proteins might also trigger fibril formation from p62.

Although there are various possible ways to explain the role of p62 in cytoplasmic inclusions, parsimony argues for the simplest explanations, especially in view of the current lack of supporting evidence for roles in mechanisms assuming more complex organization (*e.g.*, disaggregation or aggresomal responses). Thus, findings to date favor the p62-autonomous scenarios that require the minimum of novel interactions. The simplest explanation for the presence of p62 in diverse inclusions containing different pimary constituents, with or without Ub, is that the incorporation of p62 is due to binding to Ub or some other molecular determinant(s) associated with misfolded, aggregated proteins. Regardless of the identity of the target (*e.g.*, hydrophobic surfaces, oxidized residues, β-sheet conformation, chaperone proteins, or Ub-conjugated species), the binding of p62, combined with its propensity for oligomerization, would be expected to lead to strong, multivalent interaction of p62 arrays with the aggregated proteins. Some of the p62 thus incorporated might expose free binding surfaces for aberrant proteins, chaperones, or Ub, expediting the further entrapment and deposition of misfolded and/or ubiquitinated proteins in a glue-like manner. This process might be promoted by the upregulation of p62 due to proteasomal dysfunction and/or oxidative stress (study **I**). While in most types of inclusions studied, the incorporation of p62 seems to be precipitated by the primary constituent (*e.g.*, HP-tau, αS), in other types, p62 itself might constitute the initial and main component (*e.g.*, IHBs, Ub^- glial inclusions).

Taken together, the conceivable roles for p62 in cytoplasmic inclusions remain open to speculation. However, the simplest interpretations of the present findings favor the view that p62 has a structural propensity for oligomeric association with aggregates of misfolded and/or ubiquitinated proteins. Thereby, p62 likely enhances their deposition into compact masses in both cytosolic and nuclear compartments. Since mounting evidence suggests that inclusions serve as structures which sequester potentially noxious proteins, the coaggregation of p62 may promote the survival of inclusion-containing cells in tauopathies and synucleinopathies, as well as in other diseases involving protein aggregation.

7. CONCLUSIONS

This series of studies was carried out to identify a candidate protein (p62) possibly involved in neurodegenerative phenomena, and to characterize its involvement in disease-associated protein aggregation in the human brain.

The following conclusions can be drawn:

1) In neuronal culture, the expression levels of p62 mRNA and protein are prominently upregulated in response to pro-apoptotic conditions and proteasomal inhibition. At least in the former case, p62 upregulation might contribute to pro-survival signaling.

2) In human tauopathies (Alzheimer and Pick diseases) and synucleinopathies (Parkinson disease, dementia with Lewy bodies, and multiple system atrophy), the p62 protein is copiously present in the hallmark cytoplasmic inclusions and is mostly confined to perikaryal deposits.

3) In the tau pathology of Alzheimer disease, p62 is selectively incorporated into neurofibrillary tangles and is a relatively early constituent of these structures.

4) The spectrum of nigral αS pathology characteristic of Parkinson disease suggests a morphogenetic sequence in which punctate αS deposits, pale bodies, and Lewy bodies arise as successive stages of a complex aggregation process. The engagement of p62 seems to coincide with the formation of compact inclusions.

These findings are compatible with several alternative interpretations. However, combined with other relevant research, the present observations favor the view that the incorporation of p62 may enhance the deposition of aggregated proteins into compact inclusions. This presumed role of p62 encompasses both perikaryal tau- and αS-containing inclusions while appearing to be of a more general nature. In light of the mounting evidence that the sequestration of potentially noxious proteins into a discrete body may minimize their deleterious effects, p62 may thus serve to promote cell viability.

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APPENDIX: ORIGINAL PUBLICATIONS (I–IV)

I

Ubiquitin-binding protein p62 expression is induced during apoptosis and proteasomal inhibition in neuronal cells

Kuusisto E, Suuronen T, Salminen A

Biochemical and Biophysical Research Communications 2001, 280(1): 223–228.

II

Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies

Kuusisto E, Salminen A, Alafuzoff I

NeuroReport 2001, 12(10): 2085–2090.

III

Early accumulation of p62 in neurofibrillary tangles in Alzheimer's disease: possible role in tangle formation

Kuusisto E, Salminen A, Alafuzoff I

Neuropathology and Applied Neurobiology 2002, 28(3): 228–237.

IV

Morphogenesis of Lewy bodies:

dissimilar incorporation of α-synuclein, ubiquitin, and p62

Kuusisto E, Parkkinen L, Alafuzoff I

Journal of Neuropathology and Experimental Neurology 2003, 62(12): 1241–1253.

PUBLICATIONS
SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

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