Review

Aberrant signaling and senescence associated protein degradation

Gerardo Ferbeyre

Department of Biochemistry and Molecular Medicine, Université de Montréal, C.P. 6128, Succ. Centre-Ville, Montréal, Québec H3C 3J7, Canada

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ABSTRACT

Senescent cells accumulate with age and contribute to pathologies associated to old age. The senescent program can be induced by pro-cancer stimuli or is developmentally controlled. In cells forced to senesce by expression of oncogenes or short telomeres, aberrant activation of the ERK/MAP kinase signaling pathway leads to selective protein degradation by the ubiquitin proteasome system. The proteins affected by this process control key cellular processes known to be defective in senescent cells. We discuss the evidence supporting a general role for aberrant signaling and senescence associated protein degradation for organismal aging.

Contents

1. Introduction

Organisms progressively decay, wear out and die. However, the primary causes triggering these processes have been largely elusive. At the cellular level, cells can die or senesce and the mechanisms of these cellular fates can give insights into organismal aging. Cellular senescence can be greatly accelerated by oncogenic signaling which is different from normal signaling in strength and response to negative modulators. Oncogenic signaling is thus aberrant signaling. Oncogenic *ras*, for example, activates the ERK and AKT kinases leading to the phosphorylation of multiple proteins. Using proteomics analysis, we found that aberrant ERK signaling due to expression of oncogenic *ras* or short telomeres leads to degradation of specific proteins (Deschenes-Simard et al., 2013). Most of the degraded proteins contained phosphorylation motifs for proline directed kinases such as ERK1, ERK2, CDKs and GSKs. These

kinases are stimulated by oncogenic *ras* or short telomeres and provide a direct link between senescent stimuli and protein degradation. In addition, many unstable proteins in senescent cells were found to contain phosphorylation sites for basophilic kinases such as AKT and S6 kinase or acidophilic kinases such as casein kinases 1 and 2. These kinases could be stimulated as a consequence of signaling by Ras-activated kinases. Alternatively, ERK-dependent production of reactive oxygen species can enhance overall protein phosphorylation due to inhibition of protein phosphatases (Meng and Zhang, 2013). The proteins degraded in senescent cells play roles in cell cycle progression, ribosome biogenesis, cell migration, mitochondria and other functions known to be affected in these cells (Deschenes-Simard et al., 2014).

The stoichiometry of most phosphorylation events indicates that only a fraction of a particular protein is phosphorylated (Olsen et al., 2010). Therefore, during normal cell signaling the coupling between protein phosphorylation and degradation will only impact a fraction of any particular protein pool. However, during aberrant signaling, as in cells bearing constitutively active oncogenes, a larger fraction of some

E-mail address: g.ferbeyre@umontreal.ca.

particular proteins will be phosphorylated and subsequently degraded. We call this process Senescence Associated Protein Degradation or **SAPD** (Deschenes-Simard et al., 2013) and for some proteins it dominates over their biosynthesis, significantly reducing their overall levels. This mechanism is independent of cell division and could in principle explain cell dysfunctions for both dividing and non-dividing cells exhibiting aberrant signaling. However, currently the term cellular senescence applies only to dividing cells, although the process could be logically extended to non-dividing cells based on molecular instead of cellular phenotypes. Hence, non-dividing cells exhibiting dysfunctions due to the senescence associated secretory phenotype (SASP) (Oubaha et al., 2016) and SAPD could be classified as senescent.

Oncogenic mutations are not linked to normal aging but senescent cells accumulate with age in many organisms including humans (Childs et al., 2015; Tchkonia et al., 2013). On the other hand, cells could senesce in vivo in response to still unknown factors triggering aberrant signaling and SAPD (Fig. 1). The SAPD model of senescence includes: 1) a cause for aberrant signaling, 2) coupling protein modifications induced by aberrant signaling to protein degradation and 3) the cellular consequences of depletion of SAPD target proteins. Here, we review the evidence that links aging and the SAPD model.

2. Aberrant signaling during aging

Signaling includes a variety of protein modifications including phosphorylation, acetylation, methylation and sumoylation. These modifications may all contribute to aging but here we focus on protein phosphorylation which is the best understood. There is an extensive body of work on protein acetylation and aging, a pathway controlled by the protein deacetylases of the sirtuin family, which has been reviewed elsewhere (Haigis and Sinclair, 2010) and will not be included here.

2.1. Model organisms

Genetic analysis of aging in model organisms has lead to the identification of kinases that shorten life span. In *C. elegans* the insulin/IGF-1 signaling activates the PI3K/AKT cascade leading to phosphorylation and cytoplasmic sequestration of the forkhead transcription factor Daf-16 (Paradis and Ruvkun, 1998). This transcription factor is a key
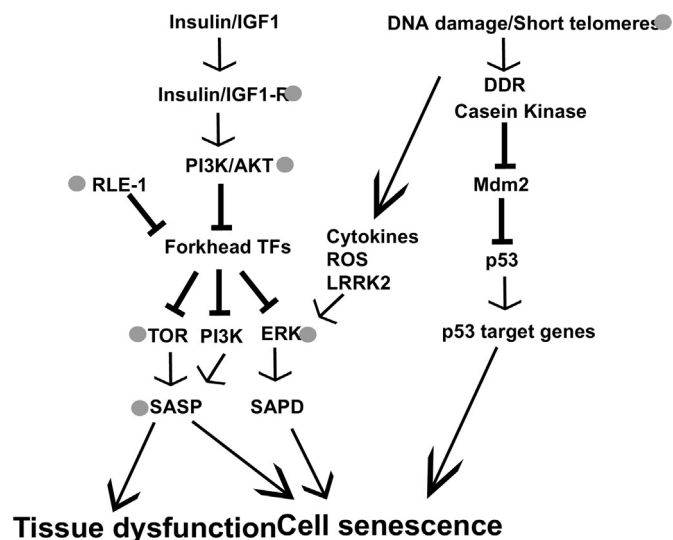
mediator of longevity in worms and it regulates the expression of genes that can increase resistance to a variety of cellular stresses (Lee et al., 2003). Intriguingly, Daf16 also acts as a transcriptional repressor of many protein kinase genes including those that inactivate its own function (Tazearslan et al., 2009). This striking attenuation of many kinase-signaling modules included the PI3 kinase pathway, the TOR pathway and the ERK/MAP kinase pathway (Tazearslan et al., 2009). Clearly, protein phosphorylation is linked to aging and its attenuation increase life span (Fig. 1).

Consistent with the work in worms, inhibitors of the ERK pathway (Slack et al., 2015) or mutations in the PI3K/AKT pathway (Yamamoto and Tatar, 2011) also prolonged life span in flies. Moreover, in agreement with the idea for a pro-longevity role for kinase attenuating mechanisms, fibroblasts obtained from long-lived mutant mice, including the Snell dwarf mice and the Growth Hormone Receptor mutant mice, displayed an attenuated ERK activation in response to oxidative stress (Sun et al., 2009).

Another protein kinase that has pro-aging functions is casein kinase 1 (Fig. 1). This kinase is controlled by the proteasome activator REGγ. Mice null for REGγ accumulate CK1, which then phosphorylates Mdm2, targeting it for proteasome-dependent degradation (Inuzuka et al., 2010). As a result, these mice experience an increase in p53 levels and activity and premature aging (Li et al., 2013). CK1 is activated by DNA damage and colocalize with p53 in PML bodies where it phosphorylates p53 at threonine 18 an event that prevents its interaction with Mdm2 (Alsheich-Bartok et al., 2008). Signs of CK1 activation in Alzheimer disease also suggest a role for this kinase in human aging (Flajolet et al., 2007; Hanger et al., 2007).

2.2. Human aging

Aberrant phosphorylation of the neuronal cytoskeleton by ERK kinases or decrease in phosphatases is associated to brain aging and human Alzheimer disease (Veeranna et al., 2004; Veeranna et al., 2011). MEK inhibitors prevent memory deficits in a mouse model of Alzheimer disease (Feld et al., 2014). The Raf-1 kinase, which acts upstream ERK1/2 in the MAPK pathway, is also increased in the brain of Alzheimer disease patients (Mei et al., 2006), and treatment with Raf kinase inhibitors protects cortical brain cells from β-amyloid toxicity (Echeverria et al., 2008). Raf inhibitors also reduced mutant huntingtin toxicity in a cell model of Huntington disease where RNAi-mediated inhibition of multiple members of the RAS-RAF-ERK pathway rescue cells and animal models form this toxicity (Miller et al., 2012). GSK3b is another kinase that can phosphorylate the cytoskeletal protein tau in neurons from patients with Alzheimer disease (Noble et al., 2005). This kinase increases with aging in the brain and accumulates in the nucleus of senescent fibroblasts (Zmijewski and Jope, 2004). The most common genetic mutation leading to Parkinson disease affects the protein kinase LRRK2, and most of its effects can be explained by activation of the ERK pathway (Fig. 1). Treatment with MEK inhibitors reversed most of the phenotypic effects of this mutation in neuron cultures (Bravo-San Pedro et al., 2013; Carballo-Carbajal et al., 2010; Reinhardt et al., 2013; White et al., 2007). As reported for Alzheimer disease, a reduction in protein phosphatase 2A activity can also underlie an aberrant protein phosphorylation state in Parkinson disease (Wu et al., 2012).

According to the SAPD model attenuation of kinase signaling should extend life-span by preventing the conversion of signaling pathways from a state of moderate signaling to a state of aberrant signaling leading to protein inactivation by SAPD (Fig. 1). Organisms need to use these signaling modules for cell proliferation and growth and they are in constant danger of passing the threshold for aberrant signaling, SAPD and senescence. A trade must be reached and longer life span can be achieved by attenuating signaling pathways. However, there is a fitness cost associated to lower activity that depends on the environmental pressures and the competition within species to pass genes to the next generation.

Fig. 1. Aberrant signaling underlies cell senescence and aging. Aberrant activation of signaling pathways due to triggers that remain to be discovered reduce cellular fitness in part by promoting protein degradation (SAPD) and reducing tissue homeostasis via the secretion of high levels of inflammatory cytokines (SASP). In cell culture and animal models, short telomeres, oncogenes and DNA damaging drugs can mimic the process. In animal models, mutations or compounds that reduce signaling through the PI3K, AKT, ERK and TOR kinases or prevent DNA damage signaling increase life span (gray circle).

2.3. Mechanism of aberrant kinase activation during aging

The reason for aberrant kinase activation in aging is not known. It is unlikely that mutations such as those found in oncogenic *ras* in tumors mediate aberrant kinase stimulation during aging. One possible explanation is the emergence of aberrant signaling by epigenetic mechanisms encoded in the dynamics of phosphorylation cascades. Protein kinases can inherently sustain a persistent activation if they autophosphorylate (Lisman, 1985; Song, 2013). This has been documented during T cell senescence where a complex of the MAP kinases ERK, p38MAPK and JNK with sestrins called sMACS (sestrin –MAPK activation complex) promote the activation of these MAPKs by autophosphorylation (Lanna et al., 2017). Calpain, a protease linked to Alzheimer disease, can generate a hyperactive form of GSK-3β after proteolytic cleavage of its C-terminus (Jin et al., 2015). Kinase signaling pathways can be locked into permanent activated states by triggering positive feedback mechanisms (Lisman and Fallon, 1999). For example, ERK can activate PKC via PLA2 and in turn, PKC can activate ERK via RAF-MEK (Lisman and Fallon, 1999; Xiong and Ferrell, 2003). Positive feedback mechanisms are ideal to mediate stable cellular states because the information is not lost when one component of the loop is degraded. Newly synthesized components are readily integrated into the mechanism by the other members of the loop. Phosphorylation-dependent feedback loops are modulated by protein phosphatases that set a threshold for their activation. Inactivation of phosphatases facilitates the establishment of kinase-based memory circuits (Sweatt, 2001) but also impair the process of erasing memories (Silva and Josselyn, 2002).

Repeated stimulation of cells with cytokines causes persistent ERK activation that depends on a positive feedback loop involving Sprouty 2. Sprouty 2 activates the tyrosine kinase Fyn, which is capable of activating ERK (Liu et al., 2010). This Sprouty-dependent activation of ERK was associated to accumulation of activated ERK in endosomes. Further identification and characterization of positive feedback modules will help discovering those driving aberrant kinase activation during aging and provide targets for pharmacological intervention. Senescent cells secrete a variety of pro-inflammatory modulators that can reinforce the senescence state in a cell autonomous manner and propagate the senescent phenotype to neighbouring cells (Acosta et al., 2013; Acosta et al., 2008a; Acosta et al., 2008b). These inflammatory cytokines act in part by inducing the generation of reactive oxygen species and stimulating signaling pathways including the ERK pathway (Fig. 1) (Acosta et al., 2008a).

It has been argued that the hyperfunctional signaling associated to senescence is the continuation of developmental programs that are not switched off (Blagosklonny, 2013). These programmes are optimized for embryonic development and growth but their persistence in the adult somehow leads to aging. In fact, cellular senescence is used as a mechanism to eliminate and replace certain embryonic structures during development (Davaapil et al., 2017; Munoz-Espin et al., 2013; Storer et al., 2013; Villiard et al., 2017) and to halt the expansion of potentially malignant cells (Braig et al., 2005; Collado et al., 2005; Serrano et al., 1997). In other words, senescence plays opposite roles in biology depending on the context. Genes do control senescence but there is no evidence that they were selected for their pro-aging functions. Aging, in this view, is a side effect of developmental programs ("a shadow of actual programs') (Blagosklonny, 2013) and/or the price of tumor suppression (Ferbeyre and Lowe, 2002).

3. Coupling protein modifications to protein degradation

In many situations, phosphorylation is coupled to protein degradation and in general phosphoproteins have a higher turnover. This coupling resets signaling modules and allows cells to respond to different stimuli or repeated stimulation (Cambridge et al., 2011). Various kinase motifs increase protein turnover. For example proteins having ERK-kinase motifs are less stable than proteins having AKT motifs (Cambridge et al., 2011). Also, multiple ubiquitin conjugating enzymes recognize only phosphorylated proteins to which they attach ubiquitin chains for proteasome-dependent degradation (Harper, 2002). Phosphorylation can also inactivate signaling pathways by controlling protein localization (Paradis and Ruvkun, 1998) or altering protein conformation (Atadja et al., 1994).

The degradation of phosphorylated proteins by the proteasome depends on E3 ubiquitin ligases that recognize phosphorylated proteins at specific motifs known as phosphodegrons. The ability of SAPD to deplete key proteins thus depends on the presence of these phosphodegrons in target proteins, E3 ligases that recognizes them and an aberrant signaling that changes the normal stoichiometry of phosphorylation labeling most of a specific protein pool for degradation. E3 ligases of the SCF family possess F-box proteins that bind directly to phosphorylated proteins. Several E3 ligases have been linked to cellular senescence. Smurf2, an E3 ligase in the TGFβ pathway, is activated by short telomeres and mediates cellular senescence (Zhang and Cohen, 2004). Smurf2 ubiquitinates Id1 and Id2, two repressors of p16INK4a-expression, explaining the widespread upregulation of p16INK4a in senescent cells (Kong et al., 2011). Smurf2 also targets the polycomb protein and epigenetic regulator EZH2 for degradation (Yu et al., 2013) a function that should also contribute to p16INK4a upregulation via erasing repressive modifications on the p16INK4a promoter chromatin (Bracken et al., 2007).

In nematodes, the E3 ubiquitin ligase RLE-1 reduces longevity by catalyzing the degradation of DAF16, a forkhead family transcription factor (Li et al., 2007). The deubiquitylase MATH-33/USP7 reverses the action of RLE-1 and promotes longevity (Heimbucher et al., 2015). Another E3 ligase component that promotes aging in nematodes is elongin c. RNAi-dependent downregulation of this gene increases life span by increasing the levels of its target HIF-1, a transcription factor with anti-aging activity (Hwang et al., 2015). There are many E3 ubiquitin ligases encoded in the mammalian genome and for the most part they remain poorly characterized. We anticipate that many more E3 ubiquitin ligases and phosphodegrons will be implicated in the regulation of senescence and aging.

4. Consequences of senescence-associated protein degradation

Studying protein turnover in several mouse models of extended longevity provided evidence linking protein degradation to aging. Longevity correlated with a reduction in protein turnover but not with a reduction in cell proliferation rates as predicted by the telomere hypothesis. The mechanisms underlying a reduction in protein turnover in long living models remain to be investigated but the authors suggested that they are likely the result in a reduction of protein damage or misfolding (Thompson et al., 2016). These authors discussed the apparent contradiction between their data and theories suggesting increased proteolytic editing of damaged proteins via autophagy in longevity models. They propose that the use of surrogate markers for autophagy rather than measuring the flux through the pathway may explain the discrepancies (Thompson et al., 2016).

Among conditions associated to aging, protein degradation has been well studied in muscle atrophy (Altun et al., 2010). Aged muscle cells contain higher levels of the ubiquitin ligase CHIP and MURF1 (Altun et al., 2010), which promote the ubiquitination and degradation of misfolded proteins. In situations leading to muscle atrophy, such as denervation and cachexia, a common transcriptional program is activated to induce the so called atrogenes (Zheng et al., 2010). Several components of the ubiquitin-proteasome system including the E3 ligases MURF-1 and atrogin are induced by activation of FOXO transcription factors (Zheng et al., 2010). Another important component of the protein degradation machinery during muscle atrophy is the p97/VCP/CDC48 ATPase, which binds multiple E3 ligases and catalyzes ATP-driven disassembly of protein complexes. This ATPase is thus important for extraction of ubiquitinated proteins from protein complexes in ER associated proteins, mitochondrial complexes, myofibrils and chromatin

(Piccirillo and Goldberg, 2012). Inhibition of p97 reduces muscle atrophy after fasting or denervation (Zheng et al., 2010) and its levels increase during aging related sarcopenia (Altun et al., 2010. The trigger of muscle atrophy during aging is unknown. However, an intriguing increase in phospho-ERK was observed in resting old muscles in comparison to young muscles (Williamson et al., 2003) and the authors suggested that old muscle are under constant stress signaling due to inflammatory cytokines such as TNF, which has been reported to increase with aging in muscles (Kirwan et al., 2001). This data from human muscles is consistent with previous reports in human senescent fibroblasts on high levels of phospho-ERK in the cytosol of these cells (Gaumont-Leclerc et al., 2004; Kim et al., 2003; Lim et al., 2000; Rahmouni et al., 2006).

A transgenic mouse reporter for the ubiquitin proteasome system revealed no significant alterations during aging (Cook et al., 2009). Intriguingly, in long living daf2 mutants of *C. elegans*, translation and proteasome components were reduced (Stout et al., 2013). Reduced protein degradation via a decrease in proteasome components allowed normal protein content in these mutants (Stout et al., 2013). These results are consistent with the SAPD model of aging. Finally, an increase in phosphorylation-dependent protein degradation may divert the proteasome from its function in the degradation of damaged proteins (Hernebring et al., 2006) further compromising the physiology of cells with aberrant signaling.

5. Concluding remarks

Aging is obviously associated with deterioration and loss of functions. Paradoxically it can be triggered at the cellular level by hyperfunctional pathways. We propose that the link between hyperfunction or aberrant signaling and the loss of function associated to aging is the degradation of aberrantly modified proteins. The SAPD model thus provides a molecular mechanism to link hyperfunction to the multiple defects associated to senescent cells. The SAPD model of aging can be tested in model organisms where longevity could be linked to downregulation of key protein kinases and/or E3 ubiquitin ligases mediating the process of phosphorylation-dependent protein degradation. Small molecules modulating this pathway could be used for anti-aging strategies. This class of anti-aging compounds will likely act in both senescent and pre-senescent cells. Since senescence plays roles in wound healing, tumor suppression and development, they should be carefully evaluated and administered in a way that they do not interfere with the positive functions of senescent cells.

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