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Protein isoprenylation in biology and disease: general overview and perspectives from studies with genetically engineered animals

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1. ABSTRACT

The posttranslational modification of proteins by lipids is a key mechanism in the regulation of protein localization and function. Isoprenylation is a process critical for the membrane association of a plethora of signalling proteins with fundamental roles in cell biology, including G proteins and nuclear lamins. Isoprenylation is irreversible but is frequently associated to reversible posttranslational modifications, such as palmitoylation or phosphorylation, that act like switches that modulate the dynamics of protein-membrane or protein-protein interactions and target the modified proteins to specific membrane compartments. The severe phenotype of animals deficient in the enzymes involved in isoprenylation and postprenylation processing highlights the significance of these processes. Moreover, alterations in the genes involved in the maturation of isoprenylated proteins have been found at the basis of some severe human diseases, like choroideremia or the premature ageing progeria syndromes. Given their critical role in the transformation potential of the Ras oncogenes, isoprenylation and postprenylation processing are targets for the development of inhibitors with antitumoral activity. The recent generation of animal models genetically engineered to target the enzymes involved in isoprenylation and associated modifications has unveiled unpredicted aspects of these modifications. Moreover, these models are proving of crucial importance for the elucidation of the mechanisms of disease, and the identification and validation of therapeutic targets. This review attempts to summarize general aspects of the posttranslational modification of proteins by isoprenylation, paying special attention to the evidences obtained from the use of genetically engineered animals and the avenues that these models open.

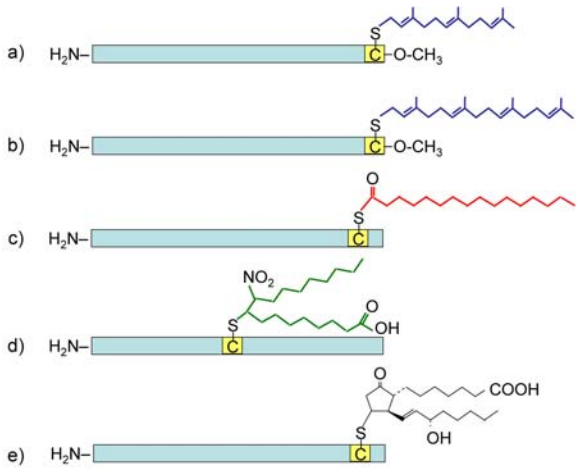

Figure 1. Scheme of various lipid modifications of proteins showing: a) farnesylation; b) geranylgeranylation; c) palmitoylation; d) addition of a nitrated fatty acid; e) addition of a cyclopentenone prostaglandin.

2. INTRODUCTION

Proteins are the direct effectors of most cellular functions. The regulation of protein function occurs at multiple levels, from the regulation of the amounts present in cells to subtle conformational changes. The physicochemical properties of proteins are tightly regulated through mechanisms among which posttranslational protein modification plays an important role. Proteins can be posttranslationally modified through the addition of a wide array of chemical species, including phosphate groups, methyl groups, glutathione, small proteins like ubiquitin, carbohydrates and lipid moieties. Proteins can be modified by various lipidic species including isoprenoids, fatty acids, like myristic or palmitic acids, glycosyl phosphatidylinositol anchors, cholesterol and electrophilic lipids. Modification of proteins by lipids can have multiple effects on protein structure and function. One of the most common consequences of the incorporation of lipid moieties is an increase in the hydrophobicity of the protein that facilitates its interaction with hydrophobic environments like cellular membranes or certain protein domains (1, 2). Lipid moieties, *per se* or in combination with other structural features, such as polybasic sequences, can determine the localization of proteins in precise subcellular structures (3). In addition, the reversibility of the lipidic modification is directly related to the dynamics of protein localization and function (4, 5). Lipid modification plays also an important role in protein-protein interactions, which, in addition to influencing subcellular localization, are critical for cell signalling (6-8). Besides its role in cellular physiology, the modification of protein by lipids has important implications in pathophysiology. Defects in enzymes catalyzing these processes are involved in several human pathologies (9, 10). In addition, the requirement for lipidic modifications of certain oncogenes makes the enzymes responsible attractive targets for the development of inhibitors with therapeutic potential (11, 12). Moreover, some widely used drugs, such as the hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors known as statins, which inhibit the synthesis of cholesterol, exert beneficial effects on cardiovascular function that are mediated in part by the impairment of the posttranslational modification of certain proteins by isoprenoid intermediates of the cholesterol biosynthetic pathway (13, 14). On the other hand, non-enzymatic addition of lipid moieties to proteins can contribute to the onset or progression of several pathophysiological processes involving protein oxidation, aggregation or accumulation, such as chronic inflammation and some neurodegenerative diseases (15, 16). In recent years, important progress has been made in the knowledge of the mechanisms and consequences of protein modification by lipids. New enzymes catalyzing the formation of the lipid-protein bonds have been identified. In addition, several experimental models based on the use of genetically engineered animals have been obtained that unveil unpredicted aspects of the modification of proteins by lipids. This is therefore, a thriving research field that is uncovering important information on fundamental biological processes and is yielding contributions important for the validation or discovery of therapeutic targets. In this review we will comment on recently identified aspects of the modification of protein by lipid moieties, devoting special attention to the use of genetically modified animal models in the unravelling of the complex implications of protein isoprenylation.

3. ISOPRENYLATION: GENERAL ASPECTS

Protein isoprenylation is a lipidic posttranslational modification which consists in the addition of an isoprenoid lipid to a cysteine residue located near the carboxyl terminal end of the protein. This is an enzymatic modification that involves the formation of a thioether linkage between the sulphur atom of the cysteine and the isoprenoid intermediate (Figure 1). In some cases, isoprenylation is followed by further processing of the protein involving proteolysis and carboxyl methylation, as it will be discussed below. The discovery of protein isoprenylation in mammalian cells was due in part to the study of inhibitors of cholesterol biosynthesis. In particular, the fungal metabolites called statins were the subject of intense study due to their potent activity as competitive inhibitors of the enzyme HMG-CoA reductase, which catalyzes the synthesis of mevalonate, the precursor of cholesterol and of isoprenoid lipids (17) (Figure 2). Soon it was found that statins exerted effects on cell proliferation and differentiation that could be prevented by supplementing cells with mevalonate but not with the known end-products of the isoprenoid pathway, such as cholesterol, dolichol or ubiquinone (18). The study of the fate of mevalonate led to the observation that products of this pathway were stably incorporated into proteins (19, 20). Since then, work from several laboratories accomplished the identification of the isoprenoid moieties involved in the posttranslational modification of proteins. The identification of the first substrates for isoprenylation in mammalian cells, which turned out to be proteins

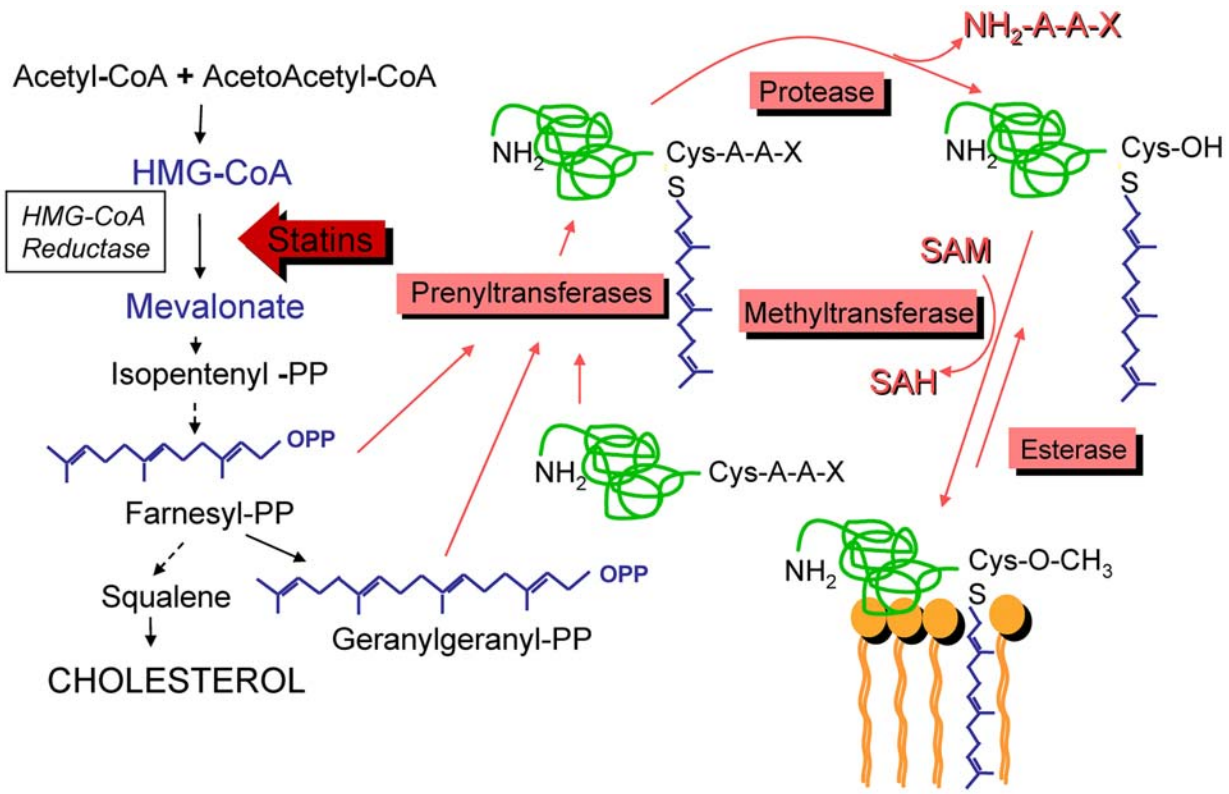

Figure 2. Schematic view of the isoprenoid biosynthetic pathway and its connection with protein isoprenylation. Isoprenoids synthesized from mevalonate are incorporated into proteins. The site of action of statins is shown. Following isoprenylation, the prenylated protein protease removes the three amino acids distal to the isoprenylated cysteine, and this residue is methylated by a specific methyltransferase. Methylation is reversible due to esterase activity. This group of modifications increases the hydrophobicity of the protein and facilitates its interaction with membranes. SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine.

important for cell proliferation, signal transduction and oncogenesis (20-22), spurred the research in this field uncovering key aspects of cell biology and human pathophysiology.

The isoprenoids attached to proteins can be the 15 carbon farnesyl or the 20 carbon isoprenoid geranylgeranyl (23) (Fig.1). Isoprenylation occurs shortly after translation and it is irreversible during the lifetime of the protein. Protein isoprenylation is a key modification involved in the correct subcellular localization of proteins and affects their activity and protein-protein interactions (24). Currently, several hundred cellular proteins are known to be isoprenylated, including key cell signalling proteins like the products of the *Ras* oncogenes H-, N-, and K-Ras (25), the proteins of the large Rho and Rab families of GTPases (26), the nuclear lamins (20, 27), proteins involved in phototransduction such as retinal cGMP phosphodiesterase and rhodopsin kinase (28) and the gamma subunits of heterotrimeric G proteins (29, 30), among others. Since the first studies reported it became clear that isoprenylation had important functional implications for the target proteins. Mutants of [Val12]K-ras 4B proteins that were not isoprenylated failed to associate with membranes and did not transform NIH-3T3 cells (21). Isoprenylation is also required for the association of lamin B with the nuclear membrane (31) and it is critical for the membrane association of heterotrimeric G proteins (32). Isoprenylation was also found to be required for full activity of rhodopsin kinase (28). A series of works established the importance of isoprenylation for the membrane localization of a growing number of members of the Ras superfamily of low molecular weight GTPases. The identification of GEFs (guanine nucleotide exchange factors) and GDIs (guanine nucleotide dissociation inhibitors) added more complexity to this picture by showing that these proteins could extract isoprenylated proteins from membranes and act as carriers in the cytosol, thus uncovering the importance of isoprenylation in protein-protein interactions and expanding the possibilities of the regulation of protein subcellular localization by isoprenylation (33). The molecular basis for this interaction has been elucidated. The crystal structures of several Rho GTPases in complex with their respective GDIs are available and demonstrate the key role of the isoprenoid moiety in the interaction between the C-terminal end of the Rho protein and a hydrophobic pocket in the GDI (2, 34). Recent works have shown that RhoGDIs play an active role in targeting Rho proteins to specific subcellular membranes, and therefore influence specific signaling

pathways (35). With respect to the Ras GTPases, several proteins that bind the C-terminal isoprenylated motif, including calmodulin (36) and galectins (6) have been proposed to play a role homologous to that of RhoGDI in the distribution of Ras proteins in specific compartments. In recent years isoprenylation has been shown to play unexpected roles in protein function and homeostasis. For instance, isoprenylation of RhoB has been shown to be required for degradation of this protein (37), through mechanisms probably dependent on protein association with specific organelles. In terms of membrane association there are important differences between both isoprenyl moieties. Geranylgeranyl moieties are more hydrophobic than farnesyl molecules, and the latter may be more dependent on additional modifications for stable membrane binding (38).

4. PROTEIN PRENYLTRANSFERASES

There are specific enzymes that catalyze the farnesylation (farnesyltransferase, FTase) or the geranylgeranylation (geranylgeranyltransferase, GGTase I and II) of proteins (39, 40). These enzymes use either farnesyl or geranylgeranyl pyrophosphate (FPP and GGPP, respectively), which are isoprenoid intermediates derived from the cholesterol biosynthetic pathway (Figure 2), as substrates. With respect to the protein substrates, FTase and GGTase I recognize proteins that possess a CAAX motif at their C-terminal end, where C is the cysteine residue that becomes prenylated, A, is an aliphatic amino acid and X is any other residue. Interestingly, the nature of the X residue determines whether a protein will be farnesylated or geranylgeranylated. Proteins ending in serine, methionine, alanine or glutamine become farnesylated, while proteins ending in leucine are geranylgeranylated. There are a few exceptions of proteins that can alternatively suffer either type of modification. This alternative prenylation can occur under situations of limited isoprenoid availability or in the presence of farnesyltransferase inhibitors (FTIs). For instance, K-Ras can be geranylgeranylated in cells treated with FTIs (41). This has important implications for the potential therapeutic applications of these inhibitors (42). The low molecular weight GTPase RhoB can naturally occur in cells in both farnesylated and geranylgeranylated forms, with the latter being more abundant (43). Several lines of evidence indicate that RhoB can play different roles depending on the attached isoprenoid (44, 45). GGTase II transfers one or two molecules of geranylgeranyl to low molecular weight GTPases of the Rab family. This is because the C terminal sequence of Rab proteins may contain CC, CAC, CCX or CCXX motifs, among others. Isoprenylation by GGTase II requires the participation of the Rab escort protein (REP) (46), an auxiliary protein involved in the recognition of Rab by GGTase II. However, several pathways for Rab prenylation have been proposed which differ in the sequence of events leading to substrate recognition and interaction with GGTase II and REP (40).

Prenyltransferases are heterodimeric enzymes. The CAAX prenyltransferases, FTase and GGTase I, are zinc metalloenzymes that share a common alpha subunit and possess a homologous beta subunit (47, 48). The beta subunit shows a barrel structure, the center of which forms a hydrophobic cavity in which the active site is located. The isoprenoid binds along one side of this cavity, with the diphosphate moiety binding in a positive charged cleft. The farnesyl moiety of FPP and the first three isoprene units of GGPP bind in an extended conformation, while the fourth isoprene unit of GGPP forms a 90º angle with the rest of the isoprenoid molecule. The binding sites for FPP in FTase and for GGPP in GGTase I are very similar, although it appears that bulky residues (tryptophan and tyrosine residues) located at the bottom of the cavity in FTase contribute to the limitation of the length of the isoprenoids that this enzyme can accommodate for catalysis (39). In fact, substitution of tryptophan102beta in FTase by threonine, changes the substrate preference of this enzyme from FPP to GGPP (49). Structural differences also determine the peptide specificity of these enzymes. The X-residue binding pocket of FTase is more polar and accepts a variety of residues (methionine, serine and glutamine, but also alanine, threonine and cysteine), while that of GGTase I possesses a more hydrophobic character and accepts leucine and phenylalanine. Importantly, FTase possesses a secondary binding site for X residues that may accommodate phenylalanine, leucine, asparagine or histidine. These structural features define rules for substrate peptide selectivity and help explain why some functional overlap exists between these two prenyltransferases (50). GGTase II shows considerable homology with the CAAX prenyltransferases, above all in the prenyl binding site. Importantly, the crystal structure of REP in complex with GGTase II has been solved providing a hypothesis for the working mechanism, according to which, the affinity of this interaction is regulated by the isoprenoid (51).

4.1. Genetic disruption of prenyltransferases

The crucial importance of prenyltransferases has been evidenced in numerous biological systems. Disruption of the genes encoding these enzymes leads to severe defects or lethality. In yeast, the genes encoding FTase alpha and beta subunits are RAM2 and 1, respectively. It is interesting to note that while RAM2 is essential for yeast, RAM1 mutants are viable, although they display growth defects (52). This may be due to the fact that RAM2 disruption affects both FTase and GGTase I. In contrast, RAM1 is essential for the human fungal pathogen Cryptococcus neoformans (53). The beta subunit of FTase has been shown to be required for development in Arabidopsis (54). The disruption of the genes encoding the prenyltransferases has been also envisaged as a potential therapeutic approach. Since the first studies in yeast it was noted that disruption of FTase altered the membrane association of Ras proteins (55). Given the importance of Ras proteins for cell proliferation and the high incidence of mutations in Ras oncogenes in human tumors, the genetic or pharmacological suppression of Ras isoprenylation was soon envisaged as an approach to investigating, and possibly controlling, Ras-mediated malignant transformations (56). Therefore, this posttranslational modification became an important target for drug discovery, in particular for the search of inhibitors of prenyltransferase with anticancer potential. In fact, several

Table 1. Some of the available models of genetically-engineered animals for the study of lipidic modifications of proteins

Disrupted gene	Function	Reference
Ftase (beta)	Farnesylation of CAAX proteins	57
GGTase II (gunmetal mouse)	Geranylgeranylation of Rab proteins	10
REP-1 (conditional)	Geranylgeranylation of Rab proteins	61
Rce1	Proteolysis of the –AAX C-terminal sequences of isoprenylated proteins	105
Rce1 (heart)	Proteolysis of the –AAX C-terminal sequences of isoprenylated proteins	115
Icmt	Methylation of the C-terminal cysteine of isoprenylated proteins after CAAX cleavage	106
ZMPSTE24	Internal cleavage of farnesylated Lamin A	116-117
PPT1	Lysosomal hydrolysis of protein palmitoyl thioester	148

FTIs are currently under clinical trials and some of them have proven clinical efficacy alone or in combination with other agents (42). However, the clinical efficacy of FTIs has been lower than expected and their mechanism of action appears to be more complex than initially thought. An important study by Mijimolle *et al.*, describing the generation of knockout mice for the beta subunit of FTase (57) (Table 1), has helped in the elucidation of the requirements for protein farnesylation in mammalian cells, and of the validity of FTase as a drug target in several models of carcinogenesis. Knockout of the FTase resulted in early embryonic lethality. FTase null embryos could not be obtained due to decreased proliferation and massive apoptosis. Interestingly, the ablation of FTase in postnatal mouse tissues did not lead to obvious histopathological alterations. Some differences were found in 6 month-old mice, which showed slightly decreased wound healing and spleen size, and maturation defects in erythroid cells. Most importantly, there were no differences in the susceptibility of adult mice to develop tumors when subjected to a protocol of skin carcinogenesis if FTase expression was ablated before the onset of the tumorigenic procedure, although a partial protection was observed when FTase was eliminated during tumor progression. Intriguingly, a substantial proportion of cellular H-Ras was associated with the membrane fraction both in papillomas from FTase-deficient animals and in FTase null cells. These results call into question the idea that farnesylation is essential for Ras membrane targeting. Many studies on the importance of farnesylation for Ras membrane association have been carried out using mutants of the CAAX cysteine (21, 25). However, the substitution of the cysteine residue at the CAAX motif could have consequences other than to block prenylation, since it could hamper membrane association by other mechanisms. Studies with FTase null cells and wild type Ras proteins will probably be necessary in order to unveil the complex determinants that may control Ras membrane association.

Alterations in the geranylgeranylation of some Rab proteins by GGTase II are associated with pathological processes. Mutations in the GGTase II gene cause a disease in mice (10, 58) that is reminiscent of the alterations in the Hermansky-Pudlak syndrome, characterized by disorders of pigmentation, prolonged bleeding, and ceroid deposition, often accompanied by severe fibrotic lung disease and colitis (59). These mice, known as *gunmetal*, present a decrease in the levels and activity of GGTase II. On the other hand, mutations affecting the protein REP-1, which is the product of the choroideremia gene (60), lead to retinal degeneration associated with a selective defect in the prenylation of some Rab proteins, particularly Rab27a (10, 58). Recently, a conditional mouse model of choroideremia has been developed that shows degeneration of photoreceptors and pigment epithelium and defects in various Rab proteins (61). This model could help in the study of the pathogenesis of this disease and in the assessment of therapeutic approaches.

4.2. Farnesyltransferase inhibitors as antiparasitic agents

Although FTIs were originally developed with the aim of blocking oncogenic transformation by inhibiting Ras processing in malignant cells, recent studies indicate that these agents may be useful tools in the fight against parasitic diseases (reviewed in (62, 63)). Parasitic diseases like malaria, caused by *Plasmodium falciparum*, or African sleeping sickness, caused by *Trypanosoma brucei*, affect hundreds of million people in certain world regions and increasing efforts are being devoted to finding new therapeutic targets. Protein farnesylation occurs in many pathogenic protozoa and appears to be essential for the survival of the parasites. Recent years have witnessed important advances in the knowledge on the protein farnesyltransferase enzymes from several eukaryotic pathogens and the nature of the isoprenylated substrates. The information acquired during clinical trials of FTIs in cancer studies is proving extremely valuable for the potential use of these compounds against parasitic diseases. Importantly, FTIs appear to be relatively non-toxic in short-term administration and display greater toxicity against parasite cultures than mammalian cells (63). The focus in this field is now the development of compounds with suitable pharmacokinetic properties that may represent effective chemotherapeutic agents.

4.3. Unexpected benefits of inhibiting isoprenoid biosynthesis: the statins

Hypercholesterolemia is at the basis of atherosclerosis and associated cardiovascular pathologies, including thrombosis and infarction. These are among the most frequent causes of death and morbidity in developed countries. For this reason, great efforts have been devoted to develop therapeutic strategies to reduce cholesterol levels. Although intended to ameliorate hypercholesterolemia, statins have shown beneficial effects in the clinic and in experimental models that occur through mechanisms independent from the reduction in cholesterol levels and are related to the ability of these drugs to reduce the pool of isoprenoid lipids involved in protein modification (64, 65). This may result in a partial inhibition of the isoprenylation of certain proteins (66). Research in this field has led to the identification of Rho proteins as important targets for the beneficial effects of statins on cardiovascular function (67). The inhibition of Rho proteins isoprenylation and function has been claimed to be

responsible for the protective effects of statins on the expression of endothelial nitric oxide synthase (eNOS) against down-regulation induced by hypoxia (68) or by exposure to oxidized LDL (64), or against cerebral-injury during experimental ischemic stroke in mice. (69). Statins have also been shown to inhibit the expression of vascular mitogens (13, 64) and to attenuate vascular smooth muscle cells proliferation (70). These and other mechanisms lead to a reduction in arterial pressure, antioxidant and antiinflammatory effects (71). In addition, a role for statins in the potential prevention of osteoporosis or Alzheimer has been proposed (72). The ability of statins to induce apoptosis in tumoral cell lines has been known for several years (73, 74). Recently, this property has received a renewed interest. The antitumoral potential of statins is being extensively studied in various models of tumorigenesis and clinical studies are under way to assess the usefulness of statins in combination with anticancer agents (75, 76).

4.4. Inhibition of protein isoprenylation as an unexpected mechanism of action of effective therapeutic agents: the bisphosphonates

Bisphosphonates are effective and widely used therapeutic agents for diseases associated with increased bone resorption, such as osteoporosis, Paget's disease and bone metastasis (77). Bisphosphonates are synthetic analogues of inorganic pyrophosphate (78). They consist of two phosphonate groups linked by non-hydrolysable phosphoester bonds to a central carbon atom. The early observation that inorganic pyrophosphate could prevent calcification by binding to newly forming crystals of hydroxyapatite led to attempts to inhibit ectopic calcification in the body by injection of polyphosphates and analogous compounds such as the bisphosphonates (79). Since then, bisphosphonates were shown not only to prevent calcification but to inhibit the dissolution of hydroxyapatite crystals and to block osteoclast-mediated bone resorption (77). Therefore, bisphosphonates became a key therapeutic tool for diseases associated with increased osteoclast activity. Bisphosphonates bind Ca^{2+} ions, and consequently, to bone mineral surfaces. This targeting contributes to their cellular selectivity *in vivo*. Further research into the remarkable effects of these compounds led to the identification of several potential mechanisms of action. While simple bisphosphonates are metabolically incorporated into non-hydrolysable analogues of ATP and inhibit ATP-dependent enzymes, causing osteoclast apoptosis, the more potent, nitrogen-containing bisphosphonates inhibit enzymes of the mevalonate pathway, in particular, farnesyl pyrophosphate synthase (80). The mode of action of these inhibitors has been recently clarified by the crystallization of bisphosphonate-bound human farnesyl pyrophosphate synthase (81). The crystal structure has shown that these compounds bind in the geranyl pyrophosphate site of the enzyme, thus competing for the binding of the substrate. The result is an inhibition of isoprenoid biosynthesis and hence, of protein isoprenylation. In fact, accumulation of unprenylated forms of several GTPases of the Ras superfamily has been demonstrated in osteoclasts. This results in an alteration of G protein-mediated signalling that most likely alters osteoclast functions essential for bone resorption (78, 82).

5. POSTPRENYLATION PROCESSING

As outlined above, isoprenylation is, in many cases, the first of a series of modifications the result of which is an increase in the hydrophobicity of the proteins that facilitates their localization in the proximity of membranes or in hydrophobic environments. The CAAX-containing proteins and some, but not all, Rab proteins (83), undergo postprenylation processing. After prenylation, a specific protease, called prenylated protein protease, FACE-2 or Rce1 cleaves the three amino acids distal to the isoprenylated cysteine residue (Figure 2). It is interesting to note that prior prenylation is required for substrate recognition by the protease. In fact, isoprenylated CAAX tetrapeptides, like N-acetyl-S-all-trans-farnesyl-cysteine-valine-isoleucine-serine are efficiently cleaved releasing the N-acetyl-S-farnesyl-cysteine moiety (84), and isoprenylated dipeptides or tripeptides can be used as substrates or inhibitors of the enzyme under in vitro conditions (85). After proteolysis, the newly exposed cysteine residue is methylated at its carboxyl group. This reaction is catalyzed by an enzyme that recognizes the isoprenylated cysteine, called isoprenylated protein methyltransferase or Icmt. Importantly, molecules as small as N-acetyl-S-farnesyl-cysteine are excellent substrates for this enzyme and competitive inhibitors of the methylation of endogenous protein substrates (86, 87). It should be noted that the same enzyme methylates all isoprenylated proteins, therefore this step does not distinguish between farnesylated versus geranylgeranylated substrates (88). Noteworthy, methylation of isoprenylated proteins and small molecule substrates is a reversible process (89), and as such, of potential regulatory significance in response to rapid signal transduction events. A methylation/demethylation cycle was early proposed for the isoprenylated nuclear lamin B in association with phosphorylation/dephosphorylation and assembly/disassembly of the nuclear lamina (90, 91). More recently, an increase in the methylation of several G proteins, including Ras, has been described in association with the activation of signal transduction pathways (92). However, information on the enzyme(s) responsible for isoprenylated protein methyl esterase activity is still scarce (93, 94), and its role in methylation/demethylation cycles with consequences for signal transduction has not been characterized.

In recent years it is becoming clear that, in addition to membrane targeting, postprenylation processing fulfils multiple roles, including protein-protein interactions and protein stability, and that these modifications are crucial for the proper function of isoprenylated proteins, including those involved in oncogenesis (12). This, together with the fact that both Rce1 and Icmt are the products of single genes in vertebrates (12), has made these enzymes important targets for therapeutic intervention and the subject of intense research both through genetic and biochemical approaches. As outlined above, several small-molecule inhibitors of these enzymes have been known for some time (85, 86, 95, 96), that alter G protein-mediated signalling, although their mechanism of action may involve in some cases the interference with isoprenylation-dependent protein-protein interactions (6, 97, 98). Nowadays, there is a renewed interest in the study of Rce1 and Icmt inhibitors as potential anti-cancer agents (12, 99).

5.1. Genetic disruption of Rce1 and Icmt

Since the earlier studies, findings from the yeast experimental system have been extremely helpful in the delineation of the complex process known as isoprenylation. Studies on the yeast a mating factor led to the identification of an attached lipid moiety that was required for function and turned out to be a farnesyl group. This provided the first clues for the definition of the CAAX motif and the search for CAAX containing putatively isoprenylated proteins. Studies performed in yeast have been also crucial for the identification of enzymes involved in postprenylation processing. The mammalian enzymes responsible for proteolysis and carboxyl methylation, both integral membrane proteins elusive to purification, were cloned (100, 101) based on their homology to their better known yeast homologues (102, 103). This has allowed the kinetic characterization of the recombinant Icmt enzyme that will be helpful in the development of inhibitors of potential use in therapeutic applications (104).

Disruption of the genes encoding Rce1 (105) or Icmt (106) causes lethality in mice, which supports the importance of these enzymes for cellular processes. Rce1-deficient mice die in late gestation or soon after birth. Icmt deficiency causes a more severe phenotype: knockout embryos die at embryonic day 10.5 and lethality is associated with extensive apoptosis and severe anemia. These alterations have been related to liver agenesis, thus indicating that this enzyme is essential for several steps of liver development (107). Importantly, cellular models (embryonic fibroblasts or embryonic stem cells) of Rce1 or Icmt deficiency have been developed (105, 108) that have allowed studying the role of these processing enzymes in the location and function of some of their targets. For instance, it has been shown that targeted inactivation of Icmt causes mislocalization of K-Ras proteins in mammalian cells and disrupts K-Ras interaction with microtubules (108, 109). Recently, mice with a conditional Rce1 allele have been generated that have allowed assessing the role of Rce1 in Ras-mediated cell transformation in cellular models (110). In this system, excision of Rce1 resulted in slower growth and suppression of Ras-induced transformation. In this context, inhibitors of Rce1 have been envisaged as potential blockers of K-Ras activity, and thus, as antitumoral agents. The advantage of these inhibitors would be to block the processing of all the protein substrates, therefore, circumventing the problem of alternative prenylation that can arise with the use of FTIs. With this view, studies have been carried out to explore the consequences of Rce1 inhibition in adult animals. Fetal liver cells from Rce1 deficient animals have been shown to support hematopoiesis when transplanted into irradiated animals (111). This suggested that pharmacological inhibition of Rce1 would not exert severe effects on hematopoiesis. However, recipients of Rce1-/- transplants showed modest proliferation of mature myeloid elements, which raised the possibility that Rce1 inhibition could have an adverse effect stimulating the growth of myeloid malignancies. Recently, an elegant study (112) has addressed the effect of inhibiting Rce1 in a mouse model of myeloproliferative disease induced by activation of a latent oncogenic K-Ras allele in hematopoietic cells. Unexpectedly, simultaneous inactivation of Rce1 and activation of K-Ras using *Cre/loxP* recombination techniques accelerated the myeloproliferative disease and led to earlier death. The mechanism for this apparently paradoxical effect remains to be elucidated. However, this observation suggests that a broad inhibition of postprenylation processing may alter the balance between various prenylated proteins needed for the homeostasis of hematopoietic elements. In the case of lamin B, postprenylation processing has been reported to be required for protein-protein interactions responsible for nuclear integrity and organization of the nuclear lamina (113). The results from various studies show that the consequences of blocking prostprenylation processing are very different depending on the target proteins. For instance, while proteolysis and methylation are essential for proper localization and function of farnesylated Ras proteins, Rho proteins and CAAX-containing Rab proteins, which are geranylgeranylated, are not affected (83, 114). Therefore, some of the severe effects observed in mice with targeted disruption of prostprenylation processing enzymes appear to be due to the lack of processing of some specific substrates. Recently, the *Cre/loxP* recombination technique has been used to generate mice lacking Rce1 in the heart (115). These mice were apparently healthy at birth but started dying at 3 to 5 months of age due to dilated cardiomyopathy. A detailed study of the phenotype of these animal models and of the mechanisms underlying the severe effects observed will shed more light on the functions of isoprenylated proteins.

5.2. The particular case of lamin A

The nuclear lamina is a lattice that lines the inside of the nuclear membrane providing structural support and fulfilling also a role in the regulation of gene expression. It is formed by the proteins called lamins, A, B and C, of which, A and B possess CAAX sequences at their C-termini and are subjected to prenylation. Lamin A presents unique characteristics: after prenylation and typical postprenylation processing it suffers a second proteolytic cleavage that removes the C-terminal fifteen amino acids giving rise to the mature lamin A protein, which is not farnesylated. Therefore, in this protein, isoprenylation directs the removal of the whole modified C-terminal segment. The function of this process has not been completely elucidated but it has been proposed that prenylation serves a nuclear membrane-targeting purpose. The internal proteolytic cleavage is catalyzed by a metalloprotease called FACE-1 or ZMPSTE24. The disruption of this gene caused a most interesting phenotype in mice, consisting of defective lamin A processing and muscular and adipocyte alterations (116). These alterations are similar to the phenotype observed in individuals with laminopathies, which are life-threatening diseases. Therefore, this mouse model was proposed to constitute an adequate experimental system for the study of the mechanisms of these diseases and the potential therapeutic approaches (116, 117). Interestingly, lamin A processing is defective in the progeria syndromes, characterized by premature ageing. In the case of the Hutchinson-Gilford progeria syndrome, lack of lamin A processing is due to a mutation in the lamin A gene that activates a cryptic splice

site leading to a mutant form of the protein containing an internal deletion of 50 amino acids comprising the site of cleavage by ZMPSTE24. This causes the accumulation of the mutant protein, as a gain-of-function lamin A isoform, in its farnesylated form. Nuclei from cells bearing this mutation present multiple defects that resemble the alterations observed in ZMPSTE 24 null cells (see (118) for review). It has been proposed that accumulation of farnesylated lamin A could play a pathogenic role in these alterations, since the presence of this mutant lamin A protein leads to the accumulation of wild type lamin A at the nuclear periphery (119). Moreover, inhibition of the cryptic splice site that leads to the mutant form of lamin A reverses the nuclear alterations in cells from Hutchinson-Gilford progeria syndrome patients (119). Most interestingly, it appears that inhibiting isoprenylation by FTIs (120) or lowering the levels of prelamin A (121) ameliorates the symptoms of ZMPSTE24 deficiency in cellular or animal models of progeria. Analysis of the transcriptional alterations related to ZMPSTE24 deficiency in mice has revealed the activation of a stress signalling pathway characterized by a marked upregulation of p53 target genes, a senescent cellular phenotype, and accelerated ageing of the whole animal (122). Morevoer, the alterations caused by ZMPSTE24 deficiency are partially reversed in ZMPSTE-/-p53-/- mice, thus suggesting that p53 hyperactivation may contribute to accelerated ageing (122). Interestingly, it has recently been shown that cell nuclei from old individuals show some characteristics similar to those of patients with the Hutchinson-Gilford progeria syndrome. This appears to be due to the sporadic use in healthy individuals of the cryptic splice site that is activated in the disease (123), thus suggesting the involvement of lamin A processing defects in physiological ageing. Therefore, in this field, the results from animal models are teaching important lessons not only about laminopathies and progeria syndromes but also about cancer and normal ageing processes (124).

6. "THE SECOND SIGNALS"

Isoprenylated proteins often bear a "second signal" that mediates and/or modulates their binding to membranes and their interactions with other proteins. This was first demonstrated for Ras proteins (3). N- and H-Ras proteins possess one and two cysteine residues, respectively, upstream of the isoprenylation site. Reversible attachment of palmitate provides an additional membrane anchoring mechanism as well as a means for the dynamic and differential regulation of the membrane binding of Ras proteins. In turn, K-Ras possesses a polybasic sequence in its C-terminal domain that stabilizes its interaction with membranes. In addition, a number of oxidative modifications, or addition of electrophilic species, have been described that could act as "second signals" in the regulation of isoprenylated protein localization and/or function.

6.1. Palmitoylation

Protein palmitoylation consists in the posttranslational addition of palmitic acid to the sulphur atom of a cysteine residue in the protein through a thioesther linkage (see Figure 1). In fact, several fatty acids can be attached to cysteine residues through this type of linkage. For this reason, although palmitoylation is the most common denomination, this modification can be described with the wider term thioacylation. This modification has been known for a long time but, given the fact that it can occur broadly spontaneously, it was thought to be non-enzymatic. Studies in yeast have greatly contributed to our knowledge about this modification. Genetic screens in yeast allowed the identification of several proteins with S-palmitoyltransferase activity. The first proteins identified, two palmitoyltransferases with activity towards Ras proteins (Erf2) and casein kinase (Yck2), respectively (125, 126), contain a sequence known as DHHC (where D is aspartate, H, histidine and C cysteine). This motif appears to play a direct role in the transfer reaction. This has triggered the search for putative, DHHC-containing mammalian protein acyltransferases. The human homologue of Erf2, DHHC9, in a complex with the protein GCP16, shows palmitoyltransferase activity toward H-Ras and N-Ras (127). Another DHHC protein, DHHC17, also known as HIP14 has shown palmitoyltransferase activity towards farnesylated Ras peptides. The number of palmitoyltransferases identified is growing rapidly (128). DHHC palmitoyltransferases act on intracellular substrates and are different from the enzymes acting on morphogens of the Wnt and Hedgehog pathways (129) that lack the DHHC sequence.

In general, palmitoylation occurs at cysteine residues close to the amino or carboxyl terminal ends of the proteins. A consensus sequence for palmitoylation has been proposed recently, which is present in some palmitoylated proteins (130). When the palmitoylated residue is the N-terminal cysteine, a rearrangement can take place giving rise to an amide linkage, and this type of modification is called N-palmitoylation. Often, palmitoylation occurs in combination with other lipidic modifications such as farnesylation or myristoylation. In fact, it is believed that prior lipidation favours the interaction of proteins with the membrane compartments where the palmitoyltransferases are located. Among the proteins known to be palmitoylated are the endothelial and inducible nitric oxide synthases (131, 132), many transmembrane proteins, like the voltage-dependent K channel (130) and G protein coupled receptors (133, 134), the scaffold protein caveolin-1 (135) and the estrogen receptor alpha (136), among many others (see (137) for a recent review). The knowledge about this modification is also benefiting from advances in proteomic research. Recently, a "biotin-switch" method for the selective detection of palmitoylated proteins has been reported (138) that has allowed undertaking a global analysis of protein palmitoylation in yeast (139). With respect to isoprenylated proteins, most of those known to be palmitoylated belong to the family of G proteins. In the low molecular weight GTPase superfamily, proteins such as H-Ras, N-Ras, RhoB, Rap2 or TC10 have been shown to be palmitoylated, although the precise function of this modification has not been elucidated in all cases. Interestingly, a member of the Rho family of GTPases has been found to possess a CAAX-like motif which is not subjected to prenylation but palmitoylation (140). The

heterotrimeric G proteins, except transducin, are also palmitoylated (141), although in this case, the palmitate is on the alpha subunit while the isoprenoid moiety is attached to the beta-gamma subunit. However, in this case, palmitoylation could also be considered a "second signal" since it can influence both Galpha-Gbeta-gamma interactions and the binding of the heterotrimer to membranes.

Palmitoylation plays a key role in the differential trafficking, localization and function of the mammalian H-, N- and K-Ras proteins. These proteins are highly homologous but differ mainly in their hypervariable domain, which is located close to the C-terminal end of the protein. H-Ras possesses two palmitoylable cysteine residues, C181 and C184 in human H-Ras, close to the isoprenylated residue, C186, and N-Ras can be palmitoylated at cysteine 181. In contrast, K-Ras does not possess cysteine residues in the hypervariable domain but a string of basic residues that contribute to stabilize its interaction with the plasma membrane. Work from many laboratories has elucidated the importance of these differential modifications. H- and N-Ras traffic from the ER to the plasma membrane through the classic secretory pathway, and this process requires palmitoylation, while K-Ras reaches the plasma membrane by other mechanisms (142, 143). Depalmitoylation of N- or H-Ras allows these proteins to undergo non-vesicular exchange with the Golgi and ER membranes (144). Repalmitoylation in Golgi redirects Ras to the plasma membrane via the secretory pathway (4). Therefore, a palmitoylation/depalmitoylation cycle regulates the localization and activity of H- and N-Ras. Furthermore, different kinetics of depalmitoylation/repalmitoylation may contribute to the Ras protein homologue-specific signal transduction events (4).

An interesting case for the importance of the second signals is provided by the Rho GTPases. RhoA and RhoB are two closely related members of this family. RhoA is involved in cytoskeletal regulation and control of gene expression, while RhoB has been involved in the regulation of endocytosis and in the induction of apoptosis. RhoA and B are 92% identical and differ in the C terminal sequence (145). While RhoA presents a polybasic sequence, RhoB possesses two cysteine residues upstream of the CAAX motif that can be palmitoylated. Interestingly, this divergence is responsible for the differential localization of RhoA and B in cells and also for the differential interaction with GDIs. RhoA interacts with RhoGDIalpha, an abundant cytosolic protein that in quiescent cells maintains most of RhoA protein in the cytosol. In contrast, RhoB interacts with a specific RhoGDI (RhoGDI3), which is a membrane protein (146). Consistent with this, RhoB is most frequently found in membrane compartments of cells. The importance of palmitoylation in this differential interaction was demonstrated by showing that insertion of a palmitoylation site into RhoA blocked RhoGDIalpha binding (147).

As stated above, S-palmitoylation is a reversible modification, its dynamics being extremely important for interaction of the modified proteins with specific membrane compartments and for signal transduction. Palmitate turnover can be regulated, thus providing potential mechanisms for the modulation of protein location and activity.

The removal of the palmitate moiety is carried out by thioesterases. Acylprotein thioesterase-1 removes palmitate from H-Ras and eNOS, among other substrates, and it is therefore involved in dynamic regulation of palmitoylation. On the other hand, the degradation of the palmitoylated proteins requires the removal of the palmitate moiety. This process is carried out by the lysosomal hydrolase known as protein palmitoylthioesterase-1 (PPT1). The importance of this step is underlined by the fact that mutations in this enzyme cause a fatal inherited neurodegenerative disease known as infantile neuronal ceroid lipofucsinosis (9). Patients with this disease develop cortical and retinal atrophy from the second year of life, leading to severe neurological alterations and death before puberty. Ultrastructurally, the disease is characterized by the accumulation of autofluorescent storage material in brain, part of which is lipidated. Genetically engineered PPT1 knockout or mutant mice are available that reproduce the symptoms of the human disease and that will hopefully help unveiling the molecular events leading to neuronal death (148). Due to the recent identification of palmitoyltransferases animal models for the study of this modification are still scarce. In a study searching for single nucleotide polymorphisms associated with susceptibility to schizophrenia Mukai and co-workers found a region related to the expression of a putative protein acyltransferase (149). Studies with mice knockout for this protein suggest a connection between protein palmitoylation in neurons and psychiatric phenotypes. Coming years will probably bring important developments in this exciting field.

6.2. The "electrostatic switch" on K-Ras

As outlined above, K-Ras relies on farnesylation of the C-terminal CAAX motif plus the presence of a polybasic sequence formed by several lysine residues present in the hypervariable carboxyl terminal domain for stable interaction with membranes. A recent work has shown that PKC can phosphorylate a serine residue located within the polybasic sequence of K-Ras and that this promotes its dissociation from the plasma membrane and association with intracellular membranes, including the mitochondrial membrane, where it can elicit cell death (150). This mechanism can have a negative effect on the growth of tumors bearing an activated K-Ras.

6.3. Non enzymatic modifications

In addition to enzymatic lipid modifications, proteins can suffer various posttranslational modifications through non enzymatic mechanisms, including oxidation of protein residues, thiolation and covalent binding of lipid moieties. These modifications may also influence localization and/or function of isoprenylated proteins. For instance, Ras proteins have been shown to be modified by nitric oxide (NO) (151) and glutathione (152). The interaction with NO or superoxide anion can result in the activation of Ras proteins through a mechanism involving

the release of GDP (153). In addition, NO donors have been also reported to increase the rate of palmitate turnover on H-Ras (154). Moreover, oxidation of H-Ras cysteine residues, including the sites for palmitoylation has been detected both *in vitro* and *in vivo*, although the consequences of these modifications have not been fully elucidated (152). Isoprenylated proteins can also be the target for the addition of electrophilic lipids. These reactive species arise as a result of the action of reactive oxygen or nitrogen species directly on the unsaturated fatty acids present in cell membranes or on the products of enzymatic pathways such as those catalyzed by lipoxygenases, cyclooxygenases or prostaglandin synthases (155, 156). The chemical nature of electrophilic lipids can be very diverse and it ranges from small reactive aldehydes, such as HNE or acrolein, to the complex oxidized phospholipids found in oxidized lipoproteins. The nature of the interaction of these lipids with proteins is also diverse. Some electrophilic lipids bearing alpha,beta-unsaturated carbonyl groups can form Michael adducts with nucleophilic residues in proteins such as cysteine, lysine or hystidine residues. These attachments can either be irreversible or suffer retro-Michael reactions in cells, probably depending on the structure of the lipidic species. In addition, reactive aldehydes can form Shiff bases with lysine residues (15, 157).

Ras proteins have been shown to be the target for the addition of electrophilic lipids of the cyclopentenone prostanoid class. The cyclopentenone prostaglandin 15-deoxy-Delta^{12,14}-PGJ_2 (15d-PGJ_2) targets H-Ras proteins selectively at cysteine 184 in human H-Ras, which is two residues upstream from the isoprenylated cysteine and it is normally one of the sites of palmitoylation (158). This modification has been shown to correlate with increased Ras activity, increased proliferation of murine fibroblasts and higher resistance to the induction of apoptosis by carcinogens in a model of skin tumorigenesis (159). Although the precise mechanism for H-Ras activation upon treatment with cyclopentenone prostaglandins remains to be elucidated, these results raise the possibility that palmitoylable cysteines in H-Ras or other proteins may be non-enzymatically modified by electrophilic lipids, thus interfering with protein palmitoylation and possibly altering Ras localization and/or function. Interestingly, other proteins like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported to be modified by covalent addition of nitro-fatty acids. In this case, an effect of lipid addition on protein function and membrane association has been reported (160). Therefore, membrane association mediated by non enzymatic addition of electrophilic lipid moieties could be an important mechanism in signal transduction, the importance of which should be elucidated in future studies.

The use of genetically engineered animals in the elucidation of the effects of oxidative modifications or of electrophilic lipid action has been limited. The non-enzymatic nature of these modifications obviously precludes direct approaches such as those targeting a precise enzyme responsible for the modification. Nevertheless, indirect approaches have been employed which target the enzymes involved in the generation of precursors of reactive lipids, like cyclooxygenases, lipooxygenases or prostaglandin synthases. For instance, PGD_2, synthesized from PGH_2 by PGD_2 synthases, is the precursor of cyclopentenone prostaglandins of the J series. Mice deficient in the lipocalin- or hematopoietic-type PGD_2 synthase have been obtained (161, 162) that could help in the delineation of the importance of these compounds as second signals. Many lipid electrophiles are detoxified through their enzymatic conjugation with glutathione, catalyzed by glutathione-S-transferase (GST) enzymes. GSH conjugates are eliminated from the cell by the action of the multidrug transporters. Therefore, genetic deletion or alteration of these systems would lead to an accumulation of reactive species in the cell. Knockout mice for various GST isoforms are available (163-165), as well as genetically-engineered animal models for the study of multidrug transporters (166-169). These could prove valuable tools to study the effects of the disrupted metabolism of electrophilic lipids. In fact, knockout mice for GSTA4-4 have been reported to be more susceptible to liver damage induced by oxidative stress and lipid peroxidation (170). In addition MEFs from alkenal/one oxidoreductase-deficient mice have been used to prove the role of this enzyme in the catabolism of 15d-PGJ_2 and its consequences on the biological effects of this prostanoid (171).

7. PERSPECTIVES

Modification of proteins by isoprenylation plays critical roles in multiple cellular processes. Disruption of the genes coding for the enzymes that catalyze these modifications in mice, results in severe phenotypes and often lethality. Recently, conditional or tissue-specific knockouts of some of these genes have been generated. These animal models are unveiling unpredicted aspects of the modification of proteins by lipids that are of great importance for the understanding of the mechanisms of disease and for the identification and/or validation of therapeutic targets. Moreover, novel enzymes are being identified that participate in the attachment of "second signals", i.e. palmitate, to isoprenylated proteins. Development of animal models targeting these enzymes will grant important advances in this field. On the other hand, understanding the mechanisms and implications of the non-enzymatic modification of proteins and its interaction or interference with enzymatic modifications will probably unveil novel possibilities for the modulation of the localization and function of isoprenylated proteins.

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Abbreviations: HMG-CoA: hydroxyl-methyl-glutaryl-coenzyme A; GEFs: guanine nucleotide exchange factors;

GDIs: guanine nucleotide dissociation inhibitors; FTase: farnesyltransferase; GGTase: geranylgeranyltransferase; FTI: farnesyltransferase inhibitor; REP: Rab escort protein; eNOS: endothelial nitric oxide synthase; LDL: low density lipoprotein; ER: endoplasmic reticulum; PPT1: protein palmitoylthioesterase-1; PKC: protein kinase C; NO: nitric oxide; HNE: 4-hydroxy-2-nonenal; 15d-PGJ_2: 15-deoxy-$Delta^{12,14}$-prostaglandin J_2; GST: glutathione-S-transferase; GSH: glutathione.

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