REVIEW ARTICLE

PROTEIN PRENYLATION — ITS GENERAL SIGNIFICANCE AND PARTICULAR RELEVANCE TO ENDOCRINOLOGY AND BONE METABOLISM

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The cholesterol synthetic pathway gives rise to farnesyl and geranylgeranyl, which are incorporated into cytosolic proteins, the prenylated proteins. The processes involved in prenylation and the functions of the prenylated proteins, including Ras, Rab, Rho and Rac, are reviewed. The use of prenylation inhibitors in anti-cancer therapy and the role of prenylation in endocrinology and bone and mineral metabolism are briefly discussed. The potential effect of the hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors on prenylated proteins and in particular the potential beneficial effect on bone is highlighted.

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The past two decades have witnessed a tremendous interest in hypercholesterolaemia as a risk factor for atherosclerosis. This interest led to the development of the hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, drugs which have been shown to be extremely effective in reducing serum cholesterol and the consequent deleterious effects of hypercholesterolaemia. The HMG-CoA reductase inhibitors were also found to be powerful tools to manipulate and investigate cholesterol biosynthesis and led to further insights into this important pathway. The reduction of cholesterol levels with HMG-CoA reductase inhibitors is widely viewed as relating only to the prevention of atherosclerosis, but it is now evident that cholesterol metabolism impinges on many aspects of molecular cell biology which in turn profoundly affect many, if not all, organ systems.

This article briefly highlights aspects of cholesterol and mevalonate metabolism and protein prenylation in particular.

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The general importance of prenylation as well as its relevance to endocrinology and bone mineral metabolism is briefly reviewed. Numerous reviews and articles have been published, to which the reader is referred for further information.[1-5]

THE CHOLESTEROL SYNTHETIC PATHWAY

It is well known that cholesterol and other sterol derivatives such as steroid hormones, bile salts and vitamin D are important products of mevalonate metabolism (Fig. 1). There are, however, less well known products of this pathway that have important physiological roles: dolichol in glycoprotein biosynthesis; the side-chain of ubiquinone, an important component of the mitochondrial electron transport chain; isopentanyl adenosine, a component of isopentanyl transfer-RNA; the farnesyl side-chain of haem a, the iron-binding nucleus of haemoglobin; and the important recently discovered prenylated proteins. The discovery of the prenylated proteins has provided many new insights into cellular biology and opened up new and novel therapeutic possibilities.

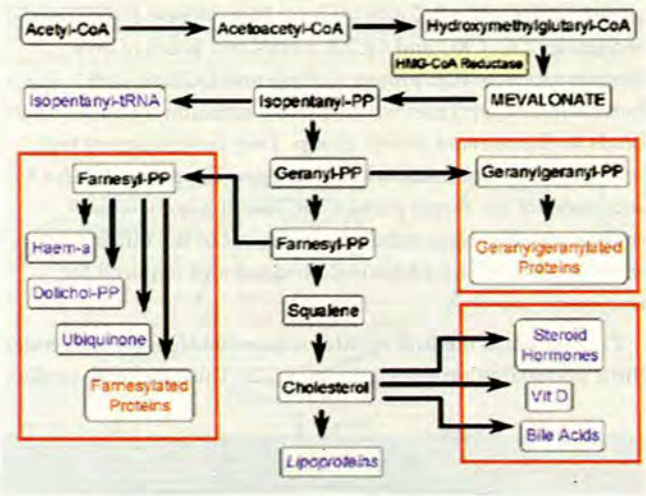

Fig. 1. The mevalonate/cholesterol metabolic pathway. Important products of this pathway include the prenylated proteins — the farnesylated and geranylgeranylated proteins to which farnesyl-PP and geranylgeranyl-PP have been added.

It is known that the inhibition of mevalonate synthesis by the HMG-CoA reductase inhibitors and the subsequent depletion of the endogenous mevalonate pool results in a cessation of cell cycling and DNA synthesis with pronounced changes in cell morphology and even suppression of tumour growth.[6] These changes could be restored by supplying exogenous mevalonate to the arrested cells or by removing the inhibitor. This restoration of cell growth and morphology could not be reproduced by adding cholesterol, dolichol, ubiquinone or isopentanyl adenosine, suggesting that some other metabolite of mevalonate was responsible for these changes. Subsequently it was demonstrated that when radiolabelled mevalonate was

added to the medium, radioactivity was incorporated into a wide range of cytosolic and membrane-bound proteins. This occurred via the covalent attachment of the isoprene products of mevalonate, farnesyl and geranylgeranyl to these proteins, a process thereafter referred to as prenylation, while the modified proteins are called prenylated proteins.[1]

THE PRENYLATION OF PROTEINS

The proteins destined to be prenylated are characterised by a carboxy-terminal CAAX box of amino acids where C represents cysteine, A an aliphatic amino acid and X any amino acid. These terminal amino acid motifs, and in some cases certain additional upstream sequences, act as recognition sites for prenyl transferase enzymes.[7,5] The prenyl transferase attaches the respective prenyl group, farnesyl or geranylgeraniol, to a carboxy-terminal cysteine of the protein. At least three prenyl transferases are known to exist and have been characterised. Farnesyl transferase (FTase) and geranylgeranyl transferase I (GGTase I) recognise a CAAX box and the terminal X of the CAAX box determines whether farnesyl or geranylgeranyl is added to the protein. Geranylgeranyl transferase II (GGTase II) recognises CC, CXC and CCXX motifs and is active on a distinct group of Rab proteins.[5] Ftase and GGTase I are heterodimeric enzymes which share a common α-subunit that binds to the relevant prenyl group. They have different but homologous β-subunits, which recognise the different CAAX sequences of the target protein. GGTase II is somewhat different and has two subunits analogous to the other transferases with an additional third subunit required for enzymatic activity.

Prenylation is the first of three sequential steps which render these prenylated proteins active (Fig. 2). This primarily confers membrane binding to the prenylated protein. Prenylation is followed by the proteolytic cleavage of the terminal 3 amino acids by a microsomal carboxypeptidase, which is then followed by the addition of a methyl group to the remaining terminal cysteine by a microsomal aminotransferase. Some prenylated proteins undergo further modification by the addition of a palmitoyl molecule to a more proximal cysteine.[8]

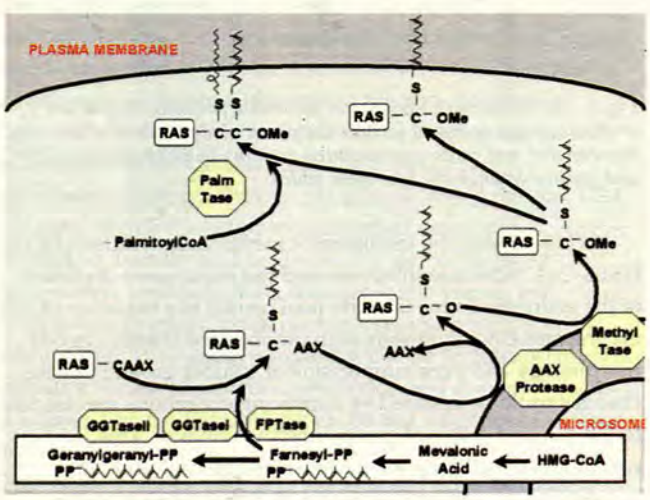

Fig. 2. The post-translational processing of the prenylated proteins. Farnesyl or geranylgernyl are added by one of three prenyl transferases followed by removal of the three terminal amino acids and the addition of a methyl and palmitoyl molecule.

In all cases prenylation is essential for the activity of all these proteins. If the terminal CAAX box is removed or blocked, if the relevant prenyl transferase is inhibited, or if the availability of the prenyl substrate is diminished, as is found with the addition of HMG-Co A reductase inhibitors, then these proteins are inactive.[1,9] The additional modifications of amino acid cleavage and methylation are also required, and sometimes essential, but mostly serve to complement prenylation in the activation of these proteins.[5] Although the bulk of the prenylated proteins are cytosolic in location, they are active only in their membrane-bound form and both prenylation and palmitoylation render these proteins lipid-soluble, thus allowing them to bind to membranes. In addition to their role in membrane binding these post-translational modifications are also important for interactions with other regulatory proteins of the small GTP-binding proteins.[10]

THE PRENYLATED PROTEINS

The prenylated proteins have diverse functions and include the nuclear lamins, the γ-subunit of the heterotrimeric G proteins, various retinal proteins and by far the largest group, the family of Ras-related small GTP-binding proteins that play an essential role in the normal function of cells.[10]

THE SMALL GTP-BINDING PROTEINS

The small GTP-binding proteins comprise a large super-family of Ras-related proteins, of which the Ras, Rab, Rho, Rac, and Rap families are prenylated. These proteins act as molecular switches and cycle between the active GTP-bound to the inactive GDP-bound form (Fig. 3). This switching is further modulated by their interaction with a large group of regulatory proteins, and this interaction of the small GTP-binding proteins with their regulatory proteins is further influenced by their prenylation state.[11,12]

The Ras family

The Ras family of small GTP-binding proteins act as important components of the cell's signal transduction pathways between receptors and the cell nucleus and other effectors, leading to, among other things, cell growth, cell differentiation and various metabolic processes (Fig. 4).

Unlike the other members of the small GTP-binding family of proteins, which are geranylgeranylated, the Ras proteins are

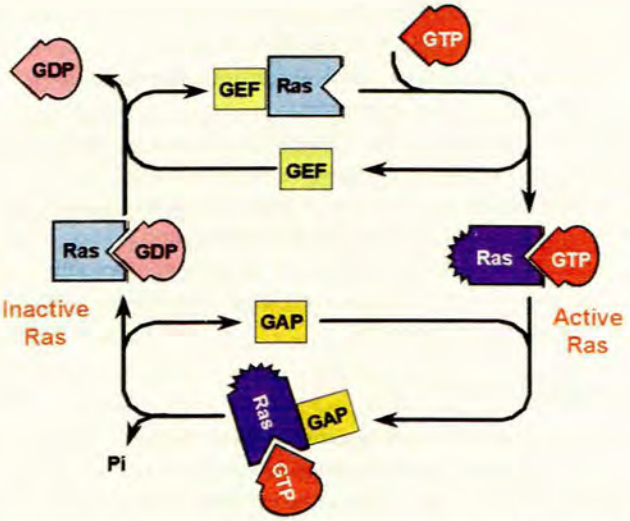

Fig. 3. The Ras-related GTP-binding proteins as molecular switches. This scheme applies to the small Ras-related GTP-binding proteins as well as the heterotrimeric receptor-associated G proteins. These proteins are only active in their GTP-bound membrane-associated form, which is modulated by many regulatory proteins. Active GTP-bound Ras has an intrinsic GTPase activity that is further enhanced by GTPase activating proteins (GAP) resulting in GDP-bound inactive Ras. Various proteins also regulate the subsequent exchange of GDP for GTP. GTP exchange factors (GEF) enhance the dissociation of GDP and exchange for GTP, thus enhancing the activation of Ras. GDP dissociation inhibitors (GDI) inhibit the exchange of GDP for GTP and therefore inhibit the activation of Ras. GDI also covers the prenylated site on Ras making it less lipid-soluble and unbinding it from the membrane with the result that the inactive GDP-bound Ras is cytosolic in position. With the removal of GDI, the prenylation site is uncovered and GDP is exchanged for GTP and the active GTP-bound Ras becomes membrane bound at its active site. Defects in this switching mechanism give rise to disease. Some mutations of Ras lack intrinsic GTPase activity and are consequently continuously active, a situation seen in numerous common cancers.

farnesylated. Without prenylation these Ras proteins are inactive and cannot perform their function.[9] Certain mutant oncogenic forms of Ras that lack intrinsic GTPase activity are consequently unable to switch to the inactive GDP-bound form and lead to the formation of a variety of human cancers.[13] When the prenylation of these oncogenic Ras forms is prevented, e.g. with the use of HMG-CoA reductase inhibitors, they lose their oncogenic capacity.[14]

The Rab family

The Rab family is for the most part involved in the regulation of intracellular vesicular transport, exocytosis, endocytosis and targeting of vesicles between different organelles (Fig. 5).[15] It is therefore to be expected that the Rab proteins will play an important role in all cells, but in particular those involved with secretion of products. The isoprenylation of these proteins is critical for their association with specific intracellular compartments and regulation of vesicular transport processes. Prenylation also plays an important role by modulating the

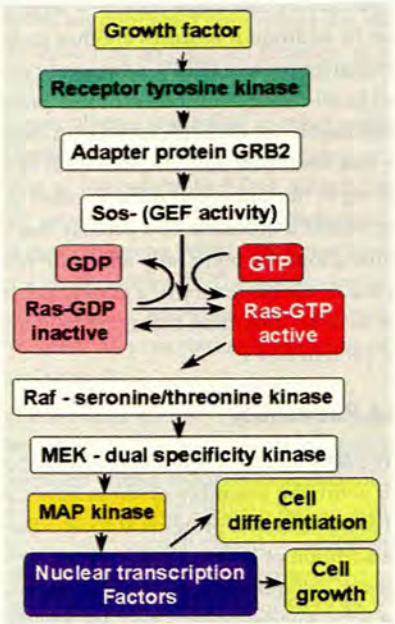

Fig. 4. Ras in signal transduction. Ras is a pivotal link between tyrosine kinase receptors and the activation of nuclear transcription factors leading to among others, cell differentiation and growth. It is via this pathway that constitutionally active forms of Ras result in cancer. Without prenylation Ras cannot participate in this pathway.

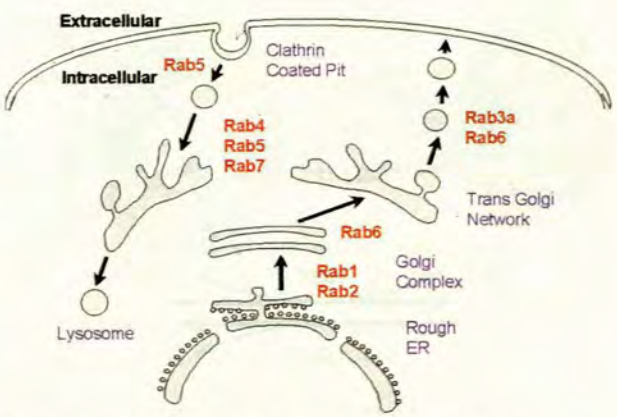

Fig. 5. The Rab proteins, members of the small GTP-binding proteins, play an important role in endocytosis, exocytosis and trafficking of vesicles between different compartments. This is crucial for the function of the endocrine pancreas and other endocrine organs.

interaction between Rab and their regulatory proteins that switch them on and off and regulate their membrane binding. GDP dissociation inhibitor (GDI) is one such regulatory protein, which regulates the GDP and GTP binding of Rab and helps to shuttle Rab between donor and acceptor membranes.

The Rab family is geranylgeranylated by GGTase II, which is somewhat different from the other prenyl transferases in that it recognises carboxy terminal sequences other than the CAAX

and raises the possibility that there may be a large family of this transferase. In addition it requires another protein for activity, Rab exchange protein (REP), which is homologous to GDI and found in all cells.[16] A mutation of this protein was found to be responsible for choroideraemia, an inherited X-linked disease that results in slow degeneration of the retina ultimately leading to blindness but with no other systemic features.[17] Why would a mutation of a protein that is widespread throughout the body only affect the retina? A further search led to the discovery of a closely related protein which is active in cells other than the retina, now named REP2, and the retinal protein REP1.[5]

The Rho and Rac family

The Rho family plays a central role in cytoskeletal organisation of polymerised actin, the assembly of stress fibres, and recruitment of focal adhesion proteins at focal adhesions (Fig. 6).[18,19] The addition of lovastatin to cell cultures results in marked changes in cell morphology, which correlate with the disassembly of actin microfilaments and are reversed by the addition of mevalonate.

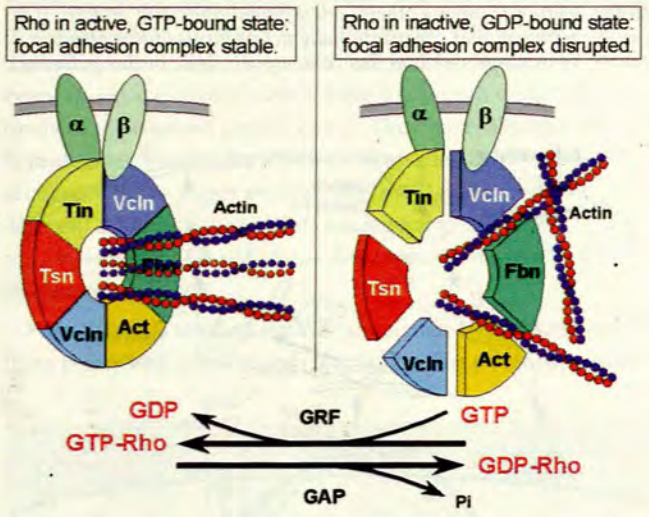

Fig. 6. Rho members of the small GTP-binding proteins play a pivotal role in the cytoskeleton via focal adhesion complex and stress fibre assembly. This explains the morphological changes observed when HMG-CoA reductase inhibitors are added to cell cultures and which can be reversed by the addition of mevalonate.

The Rac family is involved with actin filament organisation which results in the formation of lamellipodia and membrane ruffling induced by growth factors. This process can be inhibited by the micro-injection of inactive Rac mutants and prenylation inhibitors including HMG-CoA reductase inhibitors.[18,19] Rac also has an influence on the assembly of stress fibres, indicating a communication with Rho and Ras. Rac plays an essential role in the NADPH oxidase system of phagocytic leucocytes (neutrophils, macrophages, and eosinophils) which is dependent on prenylation and can also be prevented by inhibitors of prenylation.[20]

The Rho proteins act as efficient substrates for the *Clostridium botulinum* C3 ADP-ribosyltransferase exo-enzyme which ADP-ribosylates, and inactivates, Rho and produces the same cellular morphological changes as seen with HMG-Co reductase inhibitors.[21] This has been used as an additional tool in the investigation of cytoskeletal assembly. Rho is also involved in the regulation of calcium sensitivity of smooth muscle and probably other cells can also be inhibited by HMG-CoA reductase inhibitors.[22,23]

The Rap family

The Rap proteins exercise negative growth control on cells, possibly by inhibiting the function of Ras. In addition Rap, together with Rac, also plays a role in NADPH oxidase systems and function of phagocytic cells.[24]

Heterotrimeric G proteins, lamins and retinal proteins

The heterotrimeric G proteins act in concert with G protein-coupled receptors, where they function as molecular switches in much the same way as the small GTP-binding proteins and are essential for the action of many hormones including epinephrine and the peptide hormones. As the name implies, these G proteins consist of three subunits of which the γ-subunit is prenylated and responsible for membrane binding. Prevention of prenylation leads to a loss of function of these heterotrimeric G protein switches.[25]

The lamins A, B and C are filament proteins that support the nuclear membrane. Lamin B is prenylated, which facilitates its binding to the inner surface of the nuclear membrane. Prelamin A is prenylated but loses its prenylation on conversion to lamin A and plays a role in the mitotic control of the nuclear membrane assembly.[26]

Prenylated proteins play a pivotal role in signal transduction of photoreceptor systems of the retina. Prenylation is critical in the function of the γ-subunits of the heterotrimeric G protein transducin, rhodopsin kinase and retinal cGMP phosphodiesterase.[27]

Prenylation and cancer

The Ras proteins in particular, but also the other prenylated proteins including Rho and Rac, play an important role in cell proliferation. These Ras-related proteins all contain a domain that has an intrinsic GTP-ase activity, which slowly converts GTP to GDP and therefore inactivates the protein. Various GTP-ase activating proteins (GAP) which speed up this process further augment this. Certain mutant forms of Ras as well as the Ras-related proteins lack intrinsic GTP-ase activity and are

therefore continuously in an activated form. This leads to uncontrolled cell proliferation and is associated with various forms of neoplasia including those of endocrine glands.

Early on it was found that by reducing the availability of the substrate farnesyl for prenylation with the use of HMG-CoA reductase inhibitors, the prenylation of Ras could be abolished and the effect of these oncogenic forms of Ras could be inhibited.[28,2] This ability to inhibit the oncogenic activity of Ras proteins by inhibiting their prenylation with HMG-CoA reductase inhibitors led to the possibility of utilising the inhibition of prenylation as a form of anti-cancer therapy.[29,30] Various structural analogues of farnesyl act as molecular mimics and are able to inhibit FTase. Biological products that are able to inhibit FTase are under investigation, and the anti-cancer effect of taxol is partially the result of the inhibition of prenylation. Certain tetrapeptides which mimic the CAAX box are able to bind to and inhibit FTase. These have been investigated, but their clinical use is limited by the difficulty with which these compounds cross membranes and their susceptibility to proteolysis. Consequently non-peptide analogues of these tetrapeptides, which cross membranes more easily and are not prone to proteolysis, have been investigated and show great promise as anti-cancer agents.[31] Because Ras proteins are ubiquitous and play a pivotal role in cell signalling, it would be expected that inhibition of Ras would result in effects incompatible with the viability of healthy cells. However, this has not been borne out. These prenylation inhibitors appear to be relatively specific for transformed cells and normal cells relatively immune to these drugs.[29,32] The clinical use of this form of therapy in the treatment of cancer is therefore expected to become a reality. Furthermore, the concomitant use of HMG-CoA reductase inhibitors appears further to augment the effectivity of certain established anti-cancer regimens.[33]

PRENYLATION IN ENDOCRINOLOGY

The G-coupled receptors, Ras proteins and vesicular transport play a pivotal role in all aspects of endocrinology, and it is to be expected that prenylation will be equally crucial.

It has been shown that glucose-induced insulin secretion is at least partially dependent on protein prenylation, which can be inhibited by lovastatin and can be prevented by the prior administration of mevalonic acid.[34] This can therefore be expected to have an influence on beta-cell function of the pancreas and the glucose sensing mechanism. Insulin-mediated glucose transport involves the translocation of GLUT4-containing vesicles to the cell membrane. This requires a prenylated Rab4 protein that can be blocked by interfering with the prenylation process.[35] The inhibition of this process would be expected to have an influence on and increase insulin resistance. Large-scale trials of HMG-CoA reductase inhibitors in diabetic patients have not reported any changes in glycaemic control. However, no published data report the effects of HMG-CoA reductase inhibitors on beta-cell function or insulin resistance in any detail.

Insulin and prenylation are linked in other ways. Insulin increases the farnesylation of Ras via increased phosphorylation of the α-subunit FTase, which is specific for insulin and not produced by other growth factors. This is proposed as a mechanism by which insulin primes cells for the effect of other growth factors.[36,37] The insulin-induced Ras-dependent and oncogenic Ras-stimulated maturation of oocytes is dependent on prenylation that can be inhibited by prenylation inhibitors.[38]

The adrenal hormones are also influenced by prenylation. Increased dehydro-epiandrosterone sulphate (DHEAS) levels have been associated with a reduced incidence of malignancy and it has been suggested that DHEAS has an anti-carcinogenic effect. DHEAS has been demonstrated to inhibit farnesylation in normal and cancer cells, and this is suggested as a mechanism for the anti-cancer effects of DHEAS.[39] The aldosterone-induced sodium flux in polarised epithelial cells is dependent on the methylation of a geranylgeranylated protein that can be blocked by HMG-CoA reductase inhibitors but does not influence the insulin-induced flux.[40] Prenylation is also involved in the cellular biology of the adrenal medulla as demonstrated in phaeochromocytoma cells.[41]

The Rab proteins are required for exocytosis and also for prolactin secretion by the anterior pituitary.[42] There is every reason to believe that the small GTP-binding proteins play a role in the secretion of other peptide hormones, but to what degree prenylation plays a role in these processes is still to be defined.

PRENYLATION IN BONE AND MINERAL METABOLISM

The activation of cells by growth factors and cell-cell or cell-extracellular matrix contact via integrins requires the transmission of a signal from the cell surface to the cytoskeleton. This leads to changes in the cytoskeletal architecture and results in the formation of filopodia, lamellipodia (cell ruffling), and focal adhesion complexes and stress fibres. This in turn leads to changes in cell morphology and confers mobility to cells. In parallel with this certain growth characteristics of the cell are altered — some cells start proliferating or dividing and other cells undergo programmed death or apoptosis. The signal from the cell surface to the interior of the cell can follow different pathways and there is a complex cross-talk between the different pathways (Fig. 7). This means that the response to growth factors, cytokines or integrins differs in different cell types. Contact with a particular extracellular matrix protein will cause proliferation in one cell type but may cause apoptosis in another cell.

Osteoclasts are among the cells that undergo membrane

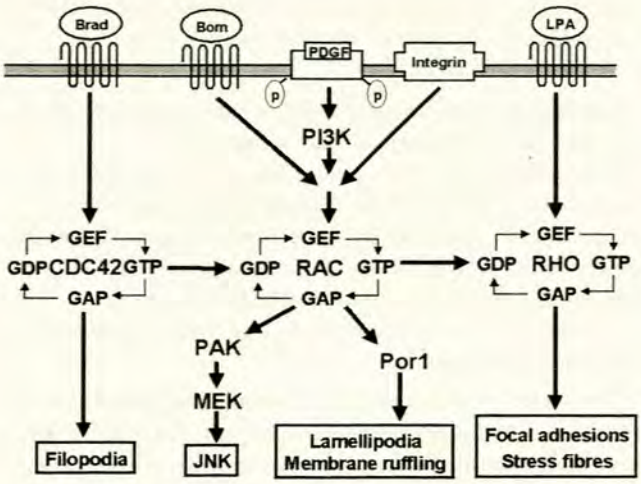

Fig. 7. The signalling pathways from the serpentine receptors, tyrosine kinase receptors and integrins which lead to cytoskeletal assembly (Brad = bradykinin; Bom = bombesin; PDGF = platelet-derived growth factor and other growth factors; LPA = lysophosphatidic acid).

ruffling prior to activation. The Cdc42, Rho and Rac proteins are pivotal intermediaries in the signal transduction between the cell membrane and actin filament organisation (Fig. 7).[19,43,44] Given the above, there is every reason to believe that inhibition of prenylation should have some effect on bone.

There is evidence that the ultimate target for bisphosphonates is the osteoclast.[45] It has been shown that the nitrogen-containing bisphosphonates including alendronate inhibit prenylation.[46] They act directly on the osteoclast by causing apoptosis via Mst1 kinase cleavage and the activation of caspases, and these effects can be mimicked by lovastatin and reversed by prenyl groups.[47,48] Osteoclastogenesis is affected in a similar fashion.[49] The ability of HMG-CoA reductase inhibitors to mimic the effect of alendronate on bone, and that this occurs via the inhibition of prenylation, has been demonstrated by other authors.[50,51] Not only is bone resorption affected but bone formation is also increased by simvastatin, and this seems to occur via an increased secretion of bone morphogenic protein-2 (BMP-2) by osteoblasts.[52] This treatment with simvastatin resulted in a 35% increase in trabecular bone volume in intact rats and a 100% increase in trabecular bone volume in ovariectomised rats. Furthermore there is evidence that steroid-induced osteoporosis[53] and other deleterious effects of steroids, including suppression of osteoblasts by steroids and osteonecrosis, can be prevented by the use of HMG-CoA reductase inhibitors.[54]

Parathyroid hormone-related peptide (PTHRP), which can be induced by various growth factors and oncogenes, is a major causal agent in the development of malignancy-associated hypercalcaemia. Prenylation inhibitors *in vitro* and *in vivo* can reduce the secretion of PTHRP and the subsequent hypercalcaemia.[55] It has been demonstrated that apoptosis can be induced in human myeloma cells *in vitro* by the use of mevastatin and bisphosphonates and that this is mediated via an inhibition of prenylation.[56]

It is clear that the widely used HMG-CoA reductase inhibitors have some effect on bone. Whether this will be evident in humans in the present format and dosages applied in the treatment of dyslipidaemia and whether it will translate into an ultimate reduction of the fracture rate remains to be seen.

Conclusion

It is clear that prenylation is critical in many spheres of medicine including endocrinology and bone and mineral metabolism. The widespread use of HMG-CoA reductase inhibitors will not only reduce serum cholesterol but can be expected to influence prenylation with subtle or overt beneficial or deleterious effects which still have to be defined.

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