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MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS IN REDOX SIGNALING

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1. ABSTRACT

It has been known for quite some time that proper cellular function requires tight control of the cellular redox state. In recent years, a growing body of literature has provided evidence of a role for reactive oxygen species (ROS) as important mediators of proliferation, acting as second messengers to modulate the activation of various signaling molecules and pathways. In contrast to high levels of ROS that may induce modifications that inhibit the activity of cellular components or result in damage, repair and cell death, the hypothesis that low levels of ROS, produced enzymatically and in a regulated fashion, are required participants of signaling pathways controlling essential cellular function is gaining grounds. The concept that ROS specifically target components of these pathways is only beginning to be examined.

The mitogen-activated protein kinases (MAPK) are a large family of proline-directed, serine/threonine kinases that require tyrosine and threonine phosphorylation of a ThrXTyr motif in the activation loop for activation. Receptor-ligand interaction leads to activation of a phosphorylation cascade where the minimal module is formed by MAPK, MAPK kinase and MAPK kinase kinase. Four separate MAPK and activating cascades have been identified, based on the TXY motif and the dual-specificity kinases that strictly phosphorylate their particular TXY sequence. They are the extracellular signal regulated kinases (ERK), c-jun N-terminal kinases (JNK), p38MAPK and ERK5. This review will summarize recent findings regarding the activation of the MAPK and the role played by ROS in their activation.

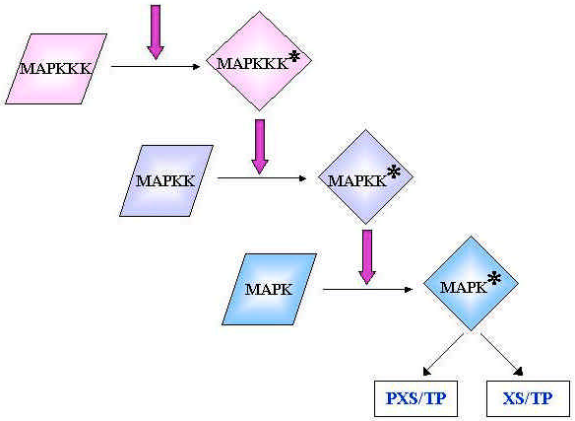

Figure 1. Three-tiered MAPK Cascades. Each MAP cascade is composed of three kinases that are sequentially activated. The change in shape is a graphical depiction for the activated form of the kinases but does not necessarily imply changes in protein conformation.

2. INTRODUCTION

In the last two decades, the field of signal transduction has thrived. As a result, our knowledge regarding the mechanisms by which cells respond to their environment has vastly increased. From the identification of large families of receptors and the production of second messengers, the field has moved to the recognition of highly regulated networks of intracellular signaling pathways that control every aspect of the cell's life and death, and are comprised of kinases, phosphatases, adapter and scaffold proteins, phospholipases and others. Among these pathways, the best characterized and most studied are those leading to the activation of a large family of serine-threonine kinases, the mitogen-activated protein kinases (MAPK), which phosphorylate various cellular targets in a proline-directed manner, including transcription factors and other kinases. Thus, the MAPK and their activation cascades are critical pathways connecting extracellular ligands to the transcriptional machinery.

Other emergent findings in recent years have changed our view on the role of oxidants in biology from that of deleterious molecules produced as a side effect of aerobic metabolism to that of critical modulators of protein phosphorylation and gene transcription. The main source of reactive oxygen species (ROS) (i.e., superoxide ($O_2^{\bullet -}$) and its dismutation product hydrogen peroxide (H_2O_2)) in cells was thought to derive from the mitochondria and from the stimulation of an NADPH oxidase found in phagocytic cells. The role attributed to ROS was mainly associated with damage, either unwanted during oxidative stress and inflammation or beneficial in the case of the phagocyte respiratory burst that participates in bacterial killing. The discovery that nitric oxide (NO), a free radical that is enzymatically produced by various cells in a regulated fashion, could participate in cellular signaling by activating guanylate cyclase and inducing smooth muscle relaxation (1,2), revived the idea previously put forth, but given scant attention, that ROS may participate in signaling for a mitogenic response. The recognition that ROS could be produced in a controlled manner by many cells other than phagocytes supported the concept that ROS can act as second messengers, affecting the activity of signaling molecules and regulating intracellular signaling pathways. In this review, we will summarize recent findings on the activation of the MAPK and on the modulation of their pathways by ROS.

3. THE MAP KINASES AND THEIR ACTIVATION MODULES

As previously mentioned, the MAPK are part of well-conserved signaling pathways that control proliferation, differentiation, embryogenesis, and cell death. Full activation of the MAPK requires phosphorylation of a tyrosine and a threonine within a ThrXTyr (TXY) motif in the activation loop. This activation step is very specific and is performed by dual specificity kinases, the MAP kinase kinases (MAPKK), which are themselves activated by phosphorylation by MAP kinase kinase kinases (MAPKKK). Thus, the MAPK are the terminal kinases of a three-tiered module of kinases that are sequentially activated by a variety of stimuli acting through diverse receptor families (figure 1). The MAPKKK are activated either by phosphorylation by MAP(4)K or by interaction with a small GTPase of the Ras or Rho family. So far, four subsets of MAPK have been identified in mammalian cells that are activated by separate kinase cascades: the classical extracellular signal regulated kinases (ERK, TEY) ERK1/2, the c-jun N-terminal kinases (JNK, TPY) JNK1/2/3, the p38MAPKalpha/beta/gamma/delta (TGY), the latter two families being also referred to as the stress-activated protein kinases (SAPK), and ERK5 (TEY), also called Big MAPK/BMK1 because its high molecular mass (reviewed in (3-5)).

3.1. The ERK Cascade

The p42MAPK was the first mammalian MAPK to be identified as a 42kD protein that increased its tyrosine phosphorylation upon stimulation with mitogens, hence the name. It was later found that stimuli other than growth factors, such as cytokines and ligands for G-protein linked receptors could also activate the p42MAPK and p44MAPK isoforms and the name was changed to ERK to reflect this diversity. The ERK module was the first pathway for which relay of the extracellular signal from the plasma membrane to the nucleus was demonstrated and it became a paradigm for other MAPK modules. All the components of the ERK module, i.e., ERK1 or ERK2, MEK1 or MEK2 and Raf isoforms have been known for over a decade. Nevertheless, questions remain as to the function and regulation of each component, in particular the respective role of the Raf isoforms and their complex mechanisms of activation by the isoforms of the small GTPase p21Ras. Interestingly, targeting of the gene for ERK1 surprisingly resulted in viable mice, indicating that ERK2 was able to compensate the loss of ERK1 but also showed a specific role for this isoform in thymocyte development (6). Recent data also suggest that the pathway may not be as linear as initially believed (see below, Figure 2 and (7,8)).

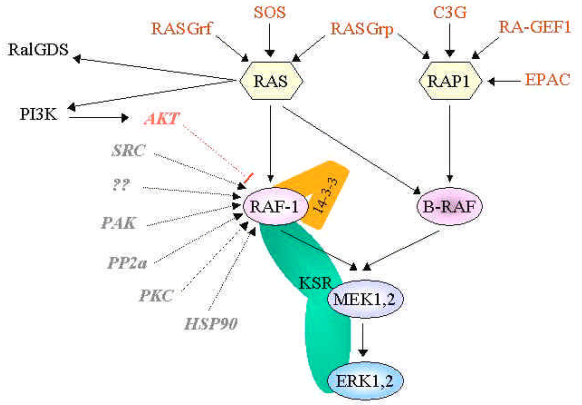

Figure 2. The ERK Cascade. The ERK cascade can be activated through activation of the small GTPases, Ras and Rap1 by various guanine nucleotide exchange factors. The activation of Raf-1 requires formation of a complex with activated Ras at the plasma membrane where further activation events, including phosphorylation, take place. The 14-3-3 proteins play an essential role in maintaining Raf-1 in an inactive conformation in resting cells and may also play a role in facilitating Raf-1 translocation and complex formation with Ras at the plasma membrane. KSR has gained credit as a scaffold protein for the ERK cascade.

3.1.1. Ras Activation

The Ras small GTPases act as molecular switches in the regulation of multiple signaling pathways, leading to various biological outcomes. Mammalian cells contain three different Ras genes that give rise to four Ras proteins, c-H, c-N, c-K_A and c-K_B, which are highly homologous, except in their C-terminal 25 amino-acids that are unique for each Ras protein. This hypervariable region also contains the CAAX motif, which signals for posttranslational lipid modifications that dictate targeting to the plasma membrane. These include CAAX-farnesylation of all Ras proteins and, cysteine palmitoylation for H- and N-Ras. A polybasic sequence stretch is thought to participate in membrane anchoring of K-Ras. Similarly to all small GTPases, Ras cycles between the inactive GDP-bound and active GTP-bound states through the controlled activity of guanine nucleotide exchange factors (GEF) and guanine activating proteins (GAP) (reviewed in (9-12)).

Ras activation has been best characterized after cell stimulation with EGF, a pathway that has served as prototype. The link between the EGF receptor and Ras is provided by the GEF SOS that exists in a complex with the adapter protein Grb2 in the cytosol through binding of the Src Homology (SH)3 domain of Grb2 to a SOS proline-rich region. Grb2 also binds to phosphotyrosine motifs via its SH2 domain, and upon EGF stimulation, it binds to the adaptor SHC, which becomes tyrosine phosphorylated when recruited to the receptor. This brings SOS to the plasma membrane in close proximity to Ras where it can promote GDP/GTP exchange. GTP-bound Ras can then bind to Raf via its effector-binding domain, resulting in activation of the ERK cascade ((13) and references therein). Variants of this activation model have been evoked to describe how other stimuli interacting with various types of receptors recruit SOS to the plasma membrane (14-17), although these linear models may have to be modified as new players are recognized. For example, other Ras GEFs have been identified, such as RasGRF 1 & 2, predominately expressed in the brain and RasGRP1-4 (figure 2). RasGRF1 is constitutively associated with the plasma membrane through its PH domain (18) and is activated by calcium and by calmodulin (19). Treatment with ionomycin activates Ras and ERK via RasGRF1 (18,20). RasGRPs are also regulated by calcium but they additionally contain a diacylglycerol-binding domain; thus, their activity is regulated by the availability of diacylglycerol and is stimulated by phorbol esters, making them non-kinase phorbol receptors (21-24). Targeting to the membrane probably occurs through the C1 diacylglycerol-binding domain, although a new member of the family was shown to be myristoylated and palmitoylated (25). Ligands that activate PLC-gamma also activate RasGRP, as observed in T-cells (26). Stimulation by PMA or by ligation of the T-cell receptor of thymocytes from the RasGRP knockout did not induce activation of Ras and ERK, resulting in a thymopoiesis defect (27). Both RasGRF and RasGRP have been suggested to provide a link between calcium signaling and the ERK pathway (28). Thus, signaling to Ras and ERK by activation of receptors other than growth factor receptor tyrosine kinases may involve alternative signaling molecules. While outside the topic of this review, it is now clearly appreciated that Ras utilizes downstream effectors other than Raf to mediate its various biological functions, among which are PI3K and the GEF RalGDS (for review, see (10,29)) (figure 2).

Because of the greater availability of reagents, most experiments have been done with H-ras, which has fed the mindset that the Ras proteins are functionally redundant. Recent data have argued against this notion, although the evidence is still limited. An early study showed that each Ras isoform differs in its ability to activate Raf-1 and to induce transformation (30). An activated K-Ras mutant was a better activator of the Raf-1 kinase than H-Ras and conversely for PI3K activation (31). N-Ras interacted with Raf-1 with higher affinity than H-Ras when low levels of Ras expression were used (32). RasGRF selectively activated H-Ras while not affecting either N-Ras or K_B-Ras *in vivo* (33). K-Ras is the isoform the most often mutated in cancers and K-Ras gene disruption resulted in embryonic death while the H-Ras and N-Ras knockout mice were viable (34,35). Altogether, these data and the knockout experiments point to specific functions and effectors for each Ras isoform. Nevertheless, the role of Ras in transformation fits with activation of the proliferative function of the ERK cascade, in support for Raf as one of the effectors of Ras.

These biological differences between isoforms may be explained in part by their distinct spatial organization in the plasma membrane (36,37). Correct membrane targeting is essential for Ras function and altering the composition of the membrane induces defects in Ras signaling (36,38). The model of simple fluid mosaic of the plasma membrane has evolved towards a more

complex structure that includes microdomains with specific lipid and protein composition. The best described of such microdomain are the liquid-ordered lipid rafts, which are enriched in cholesterol and sphingolipids and where a number of signaling proteins such as glycophosphatidylinositol-anchored proteins or myristoylated proteins appears to be located (39). Raf-1 activation was much less efficient when GTP-bound H-Ras was maintained in the lipid rafts (40). A model has been proposed where H-Ras is in a dynamic equilibrium between the lipid rafts and the disordered membrane and GTP loading favors the dissociation of H-Ras from the lipid rafts (40,41). On the contrary, K-Ras is predominantly located in the disordered plasma membrane, independently of its state of activation and interaction of its polybasic region with acidic phospholipids may create another microdomain within the plasma membrane where other signaling proteins may operate (36).

3.1.2. Raf Activation

Three genes encode for the three Raf serine/threonine kinases found in mammalian cells, i.e. Raf-1 (a.k.a. c-Raf), A-Raf and B-Raf, that contain three highly conserved regions, CR1, CR2 and CR3. The CR1 and CR2 regions of all three proteins are at the NH2-terminus, which appears to be a regulatory domain repressing their activity. Until recently, the large majority of studies regarding the role of Raf in ERK activation have been performed with Raf-1.

Activation of Raf-1 is a complex process, still not entirely understood, but clearly involving plasma membrane recruitment, protein-protein and lipid-protein interactions and, phosphorylation (reviewed in (7,8,42)). In resting cells, Raf-1 is located in the cytosol and is maintained in an inactive conformation through protein-protein interactions, stabilized by binding to the 14-3-3 proteins. These proteins belong to a large family of acidic proteins that bind to their targets in a phospho-dependent manner and sequester them in a compartment not appropriate for activation. They bind to Raf-1 via S259 and S621, which are phosphorylated in resting cells, as is S43 (43). These residues have been found to be inhibitory of Raf-1 kinase activity and their phosphorylation is increased further by PKA activation (44,45). The initial event in Raf-1 activation is the formation of complexes between Ras-GTP and inactive Raf-1 at the plasma membrane (46,47). Both the Ras-binding domain and the cysteine-rich domain of Raf-1 in CR1 are required. Binding to Ras and translocation to the plasma membrane, which may relieve the autoinhibition, are not sufficient to activate the Raf-1 kinase activity but are required for the subsequent multistep activation events to occur at the plasma membrane (7). Mitogenic stimulation induces the phosphorylation of several residues, including S338, Y341/340, T491 and S494 (48). The kinases responsible have not been clearly identified yet and may be multiple and cell- and stimulus-specific (figure 2). Phosphorylation of S338 is a good indicator of Raf-1 activation and may occur via activation of the p21-activated kinases, PAK1 and PAK3 (49-51), although this was recently challenged (52). Overexpression of Src kinases induces phosphorylation of Y341, which is required for increased kinase activity and potentiates the phosphorylation of S338, possibly by relieving the autoinhibition (47,53). Few physiologic stimuli, however, have clearly been shown to induce phosphorylation of Y341 (54). More recently, dephosphorylation of S259 has been shown to regulate Raf-1 activation (55). Displacement of the 14-3-3 from S259 makes it accessible to dephosphorylation by PP2A, which associates with Raf-1 (56). Nevertheless, Ras must induce re-phosphorylation of S259, based on the stoichiometry (55). Others have argued against a major role for dephosphorylation of S259 and proposed that the 14-3-3 proteins may be the one regulating Raf-1 activity and that efficient recruitment of Raf-1 to the plasma membrane and subsequent phosphorylation of S338 require displacement of the 14-3-3 from Raf-1, presumably by binding to Ras-GTP. In addition, S621 is also essential in this process (57).

The respective role of the Raf isoforms has only been recently explored and these studies have underscored the importance of cellular context. The phosphorylation events reported for Raf-1 do not apply to B-Raf, which lacks the equivalent to Y341 of Raf-1, replaced by an Asp and for which the counterpart to S338, S448, is constitutively phosphorylated (48). In fact, contrary to Raf-1 and A-Raf, Ras binding alone appears sufficient to activate B-Raf, which is a more potent MEK activator than Raf-1 (58). In addition, the small GTPase Rap-1, which is activated by calcium, cyclic AMP (cAMP) and diacylglycerol (59) and for which several GEF have been identified (figure 2), can activate B-Raf, resulting in ERK activation, as observed in PC 12 cells stimulated with NGF (60). PKA and cAMP exert cell-specific actions on cell proliferation and differentiation and on the ERK pathway via several mechanisms (reviewed in (61)), one of which is through cAMP activation of Rap-1. In some cells expressing low levels of B-Raf, activation of Rap-1 by cAMP may antagonize ERK activation (61,62). The role of the Raf isoforms has also been studied by gene targeting. Deletion of the A-Raf gene resulted in viable mice that died post-partum or lived to adulthood with neurological defects, depending on the background (63). The B-Raf gene deletion, however, was embryonic lethal with vascular defects and increased apoptosis, indicating an anti-apoptotic role for B-Raf (64). Different strategies were used to suppress expression of the Raf-1 gene (65-67). All resulted in embryonic lethality and placental defects, suggesting a role for Raf-1 in mouse development. The phenotypes of the different knockout mice were slightly different, in part due to the targeting strategy for one of the knockout models where residual protein expression may have confounded the results (65). It included increased apoptosis (66,67) and, vascularization defects (66), as seen in B-Raf null mice. These findings clearly indicate that the Raf isoforms are not fully redundant (68). They also make the case for a necessary role for Raf-1 and B-Raf in counteracting apoptosis. Interestingly, ERK activation by growth factors or PMA was normal in embryonic fibroblasts (MEF) from the Raf-1 knockout mice, suggesting that Raf-1 effectors other than MEK/ERK are mediating the anti-apoptotic activity of Raf-1 (66). Whether the kinase activity of Raf is necessary for this

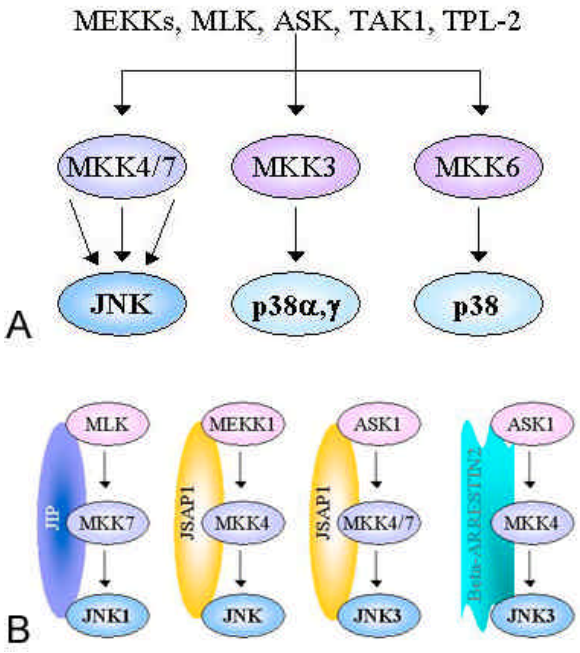

Figure 3. The SAPK Cascades. (A) The SAPK modules are not entirely identified at the level of the MAPKKK, as many kinases have been shown to activate the SAPK. (B) Depicted are some of the scaffold proteins that have been characterized for the JNK modules. See text for details.

effect is still unknown. The effectors mediating this activity are also not known. These studies may have suggested that MEK is not a substrate for Raf-1, contrary to the data obtained in *in vitro* studies of Raf-1 function. However, further work will be needed, as single gene targeting does not always give a clear answer in case of isoforms because of possible compensation. For example, ERK activation was normal in MEF from the A-Raf knockout, which could argue that A-Raf is not necessary for ERK activation but the Raf-1 and B-Raf activity towards MEK was increased in these same cells, indicating compensation by the other isoforms (69). The phenotype of the MEK-deficient mice, which died at 10.5 days of gestation, showed similar characteristics as that of the Raf-1 null mice, i.e. vascularization defects of the placenta (70), in support for MEK being a target for Raf-1. The outcomes of Raf-1 targeting more likely imply that Raf-1 has substrates other than MEK and can carry out new functions, independent of ERK. The hypothesis that Raf-1 may utilize multiple downstream effectors had previously been advanced using a different strategy where preventing the binding of Raf-1 to MEK resulted in inhibition of ERK but in the impairment of only a subset of responses (71). The identification of new substrates has been difficult because of the lack of known consensus motif for the kinase, the low specific activity of Raf-1, and the association of Raf-1 with many other kinases. Nevertheless, many binding partners have now been reported for Raf-1, which include scaffold, chaperones, regulator of subcellular location and others (for review see (7)).

3.2. The SAPK Cascades

The JNK proteins are encoded by at least three genes and, due to alternative splicing, 12 isoforms have been identified so far. Four genes are now known to encode for the various isoforms of p38MAPK. JNK and p38MAPK are primarily activated by cellular stresses such as inflammatory cytokines but also by UV, gamma-irradiation and others. The respective role of each isoform within the subfamilies has not been explored significantly as yet. The three JNK genes have been disrupted by homologous recombination and all mice are viable but are defective in apoptosis and immune responses (72). A double JNK1 and 2 knockout model resulted in embryonic death (73). Three different groups reported the results of gene targeting for p38-alpha and drew different conclusions, although all mice died during embryonic development (74-77).

The SAPK are activated through a similar kinase cascade as the ERKs (figure 3A), although the modules are not as well defined and some different mechanisms have been noted (for review see (4,72,78)). Two MAPKK have been identified as the kinases phosphorylating JNK, i.e. MKK4/SEK1 and MKK7. Disruption of either gene results in embryonic death (79-82). Interestingly, MKK4 preferentially phosphorylates Y185 while MKK7 targets only T185 (83-85). Embryonic fibroblasts isolated from mice missing the expression of both MKK4 and MKK7 exhibited no response to UV, suggesting that cooperation between MKK4 and MKK7 is required to activate JNK in response to environmental stress (82). In contrast, tumor necrosis factor (TNF) activates JNK by activating only MKK7, which was shown to be essential for this pathway by gene disruption (82). The p38MAPK are activated by the dual specificity kinases, MKK3 and MKK6, which appear to selectively phosphorylate particular p38 isoforms, with MKK3 acting on the alpha and gamma isoforms (86). Cells isolated from the MKK3 null mice showed a selective decrease in TNF-induced p38MAPK activation and cytokine expression, indicating that MKK3 is a critical component of this pathway (87).

At the level of the MAPKKK, many kinases activating either or both JNK and p38MAPK have been identified by overexpression or dominant-negative experiments and are part of three large families, i.e. the MEKKs, the mixed lineage kinases (MLK) and the thousand and ones (TAOs) (figure 3A) (4,88,89). The availability of mutated mouse models has started to provide a clearer picture of the respective role of these kinases in the SAPK cascades. Cells from MEKK1 knockout mouse and MEKK2 embryonic cells showed decrease in JNK activation to a limited number of stimuli (90-92) while MEKK3 mutation, which is embryonic lethal, did not result in defect in JNK activation (93). A recent MEKK2 mouse model described a role for this kinase in controlling the strength of the TCR/CD3 signaling in T cells (94,95), confirming previous work by others (92). Similarly, Tpl-2, another MAPKKK, does not appear to be required for JNK activation but was necessary for ERK activation (96). Apoptosis stimulating kinase 1 (ASK1) can activate the JNK and p38MAPK pathways (97). Studies with embryonic fibroblasts from the ASK1 knockout mouse

showed no effect on acute activation of both JNK and p38MAPK but sustained activation of these kinases, which is required for apoptosis, was altered (98). Upstream of the MAPKKK, other kinases have been recognized as MAP(4)K, even though it has not always been proven that they could phosphorylate the MAPKKK and the mechanisms by which they activate these cascades are unclear. Among them is the large family of Ste20 kinases that includes the germinal center kinases and the p21-activated kinases (PAK), the latter serving as effectors of the GTPases of the Rho family (4,74,99).

Some interesting findings have recently indicated that the JNK are associated with proteins that may regulate their activity. This is the case for the glutathione-S-transferase P1-1 (GSTp), the most prevalent non-hepatic isozyme of a large multigene family involved in xenobiotic detoxification. Increased expression of this enzyme has been linked to multidrug resistance and malignant phenotype of tumors. GSTp inhibits the JNK pathway and regulates proliferation, stress response and apoptosis by binding to the C-terminus of JNK1 (100-102). GSTmu, another isoform, was shown to interact with ASK1 and to repress ASK1-dependent apoptotic cell death (103). The inducible heat shock protein, HSP72, was also shown by several groups to modulate stress-induced pathways, an activity independent of its role in prevention of protein damage. TNF-alpha-induced apoptosis was regulated by HSP72 through a JNK/Bid-dependent pathway (104). The exact mechanisms by which HSP72 inhibits JNK-dependent pathways are not clearly understood, although direct binding or inhibition of dephosphorylation has been suggested (105,106). HSP72 could bind to JNK and prevented JNK activation without affecting activation of upstream kinases under mild heat shock (105). These studies clearly imply that the JNK pathway can be modulated via various protein-protein interactions that may also be relevant for other MAPK pathways.

3.3. ERK5

ERK5 and MEK5 were identified as components of a novel MAPK cascade by the yeast-two hybrid method (107). ERK5 was characterized as a redox-sensitive MAPK (108), although other stimuli such as ligands for receptor tyrosine kinases or G protein-coupled receptors can active ERK5 (109,110). The upstream kinase(s) have not been not clearly identified, but MEKK2 and MEKK3 have been implicated (111,112). Recently, mice deficient in ERK5 were generated, which died at embryonic day 9.5 (113). The ERK5 embryos had defects in cardiac development and in angiogenesis. This phenotype was highly homologous to that of the MEKK3 null mice and the transcription factor Mef2C null mice, a substrate for ERK5, suggesting the possible pathway MEKK3=>MEK5=> ERK5 => Mef2C.

3.4. MAPK and Scaffold/Linker Proteins

In yeast, the MAPK cascades are segregated and each signals for a specific cellular response (114). The protein Ste5p serves as a scaffold for the module Ste20-Ste11-Ste7-Fus3 MAPK and is absolutely required for activation of the mating cascade by pheromone while Pbs2p is a scaffold for theSte11/Hog1 MAPK in the high osmolarity response (115). The idea that this segregation may also take place in mammalian cells has gained momentum and the search has been ongoing for scaffold proteins that would participate in regulation of the specificity or increase the output of the cascade. This could be accomplished by maintaining the components of the cascade in close proximity or in the proper compartment or by stabilizing a configuration suitable for activation. The stoichiometry of scaffolds to cascade components would have to be strictly regulated and the characterization of these proteins has probably been impaired by experiments using overexpression that disrupts such stoichiometry.

No Ste5p mammalian homologue has been found but several proteins have been identified that may fulfill the characteristics of a scaffold protein. For the JNK pathway, four proteins have been found, i.e. JIP, CrkII, filamin and beta arrestins (74,116). JIP1 (JNK-inhibitor protein, a.k.a. IB1) was first identified as an inhibitor of the JNK pathway (117), and binds to MLK, MKK7 and JNK1, as do JIP2 & JIP3 (118). Results for JIP3 (a.k.a. JSAP1), which is not structurally related to JIP1 & 2 and has four splice variants, have been conflicting since it has been shown to also bind MKK4 and MEKK1 (119) (figure 3B). JIP3/JSAP1 can also be phosphorylated by ASK1 *in vitro* and *in vivo*, facilitating its interaction with the members of the JNK cascade, thus playing a dynamic, phosphorylation-dependent role in the ASK/JNK module (120). Two groups reported contradictory findings for the disruption of the JIP1 gene, resulting either in embryonic lethality (121) or viable mice where JNK activation by excitotoxic and anoxic stresses in hippocampal neurons was prevented (122). Nevertheless, these studies support a critical role for JIP1. Furthermore, overexpression of the JNK binding domain of JIP1 or the use of peptides modeled after this domain (123,124) have been shown to interfere with various JNK-dependent responses (125). The beta-arrestins participate in the desensitization of G-protein coupled receptors (GPCR) but, in addition, recent data showed that they might play a role as scaffold proteins (126). Beta-arrestin 2 maintains a JNK module composed of ASK1-MKK4 and JNK3, resulting in enhancement of JNK3 activity upon stimulation with angiotensin (127) (figure 3B). Beta-arrestin 1 & 2 can also bind the components of the ERK module upon GPCR activation, possibly targeting ERK to its cytosolic substrates (128).

The first scaffold identified for the ERK pathway was MP1, which seems to coordinate the formation of MEK/ERK complexes (129). The protein KSR for kinase suppressor of Ras was initially characterized in *C. Elegans* and *Drosophila* (130,131) and these genetic studies showed that KSR was essential for Ras signaling and appeared to function upstream or in parallel to Raf. The intrinsic kinase activity of KSR could not be conclusively confirmed but, instead, KSR has gained acceptance as a scaffold protein for the ERK pathway, stimulating Raf-1 activity in a kinase-independent manner (132,133) (figure 2). A recent study reported the results of disruption of the KSR gene. Deficient mice were viable but the absence of KSR resulted in a significant decrease in ERK activation, demonstrating

that KSR is a scaffold protein facilitating the ERK pathway *in vivo* (134). Genetic studies in *Drosophila* also led to the discovery of Sur-8 and CNK (connector enhancer of KSR) as other possible scaffolds for the ERK pathway (135). CNK appears to be directly responsible for compartmentalization of a pool of Raf kinase to the plasma membrane (136,137).

3.5. MAPK and Phosphatases

Activation of the MAPK must be terminated at a precise time for proper cell function as the duration and extent of MAPK activation, which is governed by the equilibrium between the activity of kinases and phosphatases, may determine the biological outcome (138,139). One of a major site of regulation is at the level of the MAPK, although the serine/threonine phosphatase PP2a can also dephosphorylate Raf-1 and MEK in the ERK cascade. *In vitro*, PP2a and tyrosine phosphatases can dephosphorylate the MAPK and some evidence suggests that they may also play a role *in vivo*, especially for ERK1/2 under conditions of acute activation (140), such as after stimulation by fMLP in neutrophils (141). Recently, a number of tyrosine phosphatases have been implicated in the regulation of the MAPK, including PTP-SL and HePTP (142-144).

In addition, a large family of dual specificity phosphatases (DSP) also called MAP kinase phosphatases (MKP) has been identified that dephosphorylate both phosphothreonine and phosphotyrosine residues *in vitro* and *in vivo.* In mammalian cells, up to ten family members have been described so far that all share a common structure and a signature motif in the catalytic domain that is also present in all protein tyrosine phosphatases (145,146). Most of the MKP are inducible nuclear enzymes but some are not encoded by immediate early genes and are predominantly cytosolic enzymes ((146,147) and references therein). Specific protein-protein interactions mediated by the non-catalytic domain of the MKP appear to be responsible for the distinct substrate specificity. While there is strong evidence for their role in regulating the MAPK, this physiological role has not been definitely proven in mammalian cells, contrary to in yeast and *Drosophila*.

Novel aspects of the regulation of the MAPK by DSP have recently emerged. A DSP was identified and named JSP-1 for JNK-stimulatory phosphatase-1 as it has the capacity to activate the JNK signaling pathway, albeit in co-expression studies (148). SKRP1, which is constitutively expressed in most cells, was found to selectively inhibit JNK through physical interaction with the upstream activator MKK7 (149) and, in fact, may be a scaffold for the JNK pathway as it also interacted with ASK1 (150). MKP-7, which appears to specifically dephosphorylate JNK, also functions as a shuttle protein (151).

3.6. MAPK Cascades, Substrates and Specificity

The fact that numerous and diverse cell-type specific responses are elicited upon activation of the MAPK pathways implies that recognition mechanisms must exist upstream and downstream of the MAPK to maintain accuracy and specificity ((152-155) and references therein). Activation of all MAPK results in proline-directed phosphorylation of various cytosolic and nuclear substrates at a similar sequence, i.e. S/TP or PxS/TP (figure 1). Nevertheless, some proteins are specifically phosphorylated by only one of the MAPK and not all proteins with a similar motif are substrates for the MAPK, suggesting necessary targeting domains outside of the phosphoacceptor sequence. The prototype for such domain is the delta domain in the N-terminal half of c-Jun that acts as a docking site for JNK and is absent in the oncogenic v-Jun (156,157). Analysis of other transcription factors, such as ETS family members (158,159), identified a motif that consists of a cluster of basic residues followed by either an LXL motif and/or a sequence of hydrophobic residues. This motif is conserved in many substrates and has been referred to as the D-domain (153). Another sequence, FXFP, is an evolutionary conserved docking site found in various MAPK substrates (155,160). The phenylalanine in both positions provides high affinity binding but could be replaced by a Tyr without loss in affinity, suggesting that MAPK may bind to and phosphorylate YXYP-containing substrates (160). The FXFP sequence by itself was sufficient to target phosphorylation of particular S/TP sites and it was proposed that the FXFP and D domains constitute a flexible modular system targeting the MAPK to appropriate substrates and to sites within the substrates (153,155,160).

The most specific step in the cascades is the phosphorylation of the MAPK by the MAPKK. Over the years, several studies had identified in these proteins particular sequences that were necessary for their interaction and activation. Careful alignment of reported sequences led to the identification of a well-conserved cluster of basic residues followed by hydrophobic sequences in many of the MAPK-interacting proteins such as MEK or the phosphatases, sequences that are similar to the D-domain (153,154,161). Another docking site or common docking domain (CD) that increase the efficiency of the interaction with their partners was identified in all MAPK. CD alone did not determine specificity (161) but with another domain, ED, formed a grove-like structure, which was found on the steric structure of p38MAPK and ERK2 and might affect both docking affinity and specificity (162). This groove binds to MAPKK, MAPK phosphatase and substrates in a mutually exclusive manner and is located on the opposite side to the active center (154,162,163). Thus, taken altogether, these targeting domains might be modular cassettes that regulate the affinity and specificity of the cascade. They might also play a role in determining subcellular localization and/or facilitating signaling pathway integration.

3.7. MAPK and Gene Expression

The MAPK play an important role in the regulation of gene expression either by directly phosphorylating transcription factors or by activating other kinases with transcription factors as substrates (figure 4A) (reviewed in (4,164,165)). The consequences of phosphorylation on the function of transcription factors are

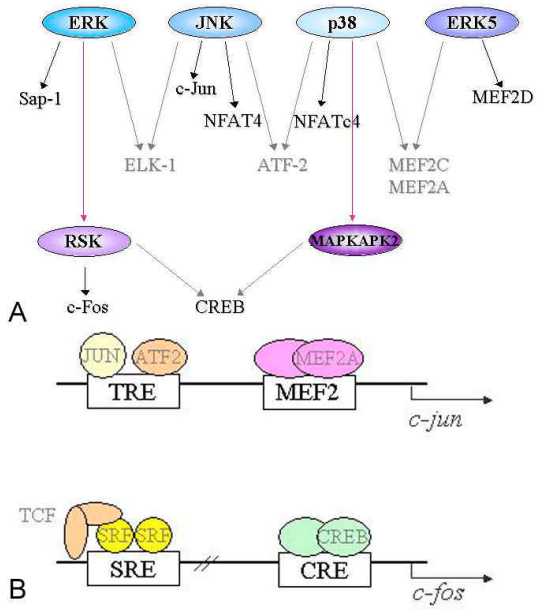

Figure 4. MAPK and Transcription factors as Substrates. (A) Each MAPK phosphorylates specific (black) but also overlapping (grey??) substrates. They also phosphorylate kinases with transcription factors as substrates, such as RSK and MAPKAPK family of proteins. Overall, the MAPK can affect SRE, CREB, MEF and AP-1 response elements that are present either alone or in tandem in the promoter of many genes such as *c-fos* and *c-jun* (B), making the MAPK essential regulators of transcription.

multiple. For example, phosphorylation of the ternary complex factors (TCFs) Elk-1, SAP-1 and SAP-2 at multiple conserved carboxyterminal S/TP motifs regulates their transcriptional activation and DNA binding abilities (166), and their association with co-regulators such as the serum response factor (SRF) at the serum response element (SRE) (167). The promoters in several genes have an SRE, as in the case of *c-fos* (figure 4B), which gene product can be part of the AP-1 complex, a transcription factor formed by homo- or heterodimerization of the Jun, Fos and ATF-2 families of basic region leucine zipper proteins. The MAPK participate in AP-1 regulation by increasing gene transcription of its components and by phosphorylating them (165,168). Phosphorylation can also regulate protein stability as in the case of the phosphorylation of c-Jun by JNK, which protects it from ubiquitination and prolongs its half-life (169). A recent study showed that stabilization of c-Fos via secondary phosphorylation by ERK serves as a mechanism by which the cells "interpret" differences in signal duration to control biological outcomes (139). Another possible consequence of phosphorylation is the control of subcellular localization. The large family of nuclear factor of activated T-cells (NFAT), which regulates cytokine expression, is maintained in the cytosol by phosphorylation, which either masks the nuclear localization signal or the site for the calcium-dependent phosphatase calcineurin. Stimulation induces calcium influx and activation of calcineurin, which dephosphorylates NFAT, resulting in translocation of NFAT to the nucleus where it binds to NFAT *cis*-acting elements. These elements are often located close to an AP-1 binding site, forming ternary complexes that integrate several signaling pathways. Several members of the NFAT family are phosphorylated by the MAPK (170).

The recognition of the role of docking domains in governing specificity has helped the understanding of some of the differences in substrate phosphorylation (153,158,159). For example, NFATc1 and c3 have a JNK docking domain that is not present in NFATc2 and c4 and the latter are not substrates for JNK. NFATc4 is targeted by p38MAPK upon growth factor induced stimulation (171). In addition, using SAP-1, Elk-1 and MEF2A, a recent study has identified critical positions and motifs within the docking domains that confer specificity and binding strength (172). Nevertheless, the activity of a promoter is usually dependent on multiple transcription factors that can either act positively or negatively and may be phosphorylated by several MAPK and by other signaling pathways. Thus, activation of several MAPK by receptor/ligand interactions may converge in the nucleus at the level of transcription, making it difficult to assess the role of each cascade on gene regulation. For example, regulation of the *c-jun* gene requires ATF-2, c-Jun and MEF2, which are all substrates for MAPK while SRF, TCF/Elk1 and CREB are involved in *c-fos* regulation, thereby requiring integration of signals transduced by several MAPK acting on more than one regulatory elements of the promoter, in concert with other kinases (109) (figure 4).

4. WHAT IS REDOX SIGNALING?

The purpose here is not to cover extensively all aspects of redox signaling as several recent reviews have done so (e.g., (173-179)) but to point out some of the major concepts in the field.

4.1. H_2O_2 is an Intracellular Messenger in Multiple Pathways

Many studies suggested that extracellular H_2O_2, which can readily cross membranes and has a relatively long half-life, could activate several pathways, although some of those initial studies used high doses that may not be relevant to signaling. Various growth factors, cytokines and agonists to G-protein coupled receptors were then shown to induce the production of ROS, leading to proliferation or apoptosis (reviewed in (180,181)). The small GTPase Rac, a component of the phagocyte NADPH oxidase that is ubiquitously expressed, appears to play a central role as a transducer of these receptor-activated redox signals (182-184). Furthermore, catalase, which metabolizes H_2O_2, inhibited the effects induced by ROS when added either extracellularly or intracellularly by pinocytosis or transfection, suggesting that H_2O_2 is most likely the ROS responsible for the effects triggered by stimulation. The identification in fibroblasts of Nox1 (previously mox-1), a homolog to the $gp91^{phox}$ subunit of the phagocyte NADPH oxidase (Nox2, see below), was followed by the cloning of several members of this new family, with various tissue specificity (185-187). Overexpression of Nox1 in NIH3T3 cells resulted in

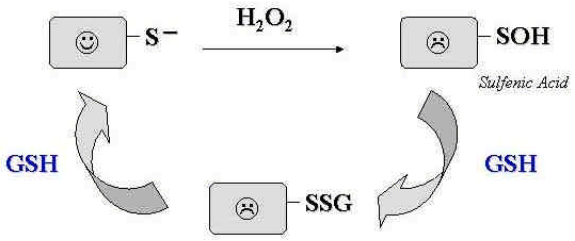

Figure 5. Mechanism of reversible protein thiol modification by ROS. PTP contain a critical cysteine in their catalytic site, which is in the form of a thiolate. Oxidation by H_2O_2 results in formation of a sulfenic acid intermediate that inhibits the enzymatic activity (☹), which is then restored (☺) through the action of thiols.

increased $O_2^{\bullet ?}$ production and transformation (185), a similar effect to that induced by oncogenic Ras in fibroblasts (188). H_2O_2 was found to mediate the effects of Nox1 (189). A few studies demonstrated inhibition of the observed effects by SOD, suggesting a role for $O_2^{\bullet ?}$ (190). While $O_2^{\bullet ?}$ may participate, it is unlikely to be the targeting ROS, based on chemistry, but it may be a necessary intermediary. Thus, so far, H_2O_2 appears to be the major ROS involved in the activation of multiple pathways.

4.2. Remember your Chemistry!

As much as many of us would like to forget about it, the chemistry of $O_2^{\bullet ?}$and H_2O_2, including their reactivity with other species such as nitric oxide (NO), leading to formation of other reactive species, will affect their reactivity with signaling molecules. Many aspects of radical chemistry have been well characterized in a test tube. While it is not always clear how much of this information is applicable to biology, there are a number of principles that should be remembered when interpreting data in a biological context. Diffusion coefficient, reaction kinetics, pH, charge, rates of destruction, all these parameters will affect which reactive species and target are capable of interaction (177,179,191). For example, $O_2^{\bullet ?}$ rapidly dismutates, either spontaneously or via superoxide dismutase catalysis, to form H_2O_2, which is not a free radical. However, NO can react 10 times faster with $O_2^{\bullet ?}$ than SOD, resulting in formation of peroxynitrite ($^?OONO^?$), a powerful oxidizing and nitrating agent. Yet, the diffusion rate of NO and the concentration of each radical will control this reaction (192). Reversibility of the interaction between target and ROS will also be dictated by chemical properties and is critical for signaling as amply demonstrated by phosphorylation/dephosphorylation events.

Identification of the precise targets for ROS in the various signaling pathways has recently started and, the general consensus is that proteins containing critical thiols are favorable targets for ROS (177,193-196). H_2O_2 will not significantly interact with thiols, unless the thiol is in the form of a thiolate ($-S^?$). Ionization of the cysteine thiol occurs only when the surrounding amino acid residues lower its pKa, which is around 8.3. Thus, only particular cysteines within a protein may be able to react with H_2O_2 (see below). Low doses of H_2O_2 will result in oxidation to a sulfenic acid (-SOH), a very unstable intermediary that has been difficult to detect. The sulfenic acid can be reduced by other thiols to form the thiolate again (figure 5) while more extreme oxidation, as with pervanadate, will give rise to compounds that are quite stable (sulfinic and sulfonic acids) (197). ROS are not the only reactive species to interact with cysteine thiolate. Peroxynitrite can also oxidize thiolates (198). In addition, NO can also interact with thiols to form S-nitrosothiols (RSNO) (199). Although their mechanism of formation is still disputed, they have been proposed as the mediators of the effects of NO in signaling (199,200). Thus, under conditions where $O_2^{\bullet ?}$ and NO are produced, chemistry and compartmentalization will determine the targeting species.

4.3. Choose your Pathway, Know your Targets

While the transcription factors AP-1 and NF-kappa B were the first signaling molecules to be considered sensitive to redox changes in the cells, the list of molecules and pathways that are affected by ROS has greatly increased in the last few years, although the exact mechanisms and/or chemical modification are not always known. For example, AKT/PKB, a serine/threonine kinase that is downstream of the lipid kinase phosphatidylinositol 3-kinase (PI3K) has been shown to be activated by H_2O_2 several years ago (201), however, it is still unclear whether H_2O_2 directly targets AKT, acts on an upstream activator such as PDK1 or on the lipid phosphatase PTEN that controls the levels of required phosphatidylinositol-3,4,5-trisphosphate (202-205).

The best described targets for ROS are the protein tyrosine phosphatases (PTP) that contain in their active site a cysteine (CX_5R) that is critical for their activity and exists as a thiolate (145,206). Low-molecular (LMW)-PTP has two vicinal cysteines but only C12 is necessary for catalytic activity. Early on, NO was shown to inhibit this PTP *in vitro* (207) and later, so was H_2O_2 (208). Denu and Tanner provided the first evidence for the reversibility of the inhibition by H_2O_2 of several purified PTP, PTP1B, VHR and leukocyte-antigen related phosphatase (LAR) and for the formation of a sulfenic acid intermediate (209) (figure 5). SHP-1 was also reversibly inhibited by H_2O_2 *in vitro* and after cell exposure (210). On the contrary, PTP1B, CD45 and LAR were irreversibly inhibited *in vitro* by peroxynitrite, either added as a bolus or produced by SIN-1 through co-generation of NO and $O_2^{\bullet ?}$ (211). S-nitrosothiols also inhibited the same PTP but the inhibition was reversed by DTT (211). Rhee's group (212) showed that PTP1B was reversibly inhibited *in vivo* by EGF stimulation, which produces H_2O_2 (213). Such reversibility has also been demonstrated *in vivo* for SHP-2 (214) and for the LMW-PTP (215). Interestingly, the two vicinal cysteines of LMW-PTP seem to form an S-S intramolecular bridge that protects the catalytic C12 from further and irreversible oxidation (215). Thus, the evidence is growing in support of an essential role for ROS modulation of PTP activity in signaling (196,216,217).

4.4. Redox Signaling Vs. Oxidant Stress

Oxidative stress has been defined as an imbalance between oxidant production and anti-oxidant

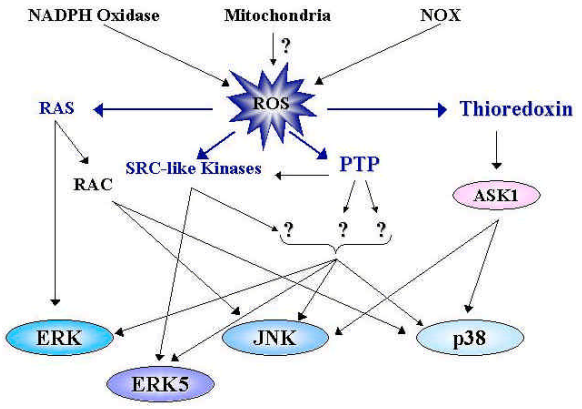

Figure 6. Proposed mechanisms for ROS-mediated activation of the MAPK. ROS are produced in the cells in a controlled fashion through the enzymatic activity of NADPH oxidase of the Nox family and possibly by the mitochondria, although the exact mechanism is unclear for the latter. Ras, Src-like kinases, PTP and thioredoxin can interact with ROS through specific cysteine residues, resulting in alteration of their activity. While the reversible oxidation of PTP by ROS is well characterized *in vitro* and *in vivo*, their targets in the various MAPK pathways have not been identified yet. See text for details.

defenses, i.e. catabolizing enzymes and redox couples such as GSH/GSSG and the thioredoxin system (191). Oxidative stress often results in cell injury and, under these conditions, most of the targets are modified irreversibly by ROS and repair mechanisms are required. However, redox signaling may refer to events that occur when low levels of ROS are produced resulting in small shift in the redox status of the cells and when the targeting by ROS of signaling intermediates is specific, required, transient and reversible, i.e. all properties characteristics of a signaling pathway. In that sense, the inhibition of SHP-2 during PDGF signaling strongly supports such definition as the ROS produced by PDGF play an integral part of the PDGF signaling and the transient and reversible inhibition of SHP-2 allows the signal both to go through and to be terminated (214). Specificity was also demonstrated because, EGF, which also induces ROS production, did not result in SHP-2 inhibition in the cells used in this study. Translocation and binding of SHP-2 to the receptor was necessary for downstream signaling, indicating that only the bound pool of SHP-2 was reversibly inhibited (214). In addition, loss of binding to the receptor of other signaling molecules such as GAP190 due to receptor dephosphorylation coincided with re-activation of the SHP-2 activity, indicating tightly controlled temporal and spatial relationships (214). This type of compartmentalization may offer an explanation for specificity. It has also been evoked by others to explain differences of outcome as a function of the production of ROS in different compartments such as the plasma membrane, cytosol or mitochondria (218).

5. EVIDENCE OF A ROLE FOR ROS IN MAP KINASE ACTIVATION

5.1. Extracellular H_2O_2 and Endogenously Produced ROS Activate the MAPK in Non-Phagocytic Cells

Many studies have shown that bolus addition of exogenous H_2O_2, and exposure to radiation or to drugs such as menadione known to induce production of H_2O_2 led to activation of the various MAP kinases (219-222), including ERK5 (223). However, extracellular H_2O_2 did activate ERK1/2 in some cells but not in others. Modulation of GSH levels also plays a role in the activation of JNK and p38 MAPK, as shown after treatment with alkylating agents (224). Furthermore, many studies have implied involvement of ROS in MAPK activation after cell stimulation with various agents based on inhibition by catalase or by compounds with antioxidants properties (reviewed in (225)). The prevention of H_2O_2 accumulation by antioxidants blocked MAPK activation after stimulation by LPA, angiotensin and serotonin, all ligands for G-protein coupled receptors (226-228). Despite this mounting evidence, the mechanisms by which exogenous or endogenously produced ROS activate the MAPK are still not well defined.

5.2. Proposed Mechanisms for ROS-Mediated MAPK Activation

5.2.1. The Small GTPases

As mentioned in the first section of this review, the small GTPases play an essential role in the activation of the MAPK. Ras, in particular, may be upstream of all cascades. Overexpression of oncogenic Ras in transformed fibroblasts induces constitutive production of large amounts of ROS that was required for the mitogenic response (188). However, activation of ERK1/2 in these transformed fibroblasts was decreased compared to controls and, while JNK activity was not increased by overexpression, the JNK activity stimulated by TNF-alpha was higher and appeared redox-regulated (188). Others showed increased $O_2^{\cdot ?}$ production by oncogenic H-Ras in keratinocytes (229) and in epithelial cells (230). A role for oncogenic Ras in DNA repair was shown to require a PI3K/Rac1/ROS pathway (231). Nevertheless, the exact mechanisms of these effects by oncogenic Ras are not known.

One of the first demonstrations of a direct effect of oxidants on endogenous Ras proteins was shown with NO in T-cells (232). Ras contains several cysteines, one of which, C118, is directly altered by NO, forming a nitrosothiol derivative. NO did not affect a C118 to S mutant (232). This cysteine is close to the guanine nucleotide binding site and, based on circular dichroism data, it was suggested that the modified cysteine induced a conformational change, resulting in enhanced nucleotide exchange (233,234). Interestingly, the three-dimensional structure of the C118S mutant by NMR showed no disruption of the protein structure and no effect on the GEF and GAP activities (235), suggesting that the mechanism of Ras activation by NO is not due to lack of interaction between the cysteine and neighboring residues but may involve a different interaction (235). Others have suggested that the modification of H-Ras by NO donors

may be more extensive and may oxidize cysteines normally targeted for lipid modification (236). NO-modified Ras activated PI3K (237) and three groups of MAPK in T-cells (238,239). H_2O_2, hemin and $HgCl_2$ also modified Ras on the same cysteine and induced an increase in ERK activity (233). Thus, these experiments have suggested that direct targeting of Ras by ROS participate in activation of the MAPK (figure 6), although the data are still limited and upstream activators have also been implicated and the consequences of activation of the MAPK may be diverse (see below and (240)).

5.2.2. The Src Kinases

The Src kinase family members all share the same structure with SH1, SH2 and SH3 domains and are post-translationally palmitoylated, which targets them to the membrane and in particular to lipid rafts with other signaling components, as well demonstrated in T-cell signaling (241). Activation of the Src kinases requires dephosphorylation/phosphorylation events (reviewed in (242)). The kinases are maintained in an inactive conformation through an intramolecular interaction between the SH2 domain and a tyrosine phosphorylated by the Csk tyrosine kinase (Y527 in c-Src). The closed conformation is stabilized by SH3/linker region interactions. Dephosphorylation of this tyrosine by PTP, such as CD45, a membrane PTP, and various cytosolic PTP, opens the structure and permits activation by autophosphorylation of a tyrosine (Y416) in the activation loop, which alters the three-dimensional structure to allow phospate transfer. Dephosphorylation of tyrosine residues in the autophosphorylation sites results in downregulation of the activity. G protein-coupled receptors, T-cell receptors and cytokine receptors require activation of the non-receptor tyrosine kinases of the Src family for their downstream signaling, including activation of the MAPK (243-245). Activation of c-Src is one of the earliest steps of the UV response leading to JNK activation (246) and several members of the family were shown to be activated by oxidation by H_2O_2, NO, peroxynitrite, diamide and others (247-250). Thus, activation of the MAPK by ROS may be the result of activation of the Src kinase family. In fact, c-Src was found to be required for the H_2O_2-induced activation of ERK5 (223,251) and JNK but not for ERK1/2 or p38MAPK (252). Another member of the Src family, Fyn, was necessary for the H_2O_2-induced activation of Ras and ERK1/2, probably through activation of JAK2 (253).

The mechanisms involved in the activation of the Src kinases by ROS are still unclear. Oxidation of specific cysteines causes activation of the kinase activity. A number of cysteines are present in c-Src, and four of them, clustered in the kinase domain, are important for protein stability and function (254) (figure 6). It was proposed that oxidation of these cysteines by ROS may destabilize the closed structure to activate the kinase (255,256), although this has not been definitely proven. Furthermore, it is unclear whether oxidation to an irreversible form (sulfonic acid) may not be required, although a study of the activation of Lyn and Hck induced by peroxynitrite, showed that the activation of Hck involved reversible cysteine-dependent oxidation while that of Lyn was cysteine-independent (257). Tyrosine nitration may be involved, as demonstrated for peroxynitrite-induced activation of c-Src (258), although the reversibility of this type of reaction and its role in signaling is unknown (259). Others proposed that clustering of membrane rafts, which occurs when cells are exposed to heavy metals, might induce cross-linking of signaling molecules that are attached to the rafts, including the Src kinases. ROS production and activation of JNK was observed in T-cells following raft clustering. This mechanism was linked to cell death by apoptosis (260). Inhibition of tyrosine phosphatases was also suggested to induce activation of the Src kinases in isolated T-cell membranes after treatment with H_2O_2 (261). Thus, while it seems that the Src kinases may play a role in activation of the MAPK by ROS, further work is needed to identify the exact mechanisms of activation, which may differ between the various members of the Src family.

5.2.3. Thioredoxin and ASK1 in JNK/p38MAPK Activation

Thioredoxin (TRX) is part of a large family of ubiquitously expressed proteins that share the same dithiol structure in their catalytic site. One of the cysteines is in the form of a thiolate and can be oxidized. Formation of a disulfide bond with the proximal cysteine in the active site can then occur. Oxidized Trx is reduced by a Trx reductase at the expense of NADPH (262). Trx is involved in the redox regulation of various transcription factors (263) and is a physiological inhibitor of ASK1, the MAPKKK upstream of JNK and p38MAPK, which is required for apoptosis (98). Trx directly binds to ASK1, an interaction that is dependent on the redox status of Trx. Oxidation of Trx by ROS releases ASK1, which is then activated (264) (figure 6). TNF-alpha activated ASK1 through ROS production, ASK-1 homo-oligomerization and interaction with TRAF-2 (265,266). It was later suggested that ASK1 forms oligomers in non-stressed cells and that H_2O_2 induces the phosphorylation of T845 in the activation loop that is necessary for ASK1 activation (267). Transphosphorylation due to changes in conformation in the pre-formed oligomers or phosphorylation by an unknown kinase may be involved (267). Interestingly, a serine/threonine phosphatase, PP5, recently identified, directly binds ASK1 and dephosphorylates T845, thereby inhibiting ASK1 activity (268). H_2O_2 induced binding of PP5 to ASK1 while releasing Trx, indicating different roles for the two inhibitors, although the doses of H_2O_2 necessary were in the high range (1 mM and above), which may be more relevant for oxidative stress than redox signaling (268). In fact, ASK1 was also found to be essential for neuronal cell death induced by endoplasmic reticulum (ER) stress and the ASK1knockout mouse was deficient in ER stress-induced JNK activity (269). ASK1 knockout mice also exhibited lower levels of JNK and p38MAPK activation in comparison to wild type after H_2O_2 or TNF stimulation (98). Further studies will be required to determine whether both the JNK and p38MAPK are required for these effects.

5.2.4. Other Mechanisms

There is little evidence for direct targeting of the MAPK by ROS. One study showed S-nitrosylation of

JNK1 by endogenously produced NO in interferon-gamma stimulated macrophages, resulting in inhibition of the kinase activity (270). However, ROS-dependent complex dissociation was demonstrated for JNK, which is inactive in non-stressed cells because of its association with the monomeric form of glutathione S-transferase Pi (GSTp) through interaction with the C-terminus of JNK (102). ROS induce oligomerization of GSTp and release from JNK, which may result in JNK activation (100). Regulation of the MAPK activity by DSP and PTP through oxidation of their critical cysteines is another mechanism that can be evoked; however, no significant data have been reported so far. While the mechanism of reversible oxidation of PTP is gaining acceptance, their particular targets in the MAPK pathways are not clearly defined. The study by Tonks's group suggests that the PDGF receptor is a target for ROS-modified SHP-2 {4268).

Two other signaling components have been shown to play a role in ROS-mediated signaling that are worth mentioning, although their role may be more relevant to oxidative stress. The alpha subunits of the G_i and G_0 families were activated by treatment of myocytes with H_2O_2, independently of receptor activation and leading to ERK activation {4503}. Studies with purified proteins showed that various cysteine residues were modified after treatment with H_2O_2, two of which, C287 and C326 being required. Nevertheless, this effect was dependent on Fe^{2+} and the formation of more reactive species, including the highly reactive hydroxyl radical (271); thus, the relevance to redox signaling still needs to be demonstrated. The SHC proteins have also been suggested to play a role in ROS-mediated events. Three genes, SHCA, B & C encode for the different proteins, of which only SHC A is ubiquitously expressed as three isoforms, p46, p52 and p66. All possess two distinct domains, SH2 and PTB, which bind proteins containing phosphotyrosine (reviewed in (272)). Their critical role in ERK activation was confirmed by a knockout mouse model where the exons encoding for the PTB domain were targeted, resulting in lack of expression of the three isoforms. The mice died in utero and dose response analyses in MEF showed that a 50- and 25-fold increase in EGF and PDGF doses respectively was required to detect ERK activation comparable to that seen in wild type (273). The p66 isoform appears to be serine-phosphorylated upon activation by stress such as H_2O_2 or UV exposure but not after EGF stimulation (273) and JNK is responsible for phosphorylation at S36 (274). MEF from mice with a targeted mutation that affected only the expression of the p66 isoform were more resistant to H_2O_2 and UV. Similarly, p66SHC knockout mice were more resistant to paraquat treatment and had increased life span (275). H_2O_2 negatively regulated the activity of the mammalian forkhead homolog, FKHRL1 through a p66 SHC-dependent pathway (276) and a p53/p66SHC pathway appears to regulate the steady-state levels of ROS and the levels of oxidative damage in mammalian cells (277). Thus, the response to oxidative stress in different tissues may depend upon the levels of expression of the p66SHC isoform.

5.3. NADPH Oxidase and MAPK Activation in Phagocytes

In phagocytes such as neutrophils and macrophages, receptor/ligand interaction during phagocytosis of bacteria or particles and upon stimulation with a variety of soluble agents regulates $O_2^{\bullet ?}$ production through activation of the NADPH oxidase. This enzyme, dormant in non-stimulated cells, requires assembly of the transmembrane flavocytochrome ($p22^{phox}$/$gp91^{phox}$} with the cytosolic proteins ($p47^{phox}$/$p67^{phox}$/$p40^{phox}$) that translocate to the plasma membrane to form a stable complex, competent for electron transfer (278). The $gp91^{phox}$ subunit is the terminal oxidase that transfers one electron to oxygen to form $O_2^{\bullet ?}$, which then quickly dismutates to H_2O_2. While some evidence indicates that assembly of the oxidase may occur intracellularly in neutrophils (279), it is currently thought that this process occurs at the plasma membrane in macrophages.

Extensive literature has documented the involvement of ROS in bacterial killing, in particular in neutrophils where myeloperoxidase, which is stored in the azurophilic granules, is essential for this process, although this has recently been challenged (280). Until recently, the potential role of the ROS produced by the phagocyte NADPH oxidase in the signaling pathways of these cells had not been explored. Early studies in permeabilized neutrophils (281) and later in intact cells (282) had shown ROS-dependent increase in tyrosine phosphorylation. Work in our laboratory demonstrated that stimulation of rat alveolar macrophages with zymosan-activated serum (ZAS), a source of C5a that binds to a G protein-coupled receptor and induces production of ROS, resulted in activation of ERK1/2 that required the presence of H_2O_2. Extracellular catalase significantly reduced the activation of ERK1/2 while marginally affecting that of p38MAPK, also activated under those conditions (283). MEK1/2 activation was also inhibited by catalase, indicating that ROS targeted an upstream component of the pathway (283). Pre-treatment of a rat alveolar macrophage cell line (NR8383) with vanadate, a well-known inhibitor of PTP, allowed activation of ERK1/2 by ZAS in the presence of catalase, indicating that vanadate relieved the block by catalase and could substitute for H_2O_2 (284). This suggests that a PTP, inhibited by H_2O_2 or vanadate allows the signal to go through and targets a component of the cascade that must remained tyrosine phosphorylated (figure 6). At this point, c-Raf and the Src kinases are the best candidates for the ROS-targeted component (284). In fact, a recent paper showed the ROS-and tyrosine phosphorylation-dependent activation of c-Raf by UV light (285). Others found that the ROS produced by phagocytosis of fibers in rat alveolar macrophages induced activation of ERK and p38MAPK (286). Recent studies have shown that LPS stimulates ROS production in macrophages via a mechanism that is partly dependent on the NADPH oxidase. LPS in these cells activated all three MAPK (287).

5.4. MAPK and Gene Expression in Redox Signaling

Although MAPK activation by endogenously produced ROS has been associated with proliferation or

apoptosis, only a few studies have so far demonstrated changes in gene expression as downstream events of their activation. The effects on gene expression of angiotension II (AngII), which induces the production of ROS, have been studied in several cell types. In cardiac fibroblasts, AngII activated all three MAP kinases in a ROS-dependent manner, resulting in increase in IL-6 gene expression through phosphorylation of the CREB transcription factor by the ERK/RSK and p38MAPK pathways (288). In cardiac endothelial cells, AngII induces expression of osteopontin through ERK activation in a ROS-dependent manner (289). Endogenous production of ROS by AngII, but also by PDGF and TNF-alpha resulted in the MAPK-dependent increased expression of monocyte chemotactic protein (MCP-1) in vascular smooth muscle cells (VSMC) (290). Expression of the early growth reponse-1 (Egr-1) transcription factor by cyclic strain was regulated by the ROS-mediated activation of the ERK pathway in endothelial cells (291). Another study reported that the endothelin-1 (Et-1) gene expression by cyclic strain in endothelial cells was also mediated through ROS activation of the ERK pathway. The Et-1 promoter has an AP-1 site and treatment with catalase reduced ERK activation and strain-induced promoter activity (292). Aortic rings from mice lacking the $p47^{phox}$ exhibited lower ROS production than wild type mice after treatment of the mice with AngII, as did cultured VSMC from the same mice treated with thrombin. Activation of p38MAPK by thrombin and expression of the redox sensitive VEGF mRNA by PDGF were decreased in these cells, indicating that a $p47^{phox}$/ROS/p38MAPK pathway was involved in VEGF expression (293). GSH depletion by diethylmaleate and ensuing oxidative stress induced cell cycle arrest at the G1 phase and the p53-independent, ERK-dependent increase in mRNA of the cyclin-dependent kinase inhibitor, $p21^{waf1/cip1}$, indicating that cells may use similar pathways in response to oxidative stress as in redox signaling (294).

Macrophages secrete various cytokines and their expression may be a function of the redox status of the cells (295). Modifying the GSH/GSSG ratio in human monocytes resulted in activation of p38MAPK by LPS and increased production of IL-12 (296). Production of TNF-alpha induced by silica in primary alveolar macrophages and in RAW 264.7 was inhibited by SOD and catalase (297). Lipopolysaccharide (LPS)/endotoxin is known to induce TNF-alpha and interleukin-1 (IL-1) production in monocytes and macrophages. Production of IL-1 in LPS-stimulated macrophages was shown to be dependent on ROS and a PTK/PI3K/Rac/p38 pathway (287). Thus, ROS-mediated activation of the MAPK pathways may play a significant role in regulation of the cytokine genes and may act in concert with the NF-kappa B pathway (298), a transcription factor that is also redox-sensitive and is critical for cytokine expression. In Kupffer cells, the resident macrophage in liver, the production of TNF-alpha was regulated by ROS-activated NF-kappa B pathway (299).

6. SUMMARY AND PERSPECTIVES

The MAPK are essential components of the complex intracellular networks that regulate gene expression and cell function. In recent years, a strong consensus has emerged that ROS play a significant role in the regulation of various signaling pathways and that MAPK activation can be modulated by ROS and other oxidants such as NO and congeners, resulting in alteration of gene expression. Nevertheless, further studies are required to determine the chemical modifications induced by ROS and their specific targets in these pathways. Based on the current knowledge, we would predict that the targeted molecules are more likely to be upstream of the MAPK themselves and may differ with cell type and stimuli or with the site of ROS production. The involvement of thiols is not surprising as thiol-containing compounds are essential in many biological functions, although how S-thiolation or S-glutathionylation affect the structure of the targeted protein and thereby its activity will need to be determined. Newly developed techniques such as mass spectroscopy/MALDI-TOF and others will greatly help in that respect.

As for all signal transduction studies, the methods used, whether it is overexpression or the breaking of the cells, may induce artifacts such as disrupting essential association or perturbing the stoichiometry of protein-protein interactions. Imaging techniques that allow studying this interaction *in vivo* would be of great benefit. Several studies have recently been initiated, using fluorescence resonance interference (FRET) to study the interactions of signaling components *in vivo* (300), for example to study the spatio-temporal activation of Ras and Rap1 (301). Fluorescence reporters have been developed to study kinase activity (302) and, recently, lipid modified monomeric green fluorescence proteins were used to study lipid partition into membrane microdomains, demonstrating that acyl modification promote clustering of lipid rafts (303). These types of technology will allow a better understanding of protein-protein and lipid-protein interactions *in vivo.* As new mouse models for signaling components are developed, their use or that of cells derived from these models in redox-related research should also benefit the field and be favored over the use of inhibitors of unclear specificity, when possible. It is also important to keep in mind that your pathway of choice, here the MAPK, is not the only one to be affected by ROS as other signaling molecules/pathways such as transcription factors, the AKT pathway, the NF-kappaB pathway, p53, caspases and others are affected by ROS. In addition, reactive nitrogen species (RNS) may also participate in redox signaling; thus, biological outcomes should be interpreted in the context of multiple ROS and RNS targets. One can foresee several more years of exciting research in this field.

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