CHAPTER II

LITERATURE REVIEW

Cytoskeleton (Norman and Lodwick, 1999; Janmey, 1998)

In eukaryotes, the cytoskeleton is a complex framework of structural protein filaments that defines the shape of cell. It is a dynamic structure, and is responsible for changes in cell shape and cell movement. It also contributes to the movement of intracellular organelles and other cytoplasmic components and is, therefore, important in organization and microcompartmentalization within the cell. In contractile muscle cells the cytoskeleton is further modified to provide the contractile machinery.

Cellular cytoskeletons can make attachments that link them to the extracellular matrix. Several specialized junctional complexes have been partially characterized that link particular cytoskeletal structures to specific extracellular structures. In each case the connection is made by a transmembranous protein that interacts with components on both sides of membrane. At the intracellular surface of the membrane, cytoskeletal elements are attached to the transmembrane proteins by accessory proteins such as α-actinin, talin and vinculin. The junctional structures are highly complex and sensitive to phosphorylation at multiple sites. Therefore, intracellular signaling pathways can impinge on cytoskeletal activity through these structures.

The cytoskeletons of all cells are based on three basic filamentous structures- **microtubules** (24 nm diameter), **intermediate filaments** (10 nm diameter) and **microfilaments** (7 nm)- in which tubulin, intermediate filament proteins and actin form the core structure, respectively. Depending on the cell type, cytoskeletons may be composed of just one filamentous element or a combination of two or three of the basic structures. In addition, a large number of accessory proteins can be involved. These confer the cell-specific and dynamic properties to the cytoskeleton. Accessory proteins can influence the position of the cytoskeleton within the cell by controlling the length of the filamentous structures and their association with other protein complexes, organelles, membranes and the extracellular matrix. In addition, they can permit modification of the

cytoskeleton in response to intracellular metabolic changes and their intracellular signaling psthways, in particular changes in $[Ca^{2+}]_i$.

Each of the core structural proteins forms helical filaments, which have a polarity due to chemically distinct heads and tails. Each protein exist in multiple isoforms, some of which are tissue specific.

Modulation of the cytoskeletal network changes the mechanical properties of the cell that are essential for functions such as locomotion and cytokinesis. The filamentous cytoskeletal network also provides a scaffolding on which motor proteins such as kinesin, dynein, and myosin can translocate to move organelles or generate internal stress. The transmembrane receptors and intracellular signals producing cytoskeletal changes in response to extracellular stimuli have been extensively studied. The cytoskeleton undoubtedly regulates cellular mechanics, but there are probably other important functions of this ubiquitous and extensive organelle. Independent of its mechanical strength, the filaments of the cytoskeleton form a continuous, dynamic connection between nearly all cellular structures, and they present an enormous surface area on which proteins and other cytoplasmic components can dock.

On one hand, some proteins bind to the cytoskeleton to alter its assembly in response to cellular signals. On the other hand, probably hundreds of proteins copurify with the cytoskeleton of detergent-permeabilized cells, often to an extent that depends on activation of specific cellular signals. Many such proteins have been shown to bind purified F-actin or other cytoskeletal filaments in vitro with affinities characterized by micromolar dissociation constants. Because the cytoplasmic concentration of actin and other cytoskeletal filaments is one the order of 10-100 μM, a major fraction of such proteins would be expected to bind the cytoskeleton in vivo.

With regard to the complexity of the cytoskeleton, the intricate association of one filament with another, and the relative high concentration of the cytoskeletal proteins, various criteria have been used to define instances where the cytoskeleton binds or otherwise alters elements of signal transduction pathways. In many cases, linkage of a signaling molecule to the cytoskeleton is detected by sedimentation from detergent-permeabilized cell extracts under conditions where it would, in the absence of the cytoskleton, remain in the supernatant. Such data are often supplemented by finding that

the coprecipitation with the cytoskeleton depends on whether the cell is activated by specific agonists, or that selective disruption of cytoskeletal elements alters its solubility. Whether coprecipitation depends only on proteins of the cytoskeleton is often difficult to ascertain because complexes such as focal adhesions, cell-cell junctions, and caveoli localize to sites involving both cytoskeletal proteins and membrane lipids where multiple signaling molecules concentrate.

Among the components localized to the cytoskeleton are ribosomes and RNA, and a few examples of proteins involved in the translational machinery that interact specifically with cytoskeletal filaments. Transmembrane protein complexes that link cells to each other or to the extracellular matrix not only link the cell membrane to the actin or intermediate filament cytoskeleton but also localize and activate signaling molecules that ultimately lead to changes in cell structure and gene expression.

Microfilaments (Norman and Lodwick, 1999)

The major constituent of microfilament is the globular protein actin, which is a component of all eukaryotic cells. The actin monomer (G-actin) possesses sites for divalent cations and nucleotides and binds Mg^{2+} and ATP. Monomers can associate to form dimers and trimers, and trimers are sufficiently stable to form nucleation centers for the further polymerization into actin filaments (F-actin). Actin filaments consists of two strings of monomers in a right-handed helical structure which achieves one turn every 14 actin monomers. Each actin monomer has a polarity and monomers associate head to tail. Polarity of the growing microfilament can be defined by the association of heavy meromyosin (a proteolytic fragment of the actin-binding protein myosin containing the two globular myosin heads), which forms an arrowhead pattern on the filament and the poles of the microfilament can be designated as the pointed (-) and barbed (+) ends. Elongation can occur at both ends but is usually faster at the barbed (+) end. G-actin contains either bound ATP or ADP. The rate of binding of G-actin ATP to growing microfilaments is much faster and binding is followed by rapid ATP hydrolysis, although this is not essential to polymerization. ATP hydrolysis allows a microfilament to treadmill, i.e. G-actin-ATP monomers are added at one end while G-actin-ADP dissociates from the other. Growing microfilaments can also break, generating new free

ends. These can behave as nuclei for further polymerization or may anneal with a second microfilament.

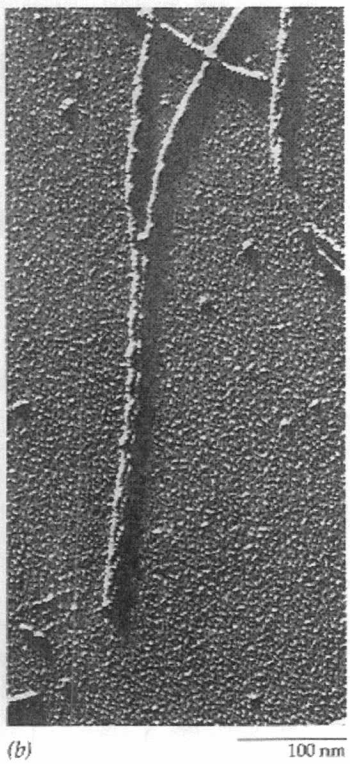

Figure 1 Electron micrograph of a replica of actin filament showing its double-helical architecture (Karp, 1999).

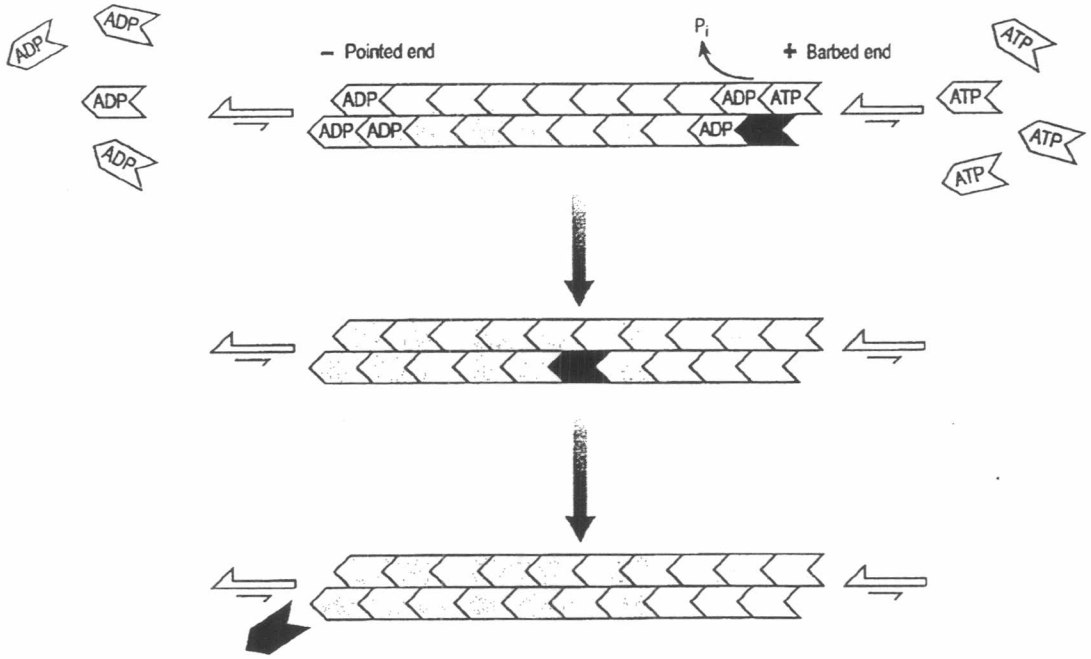

Figure 2 Treadmilling in actin microfilaments illustrated by the passage of the shaded actin molecule through the filament. Capping of microfilament ends with accessory proteins can prevent treadmilling (Norman and Lodwick, 1999).

Actin Cytoskeleton (Kreis and Vale, 1993)

Actin was originally purified from skeletal muscle tissue and further recognized as the major protein of the microfilament system of eukaryotic cells. It interacts with a large number of actin associated proteins and this results in a variety of stable and transiently regulated different supramolecular organization. Actin is also one of the key proteins in various cell motility processes which are either based on actin gel-sol transitions or ATP-dependent actomyosin interactions.

Actin is found in all eukaryotic cells examined so far. It contains 375 amino acids and has a sequence which is highly conserved throughout evolution, suggesting its central pivotal role in important cellular processes. Warm blooded vertebrates (mammals and birds) express six distinct isoproteins. They are expressed solely in a tissue specific manner, independent of the species; two striated muscle actins (skeletal muscle and

cardiac actin), two smooth muscle actins (vascular and visceral actin) and two coexpressed nonmuscle actins. Isoelectric focusing separates these isoforms into three isoelectric variants, referred to as α-, β-, γ-actin. They form a typical abundant protein triplet in a 2-D gel system (pI ≈ 5.1, M_r = 42,000). No differences were found in their polymerization properties and their segregation into different cellular structures is not clearly established. Isoactins could interact differently with enzymes such as the ADP-ribosyl-transferase or actin binding protein such as myosin.

Actins are all N-terminally acetylated and this modification follows two different pathways, depending on the actin type. Most actins also contain a 3-methyl-histidine residue located at a very conserved position (His-73). Other types of covalent modifications are ADP-ribosylation, phosphorylation, methylation, and ubiquitination. Some of these modifications affect F-actin polymerization.

The 3-D structure of actin in the actin-Dnase 1 complex has been obtained at 2.8 A° resolution. The molecule consists of two nearly equal halves each further subdivided into two subdomains. This structure is extremely similar to that of the N-terminal ATPase fragment of the 70 kDa heat shock cognate protein. Models for F-actin have been constructed in accordance with fiber diffraction patterns and image analyses. Most of the effects of chemical modification, crosslinking data and effects of site-directed mutagenesis previously obtained could be explained by these models. Another model of actin organization has been deduced from the X-ray diffraction patterns of the profilin-actin complex.

Actin gene expression is regulated in a developmental and tissue specific manner both in lower and in higher eukaryotes. Tissue specific regulatory sequences have been recognized.

Actin is an essential protein as illustrated by the lethal effect following disruption of the single yeast actin gene. Mutations of actin cause defects in muscle or cytoskeletal organization and, in some cases are accompanied by increased tumorigenicity. An unusual actin mutant is the *v-fgr* oncogene product which is a hybrid protein containing the first 129 amino acids of γ-nonmuscle actin linked to a tyrosine specific kinase.

Actin and Activities

In low salt buffers, actin is a monomeric protein (G-actin) but it associates under physiological conditions into a double helical 10 nm thick filament structure (F-actin). Actin polymerization is accompanied by hydrolysis of the actin bound ATP, although nucleotide hydrolysis is not obligatory for addition of monomers to polymers. F-actin filaments are formed by adding monomers at both ends with different kinetics. There is a fast and a slow growing end, and this polarity can be visualized by decoration with heavy meromysin into arrowhead-like structures pointing towards the slow growing end. Several drugs bind to actin and exhibit a profound effect on the polymer structure. Phalloidin binds strongly to F-actin in a ratio of one molecule of phalloidin per protomer. Another class of fungal metabolites (the cytochalasins) bind to the barbed end of the filament.

Microfilamentous Structures in Non-Muscle Cells (Norman and Lodwick, 1999)

Actomyosin structures are also found in non-muscle cells where contractile properties are required. For example, actomyosin contributes to intracellular stress fibers, which counter the forces imposed by the extracellular matrix. In cell culture, actomyosin is responsible for the flattening of attached cells and depolymerization results in the rounding up of cells after cell detachment. In locomotion a cell moves by extending flattened protrusions called lamellipodia, which form attachment sites at points of interaction with the substratum. These structures are known to contain actin and the actin-binding proteins α-actinin and vinculin. In the retreating tail of a moving cell, depolymerization of actin filaments and formation of an actin network is thought to be important. Fungal cytochalasins, which bind to the growing (+) end of actin filaments and block polymerization, also block cell locomotion. Actomyosin structures are also important during telophase in the cell cycle to form the contractile ring that begins cell division.

Unlike striated muscle the contractile apparatus in non-muscle cells is constantly dissociating and reforming depending on the state of stimulation of the cell. In the inactive, dephosphorylated form, the myosin molecule takes up an independent globular form. On phosphorylation of the myosin light chain by MLCK, the myosin molecule

extends to its rod-like form, releasing the tail domain to form bipolar thick filaments and the head domain to form cross-bridges with thin filament actin.

Controlling the Assembly of Stress Fiber and Focal Adhesions (Karp, 1999)

Focal adhesions are relatively complex macromolecular structures involving extracellular components, plasma membrane and cytosolic proteins, and actin filaments of the cytoskeleton. The study of focal adhesion assembly provides insights into mechanisms by which cells regulate shape and internal structure through directed changes in the organization of the cytoskeleton. The assembly of focal adhesions is initiated when integrins of the plasma membrane become bound by extracellular ligands. The signal for the assembly of a focal adhesion is routed from the bound integrin to a key GTP-binding protein called Rho. Rho is a small G protein that is similar in size and structure to Ras; in fact, both are members of the same family of regulatory proteins. Like Ras, Rho is a molecular switch that determines whether or not signals are transmitted along certain pathways. In this case, the signaling pathways regulate the organization of the cell's actin cytoskeleton. Cells lack both focal adhesions and organized actin filaments when the cells are quiescent (nonstimulated). When these cells are exposed to certain growth factors, signals are transmitted through Rho, leading to the assembly of focal adhesions that contain well-defined stress fibers. There are two pathways through which activation of Rho influences cytoskeletal organization. In one of these pathways, Rho activates a lipid kinase (phosphatidylinositol 5-kinase) that transfers a phosphate group onto the inositol ring of phosphatidylinositol 4-phosphate (PIP) to produce phosphatidyl 4,5-bisphosphate (PIP_2). PIP_2 acts as a second messenger by binding to a number of target proteins. Included among the PIP_2 targets are several actin-binding proteins (e.g., profilin and gelsolin) that control the state of polymerization of actin molecules. Binding of PIP_2 to these proteins stimulates the polymerization of actin monomers into actin filaments. In the other pathway, Rho activates a kinase, called Rho kinase, which phosphorylates and inactivates the enzyme myosin light-chain phosphatase. Myosin activity is stimulated by phosphorylation and inhibited by dephosphorylation. Consequently, inactivation of myosin phosphatase leads to activation

of myosin, which plays a key role in assembling actin filaments into organized stress fibers.

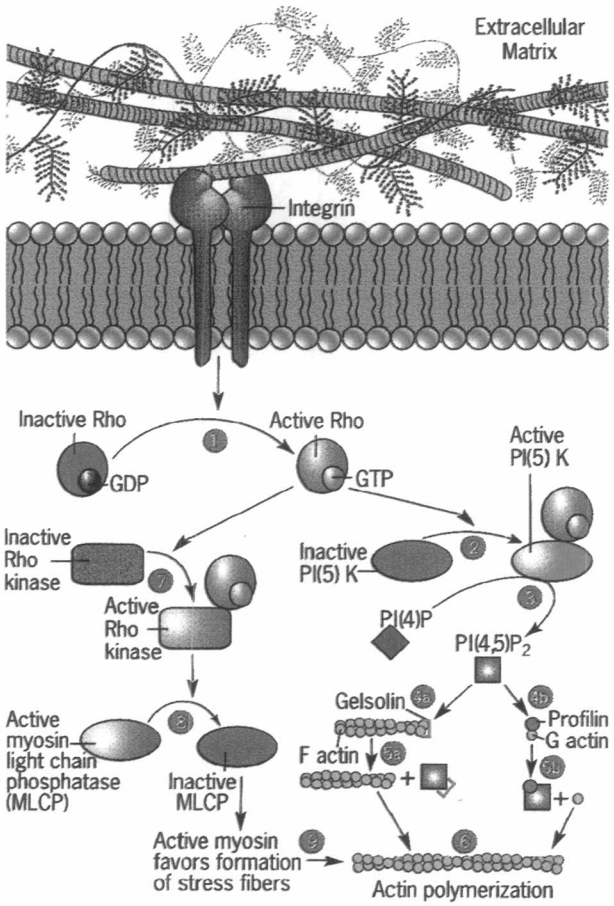

Figure 3 Signal pathways that lead to the assembly of the stress fibers of a focal adhesion (Karp, 1999).

Rho GTPases

The cell, in its tissue environment, receives many signals that act on membrane receptors. This leads to the activation of multiple signal transduction pathways and a particular cellular response is generated after integration of these via intracellular effectors. Small GTPases of the Rho family are pivotal regulators of several signaling networks that are activated by a wide variety of receptor types. When activated, Rho

GTPases affect many aspects of cell behavior, including actin cytoskeleton dynamics, transcription regulation, cell cycle progression, and membrane trafficking.

The mammalian Rho GTPases can be grouped into six different classes consisting of the following members: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG), Cdc42 (Cdc42Hs, G25K, TC10), Rnd (RhoE/Rnd3, Rnd1/Rnd6, Rnd2/Rho7), RhoD, and TTF (Aspenstrom, 1999). Like all other regulatory GTP-binding proteins, Rho GTPases act as molecular switches cycling between GDP- and GTP-bound states. When bound to GDP they are inactive; upstream events lead to the exchange of GDP for GTP and the protein switches into an active conformation. It is this form of the protein that can interact with downstream targets or effector molecules to produce a biological response. An intrinsic GTPase activity completes the cycle.

At least three classes of molecules are capable of interacting with Rho GTPases and regulating their activation state: (i) guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, (ii) GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity, and (iii) guanine nucleotide dissociation inhibitors (GDIs) inhibit the exchange of GDP for GTP and might also serve to regulate their association with membranes.

In recent years, there has been much progress in identifying cellular targets that interact with the active form of Rho GTPases and thereby mediate their functional activity.

Activation of Rho

Lysophosphatidic acid (LPA) was the first agonist identified to activate Rho (Ridley and Hall, 1992). This lipid induces actin stress fiber and focal adhesion assembly in serum-starved Swiss 3T3 fibroblasts and this can be blocked by prior incubation of cells with C3 transferase to inhibit Rho. LPA acts through one or more heptahelical G-protein-coupled receptors (GPCRs) and several other ligands acting through GPCRs are known to activate Rho, e.g. sphingosine 1-phosphate, bombesin, and endothelin (Seasholtz et al., 1999). Signal transduction through GPCRs is initiated by agonist-induced dissociation of a heterotrimeric G-protein into its Gα and Gγβ subunits, both of which can initiate downstream signals. To date, four major families of α subunits have

been identified, $G_{i/o}$, G_s, G_{q11}, and $G_{12/13}$, and the LPA GPCRs can activate several of these.

The first experiment to suggest which G-proteins might be important in Rho activation involved injection of different α subunits into fibroblasts (Buhl *et al.*, 1995). This approach revealed that $G\alpha_{12}$ and $G\alpha_{13}$ could activate Rho, whereas $G\alpha_{i2}$ could not. Constitutively active versions of $G\alpha_{12}$ and $G\alpha_{13}$ both induce a range of Rho-dependent responses such as stress fiber formation and SRF and phospholipase D activation in fibroblasts, as well as neurite retraction in a neuroblastoma cell line. Further analysis has revealed that LPA-induced Rho activation is in fact mediated by $G\alpha_{13}$, while thrombin appears to act through $G\alpha_{12}$ (Gohla *et al.*, 1998). One involves a direct link between the Gα subunit and Rho through a GEF. Hart *et al.* (1998) and Kozasa *et al.* (1998) found that p115RhoGEF can bind directly to both $G\alpha_{12}$ and $G\alpha_{13}$ through an N-terminal RGS domain that encodes GAP activity toward Gα subunits. Furthermore, $G\alpha_{13}$, but not $G\alpha_{12}$, is able to enhance p115RhoGEF-mediated GDP-GTP exchange on Rho in an *in vitro* assay. Similarly, using SRF activation as a readout, Mao *et al.* (1998) observed that p115RhoGEF worked synergistically with $G\alpha_{13}$ but not with $G\alpha_{12}$, confirming the specificity of this mechanism of Rho activation.

Although $G\alpha_{13}$ can activate Rho through p115RhoGEF additional signaling events must also be involved *in vivo*, since stress fiber formation and neurite retraction are blocked by tyrosine kinase inhibitors acting upstream of Rho (Gohla et al., 1998; Katoh et al., 1998; Nobes and Hall, 1995). One group has suggested that the tyrosine kinase involved is the EGF receptor since a dominant negative version of the receptor blocked LPA/ $G\alpha_{13}$-induced Rho activation. This mechanism thus appears to be similar to the ligand-independent activation of this receptor previously observed by others (Daub et al., 1996). Whether the EGF receptor plays a more ubiquitous role in Rho activation pathways remain to be seen. Importantly, $G\alpha_{12}$-induced Rho activation is insensitive to inhibition of EGFR activation leaving the mechanism downstream of this subunit unknown.

While the involvement of $G\alpha_{12}$ and $G\alpha_{13}$ in the control of Rho-dependent processes is well documented, the role of other Gα subunit is less clear. $G\alpha_i$ does not

seem to signal to Rho in most cell types tested (Buhl *et al.*, 1995), although it may play a role in the fMLP-induced GTP-loading of Rho in neutrophils (Laudanna *et al.*, 1996). Activated $G\alpha_q$ has been shown to induce Rho-dependent SRF activation, neurite retraction, and expression of hypertrophy-related genes (Kato *et al.*, 1998). In addition, agonists acting on the muscarinic M1 and the metabotrophic glutamate 1α receptor require $G\alpha_{q/11}$ expression for the stress fiber induction in mouse fibroblasts (Gohla *et al.*, 1999). Since $G\alpha_q$ does not interact with the $G\alpha_{12/13}$-binding Rho GEFs, a different pathway must be involved in signaling from this G protein. This pathway seems to require PKC activation and Ca^{2+} mobilization as well as the activity of a tyrosine kinase (Katoh *et al.*, 1998). The dependence on tyrosine kinase activity and the observed ability of the muscarinic M1 receptor to activate the EGF receptor in a PKC-dependent, but EGF-independent, way (Tsai *et al.*, 1997) suggest that $G\alpha_q$ could signal to Rho via an EGF receptor-dependent pathway similarly to $G\alpha_{13}$. In fact, Gohla *et al.* (1999) have presented evidence to suggest that $G\alpha_q$-dependent stress fiber formation also requires $G\alpha_{13}$ expression, suggesting that $G\alpha_q$ could induce EGFR transactivation via $G\alpha_{13}$.

The transmission of cell surface signals to Rho is not exclusively limited to GPCRs. EGF is a weak activator of Rho in serum-starved Swiss cells, an effect which has been proposed to occur via Rac activation of phospholipase A2 and subsequent arachidonic acid and leukotriene production (Peppelenbosch *et al.*, 1995). It is possible that this involves secretion of the leukotrienes followed by a new round of cell surface to Rho signaling, thus making it a rather indirect way of Rho activation, but this has not been demonstrated. Another example of Rho activation that also appears to include a Rac to Rho signaling step involves the cytokine receptors for TNF-α and IL-1. In Swiss 3T3 fibroblasts both TNF-α and IL-1 activate Cdc42, but this leads to the activation of Rac and finally Rho (Nobes and Hall, 1995; Puls *et al.*, 1999). The effect of TNF-α on Rho is most clearly seen in endothelial cells where activation of the Cdc42/Rac/Rho cascade leads to robust stress fiber formation leading to cell contraction (Wojciak-Stothard *et al.*, 1998).

Rho-Induced Actin Reorganization

At least two effectors, ROK and Dia, appear to be required for Rho-induced assembly of stress fibers and focal adhesions. P164ROKα (also known as Rho-associated kinase) and p160ROKβ (also known as Rho-assiciated coiled-coiled-containing protein kinase or ROCK) are Ser/Thr kinases, whose kinase domains resemble that of myotonic dystropy kinase (52% identity). They also contain a coiled-coil region, a ROK-kinectin homology (RKH) Rho-binding domain, a PH domain and a Cys-rich region. ROKs are activated by binding to Rho-GTP (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). Expression of the constitutively active catalytic domain of ROKα induces stellate actin-myosin filaments in HeLa and Swiss3T3 cells (Leung *et al.*, 1996; Ishizaki *et al.*, 1997). Inhibition of ROK, on the other hand, using a pharmacological inhibitor (Y-27632) causes loss of serum and [Val^{14}] Rho-induced stress fibers (Uehata *et al.*, 1997). These results indicate that ROK is necessary, but not sufficient, for Rho-induced stress-fiber assembly.

Two substrates of ROK which are likely to be key players in actin-myosin filament assembly are myosin light chain (MLC) (Amano *et al.*, 1996) and the myosin-binding subunit (MBS) of MLC phosphatase (Kawano *et al.*, 1999). MLC phosphatase is inhibited by phosphorylation, indirectly leading to an increase in MLC phosphorylation. Phosphorylation of MLC occurs at Ser^{19}, which stimulates the actin-activated ATPase activity of myosin II and promotes the assembly of actomyosin filaments (Amano *et al.*, 1996; Kawamo *et al.*, 1999; Bresnick, 1999). Interestingly Rho-GTP also binds directly to the MBS of MLC phosphatase, but why Rho should bind to both kinase and substrate is unclear at present (Kimura *et al.*, 1996).

Another ROK target is LIM kinase (LIMK), which when phosphorylated is able to inhibit (by phosphorylation) cofilin, leading to stabilization of filamentous actin structures (Maekawa *et al.*, 1999; Bamburg *et al.*, 1999). ROK has also been reported to activate a ubiquitous Na^+/H^+ exchange protein (NHE1) and this contributes, via an unknown mechanism, to stress fiber and focal adhesion formation (Hooley *et al.*, 1996; Vexler *et al.*, 1996; Tominaga *et al.*, 1998; Tominaga and Barber, 1998). Other substrates for ROK which could contribute to actin assembly are adducin, which is phosphorylated by ROK both *in vitro* and *in vivo*, causing it to bind more strongly to F-actin (Kimura *et*

al., 1998; Fukata *et al.*, 1999), and the ERM (ezrin/radixin/moesin) family of proteins, which link actin to the membrane (Bretcher *et al.*, 1999; Matsui *et al.*, 1998).

While the catalytic domain of ROK alone does not induce correctly organized stress fibers, it has been reported that, when combined with an activated (N-terminal truncation) version of Dia, stress fibers are induced (Watanabe *et al.*, 1999; Nakano *et al.*, 1999; Watanabe *et al.*, 1997). This has lead to the idea that activation of both Dia and ROK are required in order to induce stress fibers. Dia (Dia1 and 2 in mammals) is a member of the formin-homology(FH) family of proteins and contains two FH domains (Wasserman, 1998). The FH1 sequence contains multiple proline-rich motifs which bind to the G-actin-binding protein, profilin. This interaction somehow allows Dia to contribute to actin polymerization and F-actin organization into stress fibers (Watanabe *et al.*, 1999; Wasserman, 1998).

Many observations point to a role for lipids, particularly PIP_2, in actin cytoskeleton rearrangements. For instance, the binding of PIP_2 to capping proteins such as gelsolin can induce their release from actin-filament barbed ends, providing a mechanism whereby PIP_2 could increase actin polymerization (Janmey and Stossel, 1987). There are some data to suggest that PIP_2 is involved in Rho effects. Injection of antibodies against PIP_2, for example, has been reported to inhibit serum-induced assembly of stress fibers (Gilmore and Burridge, 1996). Furthermore, overexpression of phosphatidylinositol-4-phosphate 5-kinase (PI-4-P5K) (to produce PIP_2) induces massive actin polymerization in COS-7 cells (Shibasaki *et al.*, 1997). It is noteworthy that mutation of the gene for a yeast PI-4-P5K, *mss4*, results in defects in the formation of actin cables, which can be repressed by overexpression of Rho2p, a Rho-like GTPase (Desrivieres, 1998). A physical association between Rho and a type I (PI-activated) PI-4-P5K activity has been detected in Swiss-3T3-cells lysates, though this interaction is not GTP-dependent and may not be direct (Ren *et al.*, 1996). PIP_2 also binds to vinculin, increasing its talin-binding ability; therefore PIP_2 synthesis could also help to induce focal adhesion formation (Gilmore and Burridge, 1996). PI-4-P5K could, therefore, provide a link between Rho and the stimulation of both new actin polymerization and of focal adhesions assembly. Additionally, [Val14] Rho and PI-4-P5K both induce ERM phosphorylation when overexpressed in fibroblasts, suggesting that Rho activation of PI-

4-P5K may also be a mechanism by which Rho activates ERM proteins (Matsui et al., 1999).

Cytokinesis also depends on the assembly of contractile actin-myosin filaments and has been shown to be Rho-dependent in *Xenopus* and sand-dollar (flat sea-urchin) embryos (Mabuchi *et al.*, 1993; Drechesi *et al.*, 1997). Rho is recruited to the cleavage furrow of Swiss3T3 cells during cytokinesis (Takaishi *et al.*, 1995) and may utilize some of the same effector proteins required for stress-fiber formation. ROK, for example phosphorylates and disassembles the intermediate filament protein glial fibrillary acidic protein to allow cleavage to occur (Kosako *et al.*, 1997), and a dominant-negative ROK construct (containing a mutant Rho-binding domain fused to PH domain) inhibits cytokinesis when injected into *Xenopus* embryos and mammalian cells (Yasui *et al.*, 1998).

Mammalian Dia may be involved in cytokinesis, since it has been detected at the cleavage furrow in mitotic Swiss3T3 cells (Watanabe *et al.*, 1997), and, in lower eukaryotes, FH-domain proteins are commonly involved in cytokinesis. For example, the *Drosophila* homolouge, diaphanous, is essential for cytokinesis (Castrillon and Wasserman, 1994), and two yeast formin-family proteins, Bni lp and Bnrl, are both required downstream of Rho GTPases for bud formation and *bni l bnr l* double mutants are often multinucleate (Imamura *et al.*, 1997).

Citron kinase, which appears not to play a role in stress-fiber assembly, has been localized to the cleavage furrow in HeLa cells (Madaule *et al.*, 1998). Citron kinase, like ROK, contains an N-terminal myotonic dystrophy kinase-like kinase domain (46% identity with ROKα), a central coiled-coil region, a Rho-binding domain (with no homology with ROK), a Cys-rich region, a PH domain and, unlike ROK, a Pro-rich SH3 domain-binding region (Madaule *et al.*, 1995; Madaule *et al.*, 1998). Truncation of citron kinase lacking the Rho-binding domain inhibit cytokinesis in HeLa cells, producing multinucleate cells (Madaule *et al.*, 1998), suggesting that this kinase acts downstream of Rho specifically at the cleavage furrow. Whether citron phosphorylates a distinct subset of proteins from ROK, in order to fulfil its role in cytokinesis, is unknown.

Activation of Rac and Cdc42

Rac is activated by a variety of tyrosine kinase receptors; in Swiss 3T3 cells, for example, insulin, PDGF, and EGF are all strong inducers of Rac-dependent membrane ruffling (Ridley *et al.*, 1992). A key component of this pathway is the phosphatidylinositol 3-kinase (PI3-K). A role for PI3-K in Rac activation has been deduced from studies using the relatively specific inhibitors wortmannin and LY294002 as well as using dominant negative versions of the kinase (Hawkins *et al.*, 1995; Nobes *et al.*, 1995; Ridley *et al.*, 1992). Activation of Rac by PDGF and insulin is completely dependent on PI3-K and in some cell types independent of Ras (Nobes *et al.*, 1995; Ridley *et al.*, 1992). In addition, PDGF-induced GTP loading on Rac is blocked by wortmannin (Hawkins *et al.*, 1995). Since constitutively activated versions of PI3-K induce strong membrane ruffling in the absence of growth factors, other receptor-induced signals appear not to be necessary for Rac activation (Ridley et al., 1992). The role of PI3-K in the Rac pathway is still not entirely clear. One issue is whether PI3-kinase functions upstream or downstream of Rac in the signaling cascade. Several studies have demonstrated that membrane ruffling induced by activated Rac is not inhibited by inhibitors of PI3-K, while ruffling induced by activated PI3-K is blocked by dominant negative versions of Rac ((Hawkins *et al.*, 1995; Ma et al., 1998; Nobes *et al.*, 1995; Ridley *et al.*, 1992). This points toward a role of PI3-K upstream of Rac. In one study, however, it was found that PI3-K inhibitors decreased cell migration and invasion induced by activated Rac in epithelial cells (Keely et al., 1997). Thus while the importance of PI3-K in relaying cell surface signals to Rac is beyond doubt, there might also be a role for PI3-K in facilitating Rac-induced changes in cell behavior.

The p85 regulatory subunit of PI3-K contains a bcr-GAP homology domain, and although it has no detectable GAP activity, Rac and Cdc42 have been directly linked to PI3-K (Tolias et al., 1995; Zheng et al., 1994). The association is increased when serum-starved Swiss 3T3 cells are treated with PDGF indicating that it might play a role in Rac activation. More likely, however, the production of phosphoinositol 3,4,5-triphosphate (PIP_3), the plasma membrane-associated lipid product of PI3-K activity, is the signal promoting Rac activation. PIP_3 binds to the PH domain of at least three GEFs that can catalyze activation of Rho family GTPases, Tiam1, Sos, and Vav (Han *et al.*, 1998;

Rameh *et al.*, 1997). In one study on Vav, lipid binding was reported to have functional consequence and enhance GDP/GTP exchange on Rho, Rac, and Cdc42 (Han *et al.*, 1998). As the presence of the PH domain seems to exert an inhibitory function on the activity of some, but not all, GEFs (Ma *et al.*, 1998; Michiels *et al.*, 1997;Nimunual *et al.*, 1998; Olson *et al.*, 1996), it is an attractive hypothesis that PIP_3 binding relieves this inhibitory effect by eliciting a conformational change and thus activating the GEF. So far though, it remains to be shown that that is a general mechanism working on GEFs other than Vav. PIP_3 can also bind directly to Rac *in vitro* and promote GDP dissociation, raising the possibility that it might act directly on the GTPase (Missy *et al.*, 1998).

Tyrosine phosphorylation by Src-family kinases has also been shown to enhance Vav activity toward Rho and Rac, providing a possible alternative mechanism for the regulation of Rac (Crespo *et al.*, 1997; Hart *et al.*, 1998). *In vitro*, PIP_3 binding and phosphorylation appear to act cooperatively to stimulate the exchange activity of Vav, indicating that both Src-mediated and PI3-K-mediated processes might cooperate to achieve maximum GEF activation in response to growth factor stimulation. Some supporting evidence for this has come from a study of the tyrosine kinase receptor Kit (Timokhina *et al.*, 1998). Rac dependent JNK activation and membrane localization of Rac induced by the Kit ligand are impaired in cells expressing receptor mutants incapable of activating PI3-K or mutants unable to activate Src, while they are completely abolished in cells expressing receptor mutants that fail to initiate either of these pathways.

Rac is a key component in the activation of T cells induced by cross-linking of the T cell receptor (TCR) (Cantrell, 1998). There is good evidence to suggest that stimulation of the TCR leads to enhanced GDP/GTP exchange on Rac in a manner that is dependent upon tyrosine phosphorylation of Vav by the receptor-activated kinase ZAP-70 (Salojin *et al.*, 1999; Wu *et al.*, 1997). As Vav-deficient T cells fail to produce a normal response to TCR activation, this underlines, the physiological importance of this signaling pathway (Fischer *et al.*, 1998; Holsinger *et al.*, 1998). However, since Vav is expressed only in hematopoietic cells (Bustelo, 1996), other GEFs must regulate Rac in other cell types. Surprisingly, the very closely related, but more ubiquitously expressed, family member Vav-2 has been reported to activate RhoA and RhoG and so would not be expected to fulfill this role (Schuebel *et al.*, 1998). An alternative possibility is the Ras GEF, Sos,

which contains both a Ras (cdc25) and a DH/PH exchange factor domain. It has recently been shown that the full DH/PH domain is inactive as an exchange factor, while the DH domain alone is functional as a Rac GEF (Nimnual *et al.*, 1998). The ability of a Ras mutant capable of activating PI3-K, but not other Ras effectors, to induce DH/PH Sos-dependent JNK activation indicates that the Rac GEF activity might be triggered by PIP_3 binding to the PH domain. Two other Ras exchange factors, the brain-specific RasGRF-1 and the more ubiquitously expressed RasGRF2, also encode DH/PH domains and can have GFF activity toward Rac (Fan *et al.*, 1998; Kiyono *et al.*, 1999). It is an interesting that these GEFs could coordinate Ras activation of Rac through a rather direct link, similar to that formed between Gα-subunits and Rho by p115RhoGEF, but this remains to be determined. Tiam1 is also an important candidate for regulating Rac activity. Tiam1 shows intrinsic exchange factor activity specific for Rac and this is enhanced by threonine phosphorylation indicating that a different mechanism is responsible for the activation of this GEF (Fleming *et al.*, 1999; Michiels *et al.*, 1997).

Other receptor classes can also activate Rac. The chemoattractant fMLP has been shown to increase the amount of Rac GTP in neutrophils through the pertussis toxin sensitive heterotrimeric G-protein G_i (Akasaki *et al.*, 1999; Benard *et al.*, 1999). $G\alpha_{12}$ and/or $G\alpha_{13}$ have shown to induce JNK activation and phospholipase D production in a Rac-dependent manner, although as mentioned earlier, $G\alpha_{12}$ and $G\alpha_{13}$, do not induce the typical morphological changes associated with Rac activation when introduced into fibroblasts (Buhl *et al.*, 1995, Collins *et al.*, 1996; Gohla *et al.*, 1998; Plonk *et al.*, 1998). Whether this results from the strong Rho activation elicited by $G\alpha_{12}$ and $G\alpha_{13}$ masking subtler Rac-mediated effects on the actin cytoskeleton or whether it is a reflection of the existence of different Rac-containing signaling complexes with different downstream effects is unknown. Bombesin, another GPCR agonist, also induces Rac-dependent actin changes in Swiss 3T3 cells, but the G-proteins involved have not been characterized (Ridley, *et al.*, 1992).

There is some information to support a role for Gβγ subunits in Rac activation. transfection of Gβγ into COS-7 cells induces JNK activation by a mechanism requiring Rac (Lopez-Ilasaca *et al.*, 1998) and in contrast to $G\alpha_{12}$ or $G\alpha_{13}$,Gβγ also induces actin cytoskeletal changes reminiscent of those induced by Rac and susceptible to inhibition by

dominant negative Rac (Ma *et al.*, 1998). The pathway to Rac activation by Gβγ in COS-7 cells appears to be similar to that used by growth factor receptors as it is inhibited by PI3-K inhibitors and domainant negative Vav. As the pertussis toxin-sensitive fMLP-induced Rac activation in leukocytes is also PI3-K sensitive, it is possible that this is also mediated by a Gβγ pathway (Benard *et al.*, 1999).

Much less is known about how Cdc42 is activated. FMLP has been reported to induce an increase in Cdc42 GTP in neutrophils, an effect which is also pertussis toxin sensitive and therefore G_i mediated (Benard *et al.*, 1999). Bradykinin, which acts through a GPCR, induces Cdc42 dependent changes in the actin cytoskeleton (i.e. filopodia) and Cdc42-dependent activation of Rac (Kozma *et al.*, 1995). In one report, activation of the bradykinin-B2 receptor, transfected into mouse fibroblasts, induced stress fibers through $G\alpha_{13}$ – and EGF receptor-dependent signals (Gohla *et al.*, 1999). As $G\alpha_{13}$ does not seem to act via Cdc42 or Rac and as stress fibers are a Rho-regulated response (Nobes and Hall, 1995; Ridley and Hall, 1992), this indicates that bradykinin activates Cdc42 through a different pathway. One possibility is that this occurs via the Gβγ subunit of a heterotrimeric G-protein.

The cytokines TNF-α and IL-1 also activate Cdc42 as assessed by actin cytoskeleton changes (Puls *et al.*, 1999; Wojciak-Stothard *et al.*, 1998). Interestingly, activation of JNK by TNF-α and IL-1 appears to be Cdc42/Rac dependent in some cells (Auer *et al.*, 1998; Bagrodia *et al.*, 1995; Coso *et al.*, 1995, Kieser *et al.*, 1999).

Rac and Cdc42 Effectors Implicated in Actin Reorganization

Some common target proteins appear to be utilized by both Rac and Cdc42 in the induction of lamellipodia and filopodia respectively. PAK (p21-activated kinase)1,2,3 are Ser/Thr kinases, related to yeast Ste20, which have received a great deal of detailed attention (Manser, *et al.*, 1994; Manser *et al.*, 1995; Bagrodia *et al.*, 1995). *In vitro*, PAK1 and 3 bind equally well to Cdc42 and Rac, through Cdc42 stimulates PAK1 activity more strongly than does Rac (Manser *et al.*, 1995). PAK2 only inhibits the intrinsic GTPase activity of Rac, and not Cdc42, suggesting that it may be a Rac target (Zhang *et al.*, 1998). However, it is difficult to decide whether PAK1,2,3 are targets for Rac, Cdc42 or both *in vivo* from these *in vitro* interactions. There have been conflicting

reports linking PAKs to actin changes. Activated mutants of PAKs have been reported to induce both filopodia and membrane ruffles in Swiss3T3 cells and to cause neurite outgrowth in PC12 cells, similar to the effects of constitutively active Cdc42 and Rac (Sells *et al.*, 1997; Daniels *et al.*, 1998). PAK1 has been shown to localize to membrane ruffles, as well as phagocytic actin-containing cups, in N-formylmethionyl-leucyl-phenylalanine-stimulated neutrophils (Dharmawardhane *et al.*, 1997). Interestingly, PAK-induced cytoskeletal changes are partly independent of its kinase activity, but require membrane targeting (Sells et al., 1997; Daniels *et al.*, 1998; Lu *et al.*, 1997). A variety of substrates for PAKs have been identified that could affect the actin cytoskeleton. Rac, like Rho, induces phosphorylation of LIMK (Aber *et al.*, 1998), and PAK1 has been shown to phosphorylate LIMK *in vitro* (Edwards *et al.*, 1999). Also, an inactive form of LIMK has been shown to inhibit both Cdc42 and Rac induced actin changes (Yang *et al.*, 1998), suggesting that cofilin phosphorylation may be a general requirement in Rho GTPase pathways. PAKs has been reported to phosphorylate and inactivate MLC kinase, decreasing MLC phosphorylation and reducing actomyosin assembly (Sanders *et al.*, 1999). There has also been a report of Rac-induced phosphorylation of myosin II heavy chain, which potentially would also lead to loss of actomyosin filaments (Bresnick, 1999). Dominant-negative PAK inhibits this phosphorylation, but active PAK does not reconstitute the effect, suggesting that multiple effectors may mediate this activity (van Leeuwen *et al.*, 1999).

Overexpression of activated Rac and Cdc42 leads to activation of the JNK and p38 MAP kinase pathways a in variety of cell types (Coso *et al.*, 1995; Minden *et al.*, 1995; Bagrodia *et al.*, 1995). JNK activation by certain growth factors and cytokines [e.g. EGF, tumor necrosis factor α (TNFα) and transforming growth factor β) has been reported to be inhibited by dominant-negative Rac and Cdc42 (Coso *et al.*, 1995; Atfi *et al.*, 1997), but it is still not clear how general the role of Rac/Cdc42 is in JNK activation. In Swiss 3T3 cells, for example, TNFα induction of JNK is not dependent on Cdc42 (Puls *et al.*, 1999). Which Cdc42/Rac effectors mediate JNK activation in mammalian cells is still unclear. In *Drosophila* there is good genetic evidence for a link from Drac1 and DCdc42, via *mis-shapen* (PAK-like), to *hemipterous* (a JNK kinase), a pathway that is essential for dorsal closure (Su *et al.*, 1998; Glise and Noselli, 1997). In yeast, a PAK-

like kinase, Ste20, acts downstream of Cdc42 and is required for activation of the Kss1p/Fus3p MAP kinase cascade in the mating-pheromone response (Leberer *et al.*, 1992; Peter *et al.*, 1996). Mammalian PAK1 has been observed to enhance p38 activation when co-expressed with Rac and Cdc42, and constitutively active PAK1 and PAK3 have been reported to activate JNK in COS cells (Zhang *et al.*, 1995; Brown *et al.*, 1996).

Additional Rac/Cdc42 targets which may mediate JNK activation are: (1) Mlks 1, 2 and 3, which contain CRIB motifs and act as MAP kinase kinase kinases (MEKKs) [(Sahai *et al.*, 1998; Burbedo *et al.*, 1995; Nagata *et al.*, 1998; Brown *et al.*, 1996)]; (2) MEKK1 and MEKK4, which also interact with Rac and Cdc42 (MEKK4 has a CRIB domain). All are strong activators of JNK when overexpressed, and inhibitory kinase-dead MEKK1 or MEKK4 mutants block Rac/Cdc42-induced JNK (Fanger *et al.*, 1997). However, to date no physiological link has been made between Rac/Cdc42 and Mlk1, Mlk2 and Mlk3 or MEKK1 and MEKK4.

Rac is also known to be a regulator of the NADPH oxidase complex, a specialized enzyme of phagocytic cells that generates oxygen radicals to kill internalized micro-organisms (Abo *et al.*, 1991; Chanock *et al.*, 1994; Knaus *et al.*, 1991). A cytoplasmic-derived component of the oxidase complex, $p67^{phox}$, binds directly to Rac (Diekmann, *et al.*, 1994). Data suggest that the role of Rac is not to promote relocalization of this cytoplasmic protein to the membrane-bound catalytic components, as was first thought, but rather to act as an allosteric regulator by inducing a conformational change in the preformed complex to promote catalytic activity (Nisimoto *et al.*, 1997).

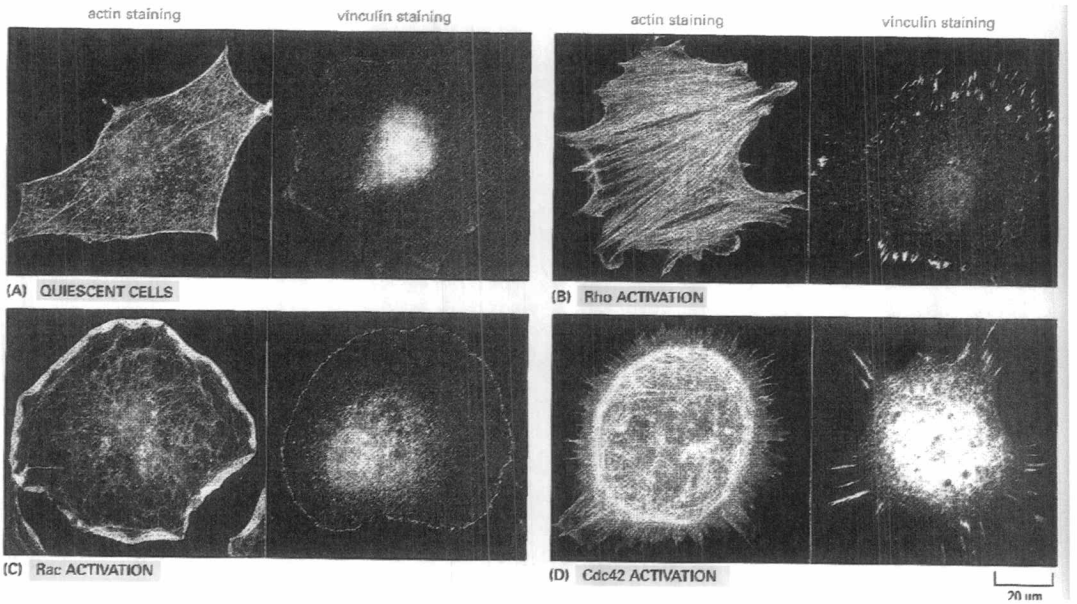

Figure 4 The dramatic effects of Rac, Rho, and Cdc42 on actin organization in fibroblasts (Hall, 1998).

Biological Roles of Rho GTPases

Rho GTPases would be expected to be involved in biological processes which require a coordinated rearrangement of the actin cytoskeleton and their role in neurite outgrowth and phagocytosis, as well as gastrulation in *Drosophiala*. In addition, other processess identified to be dependent on Rho GTPases include cytokinesis (Drechsel *et al.*, 1997; Kishi *et al.*, 1993), vascular contraction (Seasholtz *et al.*, 1999), and events that require cell motility such as transendothelial migration of lymphocytes and cellular invasion (Adamson *et al.*, 1999; Keely *et al.*, 1997; Malliri *et al.*, 1998; Michiels *et al.*, 1995; Shaw *et al.*, 1997; Stam *et al.*, 1998).

The migratory response can be induced by signals from chemoattractants and growth factors, as well as by mechanical wounding which leads to renewed integrin ligation to the extracellular matrix. The most consistent observation in such assays is that dominant negative versions of Rac inhibit cell motility. This has been observed for motility induced by HGF/SF in epithelial cells or PDGF or wounding in fibroblasts and CSF-1 in macrophages (Allen *et al.*, 1998; Anand-Apte *et al.*, 1997; Nobes and Hall, 1995; Ridley *et al.*, 1995). It appears that Rac generates the protrusive activity needed to

drive movement. The major role of Cdc42, on the other hand, is not in the migratory process per se, but in establishing cell polarity to ensure that migration occurs in a specific direction (Alen *et al.*, 1998; Nobes and Hall, 1999). Thus Rac and Cdc42 work cooperatively to promote spatially defined protrusive activity. The role of Rho appears to be more complicated. In rat embryo fibroblasts, a basal Rho activity is required for the generation of adhesive force, but excessive Rho activity inhibits migration probably through the formation of strong focal adhesions. Interestingly, in these cells focal adhesion turnover appears to be dependent on Ras, a nice example of cross-talk between Ras and Rho GTPases with physiological consequences (Nobes and Hall., 1999). It is clear, therefore, that the activity of Cdc42, Rac, Rho, and probably other small GTPases must be regulated appropriately for the cell to attain migratory behavior in response to an extracellular signal. As perturbation of Rho GTPases regulation by extracellular signaling or GEF hyperactivation has been shown experimentally to induce increased motility and invasiveness in a variety of cell types, this may have important implications in the study of human diseases such as cancer and inflammation (Malliri *et al.*, 1998; Shaw *et al.*, 1997; Stam *et al.*, 1998).

Arsenic (WHO, 2001)

Arsenic is a metalloid widely distributed in the earth's crust and present at an average concentration of 2 mg/kg. It occurs in trace quantities in all rock, soil, water and air. Arsenic can exist in four valency states: -3, 0, +3 and +5. Under reducing conditions, arsenite (As (III) is the dominant form; arsenate (As (V)) is generally the stable form in oxygenated environments. Elemental arsenic is not soluble in water. Arsenic salts exhibit a wide range of solubilities depending on pH and the ionic environment.

Effects of Arsenic on Human Health (WHO, 2001)

Soluble iorganic arsenic is acutely toxic, and ingestion of large does leads to gastrointestinal symptoms, disturbances of cardiovascular and nervous system functions, and eventually death. In survivors, bone marrow depression, haemolysis, hepatomegaly, melanosis, polyneuropathy and encephalopathy may be observed.

Long-term exposure to arsenic in drinking-water is causally related to increased risks of cancer in the skin, lungs, bladder and kidney, as well as other skin changes such as hyperkeratosis and pigmentation changes. These effects have been demonstrated in many studies using different study designs. Exposure-response relationships and high risks have been observed for each of these end-points. The effects have been most thoroughly studied in Taiwan but there is considerable evidence from studies on populations in other countries as well. Increased risks of lung and bladder cancer and of arsenic-associated skin lesions have been reported to be associated with ingestion of drinking-water at concentrations ≤50 μg arsenic/litre.

Occupational exposure to arsenic, primarily by inhalation, is causally associated with lung cancer. Exposure-response relationships and high risks have been observed. Increased risks have been observed at cumulative exposure levels ≥0.75 (mg/m^3). year (e.g. 15 years of exposure to a workroom air concentration of 50 $\mu g/m^3$). Tobacco smoking has been investigated in two of the three main smelter cohorts and was not found to be the cause of the increased lung cancer risk attributed to arsenic; however, it was found to be interactive with arsenic in increasing the lung cancer risk.

Even with some negative findings, the overall weight of evidence indicates that arsenic can cause clastogenic damage in different cell types with different end-points in exposed individuals and in cancer patients. For point mutations, the results are largely negative.

Chronic arsenic exposure in Taiwan has been shown to cause blackfoot disease (BFD), a severe form of peripheral vascular disease (PVD) which leads to gangrenous changes. The disease has not been documented in other parts of the world, and the findings in Taiwan may depend upon other contributing factors. However, there is good evidence from studies in several countries that arsenic exposure causes other forms of PVD.

Conclusion on the causality of the relationship between arsenic exposure and other health effects are less clear-cut. The evidence is strongest for hypertension and cardiovascular disease, suggestive for diabetes and reproductive effects and weak for cerebrovascular disease, long-term neurological effects, and cancer at sites other than lung, bladder, kidney and skin.

The highest toxicological relevance is attributed to organic and inorganic compounds containing trivalent arsenic with at least two reactive binding sites, such as sodium arsenite and the chemical warfare agent chlorovinylarsinedichloride (Lewisite). These compounds form stable rings with molecules bearing adjacent SH groups. The cytotoxicity of these compounds were based on their reaction with the dihydrolipoyl moiety of pyruvate dehydrogenase leading to inhibition of carbohydrate metabolism in the citric acid cycle, acetyl coenzyme A depletion and thereby reduced oxygen consumption and ATP formation (Szinicz *et al.*, 1999).

Sodium Arsenite and Toxicity

Trivalent inorganic is generally more toxic than pentavalent inorganic arsenic. The oral LD_{50} of sodium arsenite ($NaAsO_2$), a trivalent arsenical, administered to rat and mouse is following this table.

Table 1 Acute LD_{50} of sodium arsenite (WHO, 2001)

Species	Age	Sex	Route	LD50 (mgAs/kg)	LD50 (mg/kg)	Reference
Rat	adult	M/F	oral	24	42	Done and Peart (1971)
Mouse	young	M	i.m.	8	14	Bencko *et al.* (1978)
Mouse	young	M	i.m.	21	87	Bencko *et al.* (1978)
Rat	young	-[a]	i.p.	4-5[b]	9.7-10.9[b]	Frank and Moxon (1936)

[a] Sex not specified, [b] LD_{75}

The symptoms observed from arsenite intoxication include convulsions, retching and haemorrhaging in the intestinal tract.

Sodium arsenite is 3-4 times more potent than sodium arsenate in the rat after intraperitoneal administration (Frank and Moxon, 1936) and 2-3 times more potent in the mouse after intramuscular administration with respect to acute lethality (Bencko *et al.*, 1978).

In long-term exposure (119 days), the effect of sodium arsenite fed *ad libitum* to dogs were examined by Neiger and Osweiler (1989). Initially, six female dogs/group were fed 0, 1, 2, 4 or 8 mg of sodium arsenite/kg body weight per day. Two serum enzymes were elevated in dogs examined at study termination, suggesting arsenite-induced hepatotoxicity. Aspartate aminotransferase was elevated in dogs exposed to 4 and 8 mg/kg per day sodium arsenite, and alanine aminotransferase was elevated in dogs exposed to 2, 4 and 8 mg/kg per day sodium arsenite. However, no lesions in the liver were observed after gross or light-microscopic examination.

Sodium arsenite and sodium arsenate are not allergenic in the guinea-pig maximization test (Wahlberg and Boman, 1986).

Sodium arsenite is more potent than sodium arsenate in inducing a teratogenic response, and parenteral administration of arsenic is more effective than oral administration. Adminsitration of an acute oral dose of arsenite that is toxic to or near the lethal dose of pregnant mice (40-45 mg/kg) (Baxley *et al.*, 1981) or hamsters (20-25 mg/kg) (Hood and Harrison, 1982) induces a low incidence of teratogenic malformations. The major teratogenic effect induced by inorganic arsenic in laboratory animals is cephalic axial dysraphic disorder, or neural tube defect. The defect is characterized by exencephaly and encephalocele, which are non-closure and partial closure of the cephalic neural folds, respectively (Carpenter, 1987). Exencephaly and encephalocele occur rarely in laboratory rodents. Other malformations which occur to a minor extent after exposure to arsenic include fused ribs, renal agenesis, micromelia, facial malformations, twisted hindlimb, microphthalmia and anophthalmia.

Honda *et al.* (1992) observed in mice administrated sodium arsenite (19 mg/kg) intraperitoneally on day 8 and killed 1 day later, that two proteins, hsp70 and hsp105, which are produced constitutively, were increased throughout the embryo. There was a high concentration of these proteins in the neuroepithelial tissue of the embryo after treatment with arsenite.

Sodium arsenite is not mutagenic to *Escherichia coli* in tests selecting for tryptophan revertants (Rossman *et al.*, 1980). Sodium arsenite is not genotoxic to Chinese hamster ovary (CHO) cells (Rossman *et al.*, 1980) or Syrian hamster embryo cells (Lee *et al.*, 1985) when selecting for ouabain- (ATPase) or thioguanine-resistant (hypoxanthine phosphoribosyl transferase, HPRT) mutants. In the L5178Y mouse lymphoma assay, sodium arsenite is weakly genotoxic at the thymidine kinase locus without metabolic activation (Oberly *et al.*, 1982; Moore *et al.*, 1997). Sodium arsenate and sodium arsenite induce sister chromatid exchanges and chromosomal aberrations in hamster embryo cells (10^{7} mole/litre-10^{4} mole/litre) (Larramendy *et al.*, 1981; Lee *et al.*, 1985; Kochhar *et al.*, 1996). The aberrations are characterized by chromatid gaps, breaks, and fragmentation, endoreduplication and chromosomal breaks.

Sodium arsenate and sodium arsenite induce a dose-dependent transformation of Syrian hamster embryo cells (Lee *et al.*, 1985) and BALB/3T3 cells (Bertolero *et al.*, 1987). Arsenite is 4-10-fold more potent than arsenate in inducing transformation, partly because of greater cellular uptake of arsenite than arsenate (Bertolero *et al.*, 1987). BALB/3T3 cells transformed by arsenite were tumorigenic in nude mice after their subcutaneous administration (Saffiotti and Bertolero, 1989). The tumors grew rapidly and appeared as fibrosarcomas, but did not metastasize to other site within the mice. Arsenite may be the transforming agent (Bertolero *et al.*, 1987; Saffiotti and Bertolero, 1989), at least in the BALB/3T3 cells.

Inorganic arsenic induces sister chromatid exchanges, chromosmal aberrations and DNA-protein cross-links in human lymphocytes (Larramendy *et al.*, 1981) and fibroblasts (Okui and Fujiwara, 1986). These effects are dependent on dose, and sodium arsenite is more potent than sodium arsenate.

Sodium arsenite and potassium arsenite administered intraperitoneally (2.5-10 mg/kg) to mice induce a linear dose-dependent increase in micronucleated polychromatic erythrocytes (Deknudt *et al.*, 1986; Tinwell *et al.*, 1991).

Chromosomal aberrations including chromatid gaps and breaks and chromosomal rearrangement are induced in mouse bone marrow cells after oral administration of sodium arsenite (0.1-2.5 mg/kg) (Das *et al.*, 1993; RoyChoudhury *et al.*, 1996).

Several mechanisms for genotoxicity caused by arsenic have been proposed. These include reactive oxygen species and the inhibition of DNA repair. Because arsenic is not a "classified" genotoxin in that it does not directly interact with and damage DNA (Rossman *et al.*, 1980), a definitive determination of a mechanism of action has been elusive, and many proposed mechanism are still at the stage of speculation (US EPA, 1992).

Aneupoidy induced by sodium arsenite in human lymphocytes may be a result of the disruption of microtubule function and spindle formation (Ramirez *et al.*, 1997). Dose-related increases in hyperploid cells were observed after exposure to sodium arsenite (0.001-0.1 μmol/litre). Sodium arsenite inhibits tubulin polymerization and stimulates its depolymerization. The effects on tubulin may affect spindle formation, which results in altered chromosome numbers in the cells.

Long-term exposure of sodium arsenite (50 μg As/ml) in drinking-water to rats (18 months) or rabbits (10 months) alters baseline cardiovascular parameters (Carmignani *et al.*, 1985). Arsenite was associated with decreases in stroke volume and cardiac output and an increase in vascular resistance in both species. Aortic blood flow was significantly decreased in the arsenite-exposed art. Arsenite induced specific changes in the cardiovascular response in each species to neurohumoral and effector agonists. In the rabbit, blood pressure responses to phenylephrine, which stimulates α-adrenoceptors, and bilateral carotid occlusion, were reduced. In the rat, blood pressure response to tyramine was reduced. Carmignani *et al.* (1985) suggested that the reduced hypertensive responses to various agonists may be due to the increased vascular resistance induced by arsenite.

As_2O_3 and sodium arsenite induce significant dose-dependent (10-100 μmol/litre) damage to endothelial cells isolated from bovine carotid arteries after 22 h incubation (Chang *et al.*, 1991). Sodium arsenite at concentrations < 5 μmol/litre stimulates [^{3}H]-thymidine incorporation into the DNA of porcine vascular endothelial cells, which is indicative of a mitogenic response (Barchowsky *et al.*, 1996). Higher concentrations of arsenite appear to be cytotoxic to the cells. At mitogenic concentrations of arsenite, oxidants within the cells are increased for 30-60 min after exposure and the thiol levels are increased by 24 h.

Sodium arsenite is more toxic than sodium arsenate to mouse neuroblastoma cells after a 24 h exposure in culture (Repetto *et al.*, 1994). All parameters examined (cell proliferation, lysosomal function, and membrane integrity) were affected at lower doses of arsenite than arsenate and may be attributed to greater cellular uptake of arsenite (Lerman *et al.*, 1983; Bertolero *et al.*, 1987). There were differences in sensitivity of several parameters to the effects induced by arsenic. Inhibition of several metabolic processes, such as lactate dehydrogenase activity and hexosamidase activity, occurred at lower concentrations of both arsenicals than the inhibition of cellular proliferation.

Burleson *et al.* (1996) have reported that in normal human keratinocytes sodium arsenite (4 μmol/litre) stimulates transcription of c-*jun* and c-*fos*. The products of these genes dimerize, forming AP-1 transcription factors. In the arsenite-exposed keratinocytes, there is increased AP-1 DNA binding activity.

The stimulation of growth factors by arsenic may have a role in its carcinogenic effect. Sodium arsenite stimulates interleukin-8 (IL-8) gene expression and thus IL-8 secretion in cultured human keratinocytes after a 24 h incubation (Yen *et al.*, 1996). IL-8 is a peptide that may be an essential autocrine growth factor. Stimulation of IL-8 was not detected at concentrations lower than 28 μmol/litre sodium arsenite, and this was not toxic to the cells.

Other growth factors stimulated in cultured human keratinocytes by sodium arsenite include granulocyte macrophage-colony stimulating factor (GM-CSF), transforming growth factor (TGF)-α, the inflammatory and chemotactic cytokine tumor necrosis factor (TNF)-α (Germolec *et al.*, 1997) and IL-1 (Burleson *et al.*, 1996).

Expression of the proto-oncogene c-*myc*, which is associated with proliferation of keratinocytes (Pietenpol *et al.*, 1990), was also increased in the keratinocytes after exposure to arsenite (Germolec *et al.*, 1997). Proliferation of keratinocytes was increased with low doses of sodium arsenite (1-5 nmol/litre), but higher concentrations decreased cell number.

Sodium arsenite administered to mice in drinking-water (0.5, 2, and 10 mg As/litre) for 3 weeks results in immunosuppression of the humoral response (Blakley *et al.*, 1980). Both primary and secondary immune responses, with respect to the production

of immunoglobulin (Ig) M and G, respectively, were suppressed with a maximum effect occuring at 0.5 mg/litre.

There is a dose-dependent increase in urinary uroporphyrin excretion by rats after the first week of exposure to sodium arsenite (5-100 mg As/litre) in drinking water (Martinez *et al.*, 1983). There was a gradual decrease in the response over time, which was suggestive of an adaptive response.

After an acute subcutaneous exposure of rats of sodium arsenite (12.5-100 μmol/litre) or sodium arsenate (25-200 μmol/litre), hepatic activity of δ-aminolaevulinic acid (ALA) synthetase and haem oxygenase are significantly increased in a dose-dependent manner (Cebrian *et al.*, 1988). Hepatic cytochrome P-450 concentration was also decreased. Arsenite is more potent than arsenate in this response and haem oxygenase activity was increased to a greater extent than ALA synthetase.

Subchronic treatment of mice with sodium arsenite (20 mg/litre) or sodium arsenate (50 mg/litre) in drinking-water results in a time dependent porphyric response (Garcia-Vargas *et al.*, 1995). Urinary porphyrins were increased by 3 weeks after initial exposure to arsenic, which corresponded to increased activities of hepatic porphobilinogen deaminase and uroporphyrinogen synthetase. Arsenite was more potent in this response than arsenate. Significant decreases in renal uroporphyrinogen decarboxylase and hepatic and renal coporphyrinogen oxidase were also observed.

Inorganic arsenic induce apoptosis, or programmed cell death, in CHO cells (Wang *et al.*, 1996), immature rat thymocytes (Bustamante *et al.*, 1997) and human HL-60 cells (Ochi *et al.*, 1996). A characteristic marker of apoptosis is internucleosomal DNA cleavage, which was observed in all cell types after exposure to inorganic arsenic. In the HL-60 cells, sodium arsenite (0.05 mmole/litre) produced a greater response than sodium arsenate (0.1 mmol/litre). The apoptotic response by both arsenicals was increased when the cells were depleted of GSH by buthione sulfoximine (BSO). The mechanism of apoptosis in the thymocytes is unknown, but Wang *et al.* (1996) observed that in CHO cells, arsenite induces a cascade of events that leads to apoptosis. This cascade involves the genearation of reactive oxygen species, production of hydroxyl radicals via a metal-catalysed Fenton reaction, protein synthesis and activation of protein kinase. Li and Broome (1999) propose that trivalent arsenic induces apoptosis at least in

leukaemia cells, by binding to tubulin. This results in inhibition of tubulin polymerization and eventual formation of microtubules. Broome suggest that trivalent arsenic binds to cysteine residues (cis-12 and cys-13) of tubulin, which prevents GTP from binding to tubulin. This results in inhibition of microtubule formation, arresting the cells in mitosis, and activation of the genes involved apoptosis.

Scott *et al.* (1993) and Delnomdedieu *et al.* (1994a) examined the complexation of arsenic with thiols. GSH non-enzymatically reduces arsenate to arsenite. The reduction requires 2 moles of GSH to 1 mole of arsenate and also results in the formation of oxidized GSH (GSSG). Arsenite then reacts with GSH to form a GSH-arsenite complex. The complex consists of 3 moles GSH per 1 mole arsenite and is termed arsenotriglutathione. The complex is stable over a pH range of 1.5-7.5. Arsenite has a higher affinity for a vicinal thiols, such as the DMSA, than a dithiol located four carbons apart, such as that in dithiothreitol, or monothiol such as GSH (Delnomdedieu *et al.* 1993). Arsenite can also complex with GSH within intact erythrocytes (Delnomdedieu *et al.* 1994b).

Trivalent iorganic arsenicals, such as arsenite, readily react with sulfhydryl groups such as GSH and cysteine (Scott *et al.*, 1993; Delnomdedieu *et al.* 1994a). The complex between arsenic and vicinal sulfhydryl reagent reagent is particularly strong. The activity of enzymes or receptors is due in part to the functional groups on amino acids such as the sulfhydryl group on cysteine or coenzyme such as lipoic acid, which has vicinal thiol groups. Thus, if arsenite binds to a critical thiol or dithiol, the enzyme may be inhibited (Aposhian, 1989). Arsenite inhibits pyruvate dehydrogenase (Peters, 1955; Szinicz and Forth, 1988), a lipoic-acid-dependent enzyme involved in glucogenesis. The acute toxicity of inorganic arsenic may result in part from inhibition of glucogenesis and ultimately depletion of carbohydrates from the organism (Reichl *et al.*, 1988; Szinicz and Forth, 1988). However, binding of arsenite to protein at non-essential sites may be a detoxication mechanism (Aposhian, 1989). Arsenite inhibits the binding of steroids to the glucocorticoid receptor, but not other steroid receptors (Lopez *et al.*, 1990; Simons *et al.*, 1990). The glucocorticoid receptor has vicinal thiols that are involved with steroid binding (Simons *et al.*, 1990).

Mechanism of Arsenite in Relation to Cell Signaling

In multicellular organisms where different functions are carried out by differentiated cells, mechanisms for intercellular communication are required to ensure the efficient integration of cellular activities or **homeostasis**. To survive, cells must communicate with the neighbors, monitor the conditions in their environment, and respond appropriately to a host of different stimuli that impinge on their surface. Cells carry out these interactions by a phenomenon known as **cell signaling**, in which information is relayed across the plasma membrane to the cell interior and often to the cell nucleus.

One of the fundamental mechanisms by which cells in multicellular organisms communicate is the binding of polypeptide ligands to cell surface receptors that possess tyrosine kinase catalytic activity. Receptor tyrosine kinase (RTKs) are transmembrane glycoproteins that are activated by the binding of their cognate ligands, and they transduce the extracellular signal to the cytoplasm by phosphorylating tyrosine residues on the receptor themselves (autophosphorylation) and on downstream signaling proteins. RTKs activate numerous pathways within cells, leading to cell proliferation, differentiation, migration, or metabolic changes (Schlessinger and Ullrich, 1992).

It has been shown that PI3K activation appears to be an essential condition for reorganization of actin filaments in a cell. Oncoprotein rac, which belongs to the small G-protein group and is involved in signal transduction via the stress-activated kinases of the JNK family, is considered as a possible effector of PI3K. The other possible pathway for PI3K to control actin assembly is based on the lipid kinase activity of PI3K.

Rho GTPases (Rho, Rac and Cdc42), downstream effectors of protein tyrosine kinase and phosphotidylinositol-3 kinase (PI3K), would be expected to be involved in biological processes which require a coordinated rearrangement of the actin cytoskeleton and neurite outgrowth and phagocytosis.

Epidermal growth factor (EGF) and transforming growth factor alpha (TGF alpha) are important keratinocyte mitogens. Their effects are mediated by a cell membrane receptor (EGFR) (Groves *et al.*, 1992).

EGFR tyrosine kinase moiety provides protection to normal human keratinocytes against apoptosis. This protection is at least in part, due to EGFR-dependent expression of the antiapoptotic Bcl2 family member, (Bcl-XCL) (Jost *et al.*, 2001).

The Rho GTPase and Fyn tyrosine kinase have been implicated previously in positive control of keratinocyte cell-cell adhesion (Galautti et al., 2002).

Ultraviolet (UV) irradiation causes photoaging through induction of matrix-degrading metalloproteinase (MMP), which is upregulated by activator protein-1 (AP-1) (Jun/Fos). The c-Jun kinase activity proves to be critically important in the regulation of AP-1 activity. UV irradiation activates epidermal growth factor receptor (EGFR) and cytokine receptors leading to the activation of c-Jun kinase in cultured human skin keratinocytes in vitro and in human skin in vivo. UV induced autophosphorylation of interleukin 1 receptor (IL-1) associated kinase (IRAK) in a manner analogous to IL-1 beta or EGF. EGFR plays a dominant role in the crosstalk (EGFR transactivation) among growth factor receptor and cytokine receptors leading to the activation of c-Jun kinase upon UV irradiation in keratinocytes (Wan *et al.*, 2001).

Li and Chou (1992) reported that exposure of Swiss 3T3 cells to a low dose of As^{3+} (2.5 μm) results in apparent cell retraction and loss of thick cables of actin filaments. At high doses (≥ 20 μm), As^{3+} treatment caused a severe loss of microtubules. Treatment of cells with As^{3+} also induced a dose-dependent inhibition of cytoskeletal protein synthesis.

Vega *et al.* (1999) reported that arsenic-exposed human peripheral blood mononuclear cells (PBMC) showed that proliferative inhibition was due to a suboptimal level of IL-2 secreted by lymphocytes. Electron microscopy studies revealed that cellular ultrastructure in golgi apparatus, mitochondria, cytoskeleton, and perinuclear membrane were altered.

Arsenite-induced stress at the molecular level shares many features with the heat shock response. This includes the differential sensitivity of the stress signal pathway elements to the magnitude of the stress, stressor-specific activation of the response elements, and the protective role of the heat shock response. Oxidative stress, the central component of heat shock response, is typical of arsenic-related effects that are, in fact, regarded as the chemical paradigm of heat stress. Similar to heat stress, arsenite induces

heat shock proteins (HSPs) of various sizes. The signal cascade triggered by arsenitelike heat stress induces the activities of the mitogen-activated protein (MAP) kinases, extracellular regulated kinase (ERK), c-jun terminal kinase (JNK), and p38. Through the JNK and p38 pathways, arsenite activates the immediate early genes c-fos, c-jun, and egr-1, usually activated by various growth factors, cytokines, differentiation signals, and DNA-damaging agents. Like other oxygen radical-producing stressors, arsenic induces nitric oxide production at the level of transcriptional activation along with induction of poly(ADP)-ribosylation, NAD depletion, DNA strandbreak, and formation of micronuclei (Bernstam and Nriagu, 2000). Porter *et al.* (1999) also reported that arsenate and arsenite activate JNK, however, the mechanism by which this occurs is not known. They showed that both arsenate and arsenite activation of JNK requires Rac and Rho. Arsenite stimulation of JNK requires PAK, whereas arsenate-mediated activation of JNK was unaffected by inhibitory mutant PAK.

Smith *et al.* (2001) also reported that low-level arsenite (5μM) treatment of porcine aortic endothelial cells (PAEC) stimulated superoxide accumulation that was attenuated by an inhibitor of NADPH oxidase, diphenyleneiodonium chloride. Analysis of NADPH oxidase components revealed that p67-phox localized exclusively to membrane of both control and treated cells. In contrast, cytosolic Rac1 translocated to the membrane fractions of cells treated with arsenite or angiotensin II but not with tumor necrosis factor. Immunodepletion of p67-phox blocked oxidase activity stimulated by all three compounds. However, depleting Rac1 inhibited responses only to arsenite and angiotensin II. They demonstrated that stimulus-specific activation of NADPH oxidase in endothelial cells was the source of reactive oxygen in endothelial cells after non-cytotoxic arsenite exposure.

Souza *et al.* (2001) reported that arsenite induced the activation of Akt at both Ser473 and Thr308, and its downstream effector eNOS in cultured human keratinocytes. Arsenic also induced phosphorylation of p38. PI3-K inhibitors, wortmannin and LY394002 inhibited arsenite-induced phosphorylation of Akt and eNOS but had no effect on phosphorylation of p38. Interestingly, SB203580, a known p38 inhibitor, completely inhibited arsenite-induced phosphorylation of Akt and eNOS. They indicated that arsenite induced activation of Akt and eNOS, via PI3-K and p38 pathway.

Recently, Simeonova *et al.* (2002) reported that arsenite activates EGFR and ERK in a human uroepithelial cell line. Arsenite-induced EGFR phosphorylation was independent of autocrine EGF, was sensitive to N-acetyl-cysteine (NAC), and did not involve the major autophosphorylation site, Tyr1173 and the inhibitor of Src activity, PP1, inhibited arsenic-induced EGFR and ERK phosphorylation. They indicated that c-Src was also induced by arsenite and was a prerequisite for the EGFR and ERK activation. In vivo, they found that exposure of mice to arsenite in drinking water, which had been found previously to be associated with AP-1 activation and epithelial cell proliferation, induced EGFR and ERK activation in the urinary bladder.