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THE ROLE OF REACTIVE OXYGEN SPECIES IN THE
ACTIN CYTOSKELETON REORGANIZATION
AND CELLULAR MOTILITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Leni Moldovan, M.S.

The Ohio State University
1999

Dissertation Committee:
Dr. Pascal J. Goldschmidt-Clermont, Adviser
Dr. Nicholas A. Flavahan
Dr. John M. Robinson
Dr. Arthur R. Strauch

Approved by

Molecular, Cellular and Developmental Biology
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Leni Moldovan

1999
ABSTRACT

The small GTPase rac1 (rac) controls actin redistribution to membrane ruffles and lamellipodia in fibroblasts and other cell types, as well as the activation of the NADPH oxidase in phagocytes. We explored the possibility that the two processes could be related. Specifically, we hypothesized that: (1) A constitutively activated mutant of rac, rac\textsuperscript{V12}, induces the activation of a non-phagocytic NADPH oxidase. (2) The resultant production of superoxide and derived radicals contribute to the reorganization of the actin cytoskeleton within membrane ruffles. (3) As a consequence, superoxide, or its metabolites, is involved in cellular motility, for which actin reorganization is a prerequisite. We used a replication deficient adenoviral vector (Ad-rac\textsuperscript{V12}) to overexpress with high efficiency rac\textsuperscript{V12} in human and mouse aortic endothelial cells. We showed that, in addition to the formation of membrane ruffle, rac\textsuperscript{V12} induced an increase in the total amount of F-actin within endothelial cells. Concurrently, rac\textsuperscript{V12}-overexpressing cells produced significantly higher amounts of free radicals than cells infected with a control virus, encoding the bacterial β-galactosidase. In order to assess whether the effect of racV12 on actin reorganization is mediated by superoxide or by its derived radicals, we co-expressed the human enzyme Cu,Zn-superoxide dismutase (SOD), by means of another adenoviral vector construct. Overexpressed SOD reduced the concentration of superoxide detected in Ad-rac\textsuperscript{V12} transfected cells, and reversed the effects of Ad-rac\textsuperscript{V12} on
the content of filamentous actin. MnTMPyP, a SOD mimetic, had similar effects, in that it reduced superoxide and derived free radicals concentration, as well as ruffle formation and the amount of filamentous actin within rac\(^{12}\)-overexpressing endothelial cells. These data support the hypothesis, that superoxide is one of the important mediators acting downstream of rac1 on the pathway of actin cytoskeleton remodeling in endothelial cells.

Furthermore, we studied the association between production of reactive oxygen species (ROS), actin organization, and cellular motility. We have used an endothelial cell monolayer wounding assay to demonstrate that the cells at the margin of the wound thus created, produced significantly more free radicals than cells in distant rows. The rate of incorporation of actin monomers into filaments was fastest at the wound margin, where heightened production of free radicals was detected. We have tested the effect of decreasing ROS production on migration of endothelial cells and on actin polymerization. The NADPH inhibitor diphenylene iodonium, as well as MnTMPyP, virtually abolished cytochalasin D-inhibitable actin monomer incorporation at the fast-growing, barbed, ends of filaments. Moreover, endothelial cell migration within the wound was significantly retarded in the presence of both DPI and MnTMPyP. Thus, migration of endothelial cells in response to loss of confluency includes the intracellular production of ROS, which contribute to the actin cytoskeleton reorganization required for the migratory behavior of endothelial cells. We conclude that ROS - i.e., superoxide or one of its metabolites, are necessary for the maintenance of a "activated" actin cytoskeleton, with fast polymerization-depolymerization occurring within membrane ruffles, during cellular migration.
I dedicate this work to those to whom I owe my becoming:

my parents, my husband, my children.
ACKNOWLEDGMENTS

The most special thanks to my adviser, Dr. Pascal J Goldschmidt-Clermont, who trusted me a priori and often believed my results more than myself, who introduced me into this great area of research - free radicals and actin - and who helped me when confusion was about to overtake, by pointing towards the right way.

I am also grateful to Drs. Nicholas Flavahan, John Robinson and Arthur Strauch, from the Molecular, Cellular, and Developmental Biology program of The Ohio State University, members of the thesis committee, with whom I had helpful discussions during the preparation of this work.

I am thankful to all those who, in different ways, contributed to my formation as an intellectual and scientist: first and above all, my parents; the professors from the School of Biology, University of Bucharest, Romania; the late Dr. Nicolae Simionescu, and Dr. Maya Simionescu, from the Institute of Cellular Biology and Pathology, Bucharest, Romania; my professors from the Department of Biochemistry, SUNY at Buffalo, especially Drs. Dan Kosman, Edward Niles, Cecile Pickart, Murray Ettinger, and Te-Chung Lee; the professors from the Cellular and Molecular Medicine program from Johns Hopkins University, especially Drs. Douglas Murphy and Garry Pasternak.

Also many thanks to the colleagues and investigators with whom either I collaborated during this work, especially Drs. Kaikobad Irani, from the Johns Hopkins...
University, Toren Finkel, from the Heart, Blood and Lung Institute, N.I.H., and Hamdy Hassanian, from the Heart and Lung Institute, O.S.U., or with whom I had interesting and fruitful discussions concerning this work, especially Drs. Christine Roos and Herve Kovacick, from the Heart and Lung Institute, O.S.U., as well as Mr. Mark Kotur, from the Flow Cytometry Facility of the H.L.I.

Last but not the least, without the constant trust, encouragement, understanding, and support, in addition to the endless patience of my husband, Nicanor Moldovan, I would have probably accomplished much less.

This work was supported by National Institutes of Health grants HL52315 and GM053236-03, by an American Heart Association award (Established Investigator) to Pascal J. Goldschmidt-Clermont, and by the Scleroderma Research Foundation.
VITA

July 13, 1955............................... Born – Bucharest, Romania

June 1978................................. M.S. Biology, University of Bucharest

1978 - 1981............................... Teacher of Biological Sciences, School No. 12,
Bucharest, Romania

1981 – 1994............................... Research investigator, Institute of Cellular Biology
and Pathology, Bucharest, Romania

1994 – 1996............................... Graduate Research and Teaching Associate, Dept.
of Biochemistry, SUNY at Buffalo, Buffalo, NY

1996 – 1997............................... Graduate Research Associate, Cellular and
Molecular Medicine Program, Johns Hopkins
University, Baltimore, MD

1997 – present............................. Graduate Research Associate, Molecular, Cellular,
and Developmental Biology Program, The Ohio
State University, Columbus, OH
PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular, Cellular, and Developmental Biology
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The tertiary structure of proteins enables them to perform the vast array of specific functions necessary for the maintenance of the living state. Their structure can oscillate between two extreme conformations, from unfolded to optimally folded, with a spectrum of different relevant states, usually stabilized by a given set of environmental factors. Certain proteins can also self-associate in physiological conditions. Actin is such a protein, which, upon self-association, can form a sophisticated network, further elaborated by the addition of more than 30 variants of the actin-binding protein family.

Protein-protein interactions are usually transient. Nevertheless, when such interactions result from the oxidation of sulfhydryl groups, linking cysteine residues through disulfide bridges, they require relatively large activation energies to be disjoined. At the same time, intra-protein disulfide bonds stabilize the three-dimensional structure of the proteins that possess them. Extracellular proteins need such a structural "reinforcement", to withstand the harsh and perpetually changing conditions, and making them less susceptible to degradation (Branden & Tooze, 1991). However, intracellular proteins require a higher flexibility, since they are involved in a dynamic network of structural, metabolic, and signaling interactions. Therefore, cells are equipped with an
important set of anti-oxidant systems, that keep the proteins mainly in a reduced form, to prevent unnecessary intra- and inter-protein disulfide bond formation.

Our laboratory has been involved in the study of reactive oxygen species (ROS) as signaling molecules, for the past 7 years. ROS are the highly reactive molecules that contribute largely to the oxidized state of proteins. Our work, and work from several other laboratories, has brought an important change in the framework wherein free radicals were considered solely a harmful molecular species, which, through their oxidizing activity, impair normal cellular functioning. Previous reports had already suggested that the effects of ROS might be more complex (Halliwell, 1991; Barja, 1993). It was more recently established, though, that ROS can be important mediators for multiple processes that include the regulation of transcription (Westwick et al., 1997a; Suzuki et al., 1997; Ono et al., 1993), cell proliferation and apoptosis (Moriyama et al., 1992; Irani et al., 1997; Polyak et al., 1997; Edwards, 1996; Jabs, 1999), and cellular activation (Goldstone et al., 1995; Goldstone et al., 1995; Goldstone et al., 1996; Guyton et al., 1996). A recurrent quest in the field of biology is the deciphering of the mechanisms that explain how signals originating from outside the cells can trigger specific cellular behaviors. One protein that corresponds to a cornerstone for many signaling processes is p21\textsuperscript{ras} (ras). We and others have recently discovered that ras can regulate cascades of signaling reactions through the production of oxygen radicals by specialized enzymes (Kheradmand et al., 1998; Doanes et al., 1998; Yeh et al., 1999). This thesis will focus on how ras and ROS contribute to the organization of the actin cytoskeleton, and on the resultant cellular locomotive behavior.
CHAPTER 2
LITERATURE REVIEW

2.1. THE ACTIN CYTOSKELETON

2.1.1. Structure and organization of the actin cytoskeleton

Comprising up to 10% of the total cellular protein, there is no wonder that actin is one of the most studied proteins, and that the mechanisms that control the organization of actin within cells are under active investigation. There are important differences between the organization of actin filaments within muscle and non-muscle cells. About half of the actin pool in any non-muscle cell is unpolymerized (Pollard & Cooper, 1986). Upon polymerization, actin monomers associate head-to-tail and form filaments (F-actin). These filaments further associate in various higher-level structures. Since our experimental system consists of non-muscle cells, we will focus on actin (re)organization within these cells.

Actin can exist either as a monomeric molecule, G-actin, or as a filamentous polymer, F-actin. It binds one molecule of ATP or ADP and has a single high affinity and several low affinity binding sites for divalent cations (Mg$^{2+}$) (Kabsch & Vandekerckhove, 1992) (for structure, see Fig. 1). After incorporation of an actin monomer into a filament, the bound ATP is slowly hydrolyzed to ADP. Interestingly, the analysis of the X-ray structure of actin, of the N-terminal domain of the heat-shock cognate protein Hsp70, and
These structures were obtained with the RasMol molecular imaging program, using the published coordinates of the proteins. In both images, actin has the same orientation, and the barbed and pointed ends are indicated. The four subdomains are numbered 1 through 4. C, nucleotide-binding cleft. Red: α-helices; yellow: β-sheets; blue: loops.

**Figure 1.** The structure of α-actin complexed with DNAse I (a) and β-actin complexed with profilin (b).
of hexokinase, has revealed an unexpected extensive similarity in tertiary structure between these molecules. Despite the absence of significant similarity of their amino acid sequences, all three proteins share the same core architecture and a common nucleotide binding site (Kabsch & Holmes, 1995). All three are ATPases or kinases and bind ATP in association with Mg$^{2+}$ or Ca$^{2+}$. We will discuss the importance of ATP binding and hydrolysis for actin function later in the chapter.

F-Actin forms a network throughout the cell, however the highest density is in the cortex, which is the region just beneath the plasma membrane (Alberts B et al., 1994). Many quiescent cultured cells, such as endothelial cells (the cells lining the inner surface of blood vessels) and fibroblasts (the cells responsible for wound healing), display bundles of actin filaments associated with actin-binding proteins such as myosin and α-actinin, called stress fibers (Kreis & Birchmeier, 1980; Byers & Fujiwara, 1982; Wong et al., 1983; Gabbiani et al., 1983). At one or both ends (Kano et al., 1996) stress fibers come in tight contact with specialized domains of the plasma membrane, named focal adhesions, through which cells adhere to the extracellular matrix. These are complex structures, the main component of which are transmembrane proteins of the integrin family (Tamkun et al., 1986). Integrins have an extracellular domain which specifically binds components of the extracellular matrix, such as fibronectin, and a cytosolic domain, connected to actin stress fibers through interaction with several ligand proteins: vinculin, talin, α-actinin, tensin, radixin (for review, see Jockusch et al., 1995)(Fig. 2). Focal adhesions are highly dynamic structures, as they continuously remodel, and their distribution is responsive to environmental signals (Davies et al., 1993).
Figure 2. Schematic representation of the structure (a) and the assembly (b) of focal adhesions.
A dramatic change in the appearance of the actin cytoskeleton occurs when the cells are stimulated. Reendothelialization following injury to the endothelium (Gotlieb, 1990), growth factors, e.g., platelet-derived growth factor (PDGF) (Herman & Pledger, 1985), tumor promoters (Schliwa et al., 1984), chemotactic proteins (Norgauer et al., 1994; Westphal et al., 1997), as well as other physiologic or experimental stimuli, induce the redistribution of actin filaments. Actin remodeling (Stossel et al., 1999) is the major intracellular change responsible for the motile responses to the mentioned factors, including the intense membrane activity known as "ruffling", shape changes and directional locomotion (Cooper, 1991). Advancing cells extend in the direction of their movement a flat process, the lamellipodium, or thin, cylindrical projections, named filopodia (Condeelis, 1993; Lauffenburger & Horwitz, 1996; Stossel et al., 1999) (Fig. 3). Actin fibers are continuously assembled at the plasma membrane in these regions, and are stabilized by cross-linking proteins such as ABP-280 (Stossel et al., 1999b). The dynamic equilibrium between polymerization and depolymerization of actin fibers is maintained (and controlled) by the redistribution of different actin-binding proteins (Theriot & Mitchison, 1992; Cunningham, 1992; McGrath et al., 1998).

The organization of actin filaments within these structures is different from that in stress fibers, and, accordingly, the actin-binding proteins that contribute to their stabilization are different. Within stress fibers, filaments are arranged with opposite polarities, the loose packing is maintained by α-actinin cross-bridges, and their contractility is supported by the presence of myosin (Katoh et al., 1998). The leading edge of an advancing cell is composed of thin protrusions of membrane, which continuously extend and retract. Actin is organized in bundles (in filopodia) or forms a
Figure 3. Subconfluent MAEC displaying prominent membrane ruffles (arrows)
three-dimensional network of orthogonally arrayed short filaments (in lamellipodia) (Hartwig, 1992; Welch et al., 1997a). These actin filaments have their fast growing ends oriented towards the membrane and undergo a continuous cycle of assembly towards the plasma membrane, followed by depolymerization (Welch et al., 1997a). The lamellipodium contains specific proteins that cross-link actin into this network, such as Arp2/3 (Mullins & Pollard, 1999), or fimbrin and filamin within filopodia (Matsudaira, 1994; Ohta et al., 1999). These cellular structures, specific to motile cells, thus contain a large number of actin-binding proteins that contribute to the highly dynamic behavior of the actin cytoskeleton. We will discuss in more detail several of these proteins in the next section.

2.1.2. Dynamics of the actin cytoskeleton in relation to the cellular motility

Although the term “actin cytoskeleton” has a connotation of rigidity, suggesting that it mainly contributes to the mechanical support of the cell, this is by no means the case. The actin cytoskeleton is highly dynamic, since only such dynamic actin-actin and actin-binding proteins interactions can support cellular motile responses on time scales ranging from sub-seconds to hours.

Actin filaments are polar structures, with two structurally and functionally different ends: a slow growing “minus end” and a faster growing “plus end”. Because of the oriented “arrowhead” appearance of the complex formed between actin filaments and myosin heads, the “minus end” is also called the “pointed end”, while the “plus end” is referred to as the “barbed end” (Albers et al., 1994). The first step in the formation of an
actin filament is nucleation. It involves the association of actin monomers to form dimers, and then trimers, which then act as templates for elongation (Wegner & Engel, 1975). This is an extremely unfavorable reaction (having a high activation energy), which minimizes the probability of random formation of new filaments (Pollard, 1988). It is the rate-limiting step in actin polymerization, and it is also an important point of control for actin assembly. Polymerization of pure actin \textit{in vitro} requires ATP and the cations $K^+$ and $Mg^{2+}$. $Ca^{2+}$ may replace $Mg^{2+}$, however with lower efficiency: $Mg^{2+}$-actin nucleates and polymerizes more readily than $Ca^{2+}$-actin, and the polymer of $Mg^{2+}$-actin is more stable than that of $Ca^{2+}$-actin (Selden \textit{et al.}, 1983). The polymerization rate is different at the two ends of the actin filament: at the barbed end, actin polymerizes at up to 10 times the rate at the pointed end, with $K_d$ of 0.1 $\mu$M and up to 1 $\mu$M, respectively. A \textit{critical concentration} was defined, as the free actin monomer concentration at which the rate of subunit addition equals the rate of subunit depolymerization; in other words, the proportion of actin polymer stops increasing (Oosawa & Asakura, 1975; Alberts \textit{et al.}, 1994). The critical concentration is different at the minus and plus ends of an actin filament, and is around 0.2 $\mu$M, much lower than the concentration of monomeric actin in cells [10-15 $\mu$M in non-motile cells, and up to 280 $\mu$M in motile cells (Devineni \textit{et al.}, 1999)]. As a consequence, cells have evolved various mechanisms to prevent the actin from the G-actin pool to polymerize.

As polymerization proceeds, actin hydrolyzes bound ATP in an irreversible reaction, that depends on the concentration of polymerized ATP-actin, not on the rate of incorporation of ATP-actin into the polymer (Pollard & Weeds, 1984). Hydrolysis is not required for polymerization. After ATP cleavage, $P_i$ is not immediately released, but
remains bound to F-actin in an F-actin-ADP-P\textsubscript{i} transient (Carlier & Pantaloni, 1986). This intermediate is a highly stable filament, and the release of P\textsubscript{i} destabilizes it through a conformational change of actin monomers, that weakens the bonds within the polymer and thereby promotes depolymerization (Carlier, 1989; Carlier, 1990). Compared with ATP-actin, ADP-actin polymerizes slower at both ends, dissociates faster from the barbed end, but dissociates slower from the pointed end (Pollard, 1986a). At steady state, when actin is continuously added at the barbed ends, and continuously dissociates from the pointed ends, a dynamic process occurs, called \textit{treadmilling}, maintained due to the ATP hydrolysis (Selve & Wegner, 1986). The distinct properties of actin molecules containing different intermediates of ATP hydrolysis is important, for instance, in maintaining high rates of net polymerization or net depolymerization at different sites within a cell (Fechheimer & Zigmond, 1993). Thus, the role of ATP hydrolysis is to maintain the dynamic state and polarity of filamentous actin, which is able to respond within seconds to environmental challenges.

We already mentioned that the concentration of monomeric actin within cells is above the critical concentration. This raises the question of how cells prevent excessive actin polymerization, and also leads to the more complex problem of the control of actin organization. The discovery of numerous proteins that interact with both G- and F-actin provided an answer to these questions. The interaction between actin and actin-binding proteins controls different steps of the process of actin fiber assembly and disassembly. Although specific, many of these interactions are weak, (K\textsubscript{d} in the range of $10^{-5}$M; (Pollard, 1988). Many various proteins regulate the pool of monomers available for polymerization; they also cap, sever, and cross-link filaments, and many of these
interactions comprise very low affinity bonds between the involved molecules (Pollard, 1986b). An obvious advantage of such weak interactions resides in the rate with which they are formed and broken, which, in turn, is essential for the dynamic properties of the actin cytoskeleton and for the promptitude with which cells respond to stimuli. As a corollary, one would expect that signaling pathways exert control upon the interactions between actin and its regulatory proteins (Pollard, 1988).

Actin polymerization plays a major role in cell movement. The controls of actin sequestration/desequestration and of filament turnover are two important features of cellular motility. Actin binding proteins use properties derived from the steady-state monomer-polymer cycle of actin in the presence of ATP, to control the F-actin/G-actin ratio and the turnover rate of actin filaments. Capping, severing, and sequestering proteins regulate the size of the pools of F-actin and unassembled actin by affecting the steady-state concentration of ATP-G-actin (Carlier & Pantaloni, 1997).

We shall first briefly examine how actin-sequestering proteins contribute to the control of actin polymerization/depolymerization cycle. An important limitation in the generation of new filaments is achieved through the binding of actin monomers to protein ligands. G-actin binds stoichiometrically to G-actin-sequestering proteins such as thymosin β₄ (Cassimeris et al., 1992; Carlier et al., 1996; Ademoglu et al., 1998), and profilin (Carlsson et al., 1977; Carlier & Pantaloni, 1994). When the cell is stimulated, actin is released, the rate of exchange of ADP/ATP increases (Goldschmidt-Clermont et al., 1992a), and F-actin is formed. Further, the steady state of actin assembly is controlled by the interaction of F-actin with capping proteins (Carlier & Pantaloni, 1997).
For a while after its discovery, the small (5 kDa) protein thymosin β4 was considered an exclusive actin-monomer-binding protein (Sun et al., 1995). Platelets and leukocytes contain high amounts of thymosin β4, around 500 μM (Weber et al., 1992), and 170 μM (Cassimeris et al., 1992), respectively. It forms a 1:1 complex with actin, and thus sequesters a large pool of G-actin. However, this is a readily accessible pool, because the affinity of non-muscle G-actin for thymosin β4 is comparable to that for the pointed ends of the actin filaments. Hence, thymosin β4 maintains an equilibrium between a small pool of free actin monomers (< 1 μM), the ends of actin filaments, and a large pool of actin-thymosin β4 complex (Weber et al., 1992). Interestingly, thymosin β4 binds ATP-G-actin about 50-fold stronger than ADP-G-actin (Carlier et al., 1993), and it inhibits the exchange rate of the actin-bound nucleotide by a factor of $10^{10}$ (Goldschmidt-Clermont et al., 1992a), thus contributing to the maintenance of the more readily polymerizable ATP-G-actin pool. Nevertheless, most of the in vitro studies were done with low concentrations of thymosin β4. When investigators looked at the effects of higher thymosin β4 concentrations, both in vitro and in vivo, it turned out that this protein interacted with F-actin as well. As a matter of fact, thymosin β4 was incorporated into the filaments, and cells overexpressing this protein had more F-actin than control cells (Sun et al., 1996; Carlier et al., 1996). Thus, the role of thymosins is clearly more complex than just sequestering actin monomers.

Another actin-binding protein that belongs to the sequestering proteins group is profilin (Fig. 1b). It is also a G-actin-binding protein, and has some of the properties of a sequestering protein. It binds to actin monomers in a 1:1 complex, thereby inhibiting the spontaneous nucleation of actin filaments (Pollard & Cooper, 1984). As opposed to
thymosin β4, profilin catalyses the exchange of adenosine nucleotides bound to actin monomers by increasing the off-rate by $10^4$ (Mockrin & Korn, 1980; Goldschmidt-Clermont et al., 1992a). This effect might potentiate filament formation in physiological conditions, a conclusion also supported by the fact that profilin localizes in regions of the cells where rapid actin assembly occurs (Buss et al., 1992). Another conceptually important finding is that the effect of profilin depends on the presence of other actin-binding proteins (Pantaloni & Carlier, 1993). If F-actin barbed ends are capped, the sequestering activities of profilin and thymosin β4 are additive. However, in the presence of uncapped barbed ends or thymosin β4, profilin promotes actin assembly. More recent studies addressed the interaction of profilin and other actin-binding proteins, which will be discussed later in the chapter.

The analysis of the interactions between F-actin and its binding proteins showed that they are extremely complex: capping, promoting assembly, promoting disassembly, severing, stabilization, different patterns of actin filaments cross-linking, anchoring (connecting with various membrane structures, such as focal adhesions and intercellular junctions). We shall focus upon several better studied proteins, proven to play important roles in cell motility.

Some actin-binding proteins can provide more than one function. Gelsolin is a paradigm for a family of filament-severing proteins that also includes villin, severin, adseverin, and others. In the presence of micromolar concentrations of calcium, it severs actin filaments, thus reducing their length (Yin & Stossel, 1979). [Interestingly, the effect of calcium on actin-bundling proteins (α-actinin, fimbrin, 30-kDa bundling protein) is to promote dissociation, therefore reversal of bundling (Janmey, 1994)]. Gelsolin also has
an apparently paradoxical effect: it binds actin monomers and oligomers, forms new nucleation sites and thereby promotes polymerization (Matsudaira & Janmey, 1988). After severing, gelsolin caps the barbed end of a free or a newly severed filament, thus preventing polymerization at the fast-growing ends (Kwiatkowski, 1999). Gelsolin is also regulated by plasma membrane molecules, namely the inositol phospholipids (PPIs). The best characterized signaling role for PPIs is as a source of inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI (4,5)P$_2$] by phospholipase C (PLC). Binding of gelsolin to PPIs appears to reverse its interaction with actin, thus regenerating the ligand for more interaction with new actin molecules (Janmey & Stossel, 1989). [It is noteworthy that profilin interaction with actin is also regulated by PPIs (Lassing & Lindberg, 1985)]. Interestingly, there are two forms of gelsolin: one is the intracellular isoform, that performs the functions above mentioned; there is also an extracellular, plasmatic form of gelsolin. An important difference between these two forms is that the extracellular one has an intramolecular disulfide bond between cysteines 188 and 201 in domain 2, while the intracellular gelsolin is in reduced form (Wen et al., 1996).

A remarkable structural similarity was found between gelsolin and another, unrelated, F-actin binding protein, cofilin, which is a member of the actin depolymerizing factors (ADF) family (Van Troys et al., 1997; Wriggers et al., 1998; Maciver, 1998). It was first considered an actin severing protein, however it was recently discovered that the mechanism, by which it enhances actin depolymerization, is to increase the filament twist, thereby weakening the intrafilament interactions between actin monomers (McGough et al., 1997). The result is a 25-fold increase of actin dissociation from
pointed ends (Carlier et al., 1997), a process which is cooperative (Ressad et al., 1998).

An unusual feature of this protein, that makes it unique among actin-binding proteins, is its regulation by serine phosphorylation (Davidson & Haslam, 1994; Suzuki et al., 1995): it is activated by dephosphorylation and inactivated by phosphorylation. Moreover, when actin dynamics are measured in the presence of both ADF/cofilin and profilin, the following changes are observed: (1) although actoferin (Acanthamoeba cofilin) inhibits ADP exchange and forms a stable complex with actin, profilin overcomes this effect and accelerates the recycling of both proteins (Blanchoin & Pollard, 1998); (2) profilin synergises with ADF in increasing actin filaments treadmilling (Didry et al., 1998).

ADF/cofilin proteins are not the only actin binding proteins with which profilin interacts: the actin-related protein (ARP) 2/3 complex is involved in the actin cytoskeleton organization, and is also a partner of profilin in this activity. In fact, the complex was first purified from Acanthamoeba castellanii by profilin affinity chromatography (Machesky et al., 1994). The complex is formed of two actin-related proteins, Arp2 and Arp3, as well as five new proteins (p40, p35, p19, p18, p14) (Mullins et al., 1997). The complex was detected within lamellipodia (Welch et al., 1997b; Machesky et al., 1997), a finding that suggested it might be an important organizer of the specific actin structures found at the leading edge of migrating cells. And indeed, it was subsequently found that Arp2/3 complex caps the pointed ends of actin filaments, and the ensemble of Arp2/3 and actin forms a nucleation site for actin polymerization only from barbed ends (Mullins et al., 1998). Moreover, it also binds to the sides of actin filaments, at a constant angle of about 70°, and this remarkable feature supports formation of a
branching network of actin filaments at the leading edge of motile cells (Mullins et al., 1998) (Fig. 4).

We have briefly reviewed some of the most studied actin-binding proteins involved in actin (re)organization. There are obviously many more, less well understood proteins, and whose roles begin to be recognized. It is important to understand the complexity of actin interactions with its binding proteins, as the control of cytoskeleton remodeling happens via these interactions, and thereby the control of actin polymerization/depolymerization parameters. Moreover, some of these actin-binding proteins, and even actin itself, have the potential of being modulated by redox conditions, an important feature to take into consideration, if we are to understand how actin reorganization takes place in cells, and the contribution of ROS in this process.

2.1.3. Signaling pathways for the actin cytoskeleton reorganization

The nature of the signaling pathways that control actin reorganization in non-muscle cells has been long sought. However, despite efforts of many laboratories, the biochemical steps connecting extracellular signals to the reorganization of the actin cytoskeleton are not yet completely characterized. There are many points of control along these pathways, and the pathways themselves are often redundant.

We have described some of the protein players that control the ratio of polymerized/unpolymerized actin. The next problem is to define how these proteins are controlled, and in response to what extracellular signals. A very important discovery for the field was that the small GTP-binding proteins from the rho family, part of the bigger
Inactive or sequestered Arp2/3 complex (a) is recruited to the leading edge of the cell by an unknown mechanism (b). The complex nucleates new actin filaments either while free (c) or while bound to the side of existing filaments (d). The complex anchors the branch at a fixed angle of 70° relative to the barbed ends of the two filaments. Rapid growth of new filaments in the barbed direction, expands the actin filament network. Subsequent disassembly of the network deep in the cytoplasm regenerates subunits for later growth of the cortical network.

**Figure 4.** Dendritic nucleation model for actin polymerization, capping, and network formation at the leading edge of a motile cell.
ras superfamily of proteins, namely rho, rac and Cdc42. represent a cornerstone of actin cytoskeleton control (see also next section). The activity state of these proteins is determined by the bound nucleotide: GTP in the active form, GDP in the inactive form (Fig. 5). The presence or absence of an extra phosphate group on the guanosine nucleotide regulates the conformational changes of rho proteins responsible for the interactions of the GTPases with downstream effector ligands (we will analyze in more detail this mechanism in the next section).

In Swiss 3T3 cells, (1) rho proteins were shown to mediate the lysophosphatidic acid and bombesin-induced formation of focal adhesions and actin stress fibers (Ridley & Hall, 1992a); (2) rac proteins are required for the platelet-derived growth factor-, insulin-, GTPases cycle between GDP-bound (inactive) and GTP-bound (active) forms. Interacting proteins control each of the three steps: GDP - dissociation inhibitor (GDI), guanine nucleotide exchange factor (GEF), and GTPase-activating protein (GAP).

**Figure 5.** The principle of GTPases functioning.
bombesin- and phorbol ester (phorbol 12-myristate 13-acetate, PMA)-stimulated actin polymerization at the plasma membrane that results in membrane ruffling (Ridley et al., 1992); and (3) Cdc42 controls the formation of filopodia induced by bradykinin receptor activation (Nobes & Hall, 1995a, Kozma et al., 1995). Moreover, in these cells, a sequential activation of Cdc42 → rac → rho was described (Nobes & Hall, 1995), although this conclusion cannot be extrapolated to other cells (Fig. 6).

The extracellular triggers of actin remodeling are mostly hormones, such as growth factors [platelet-derived growth factor (PDGF) (Nobes et al., 1995); insulin-like

![Diagram of actin remodeling pathways](image)

*Figure 6.* The members of the rho family of small GTPases control different patterns of actin cytoskeleton reorganization in various environmental conditions.
growth factor, IGF (Vollenweider et al., 1999); insulin (Tsakiridis et al., 1997), and bradykinin, as already mentioned (and described in the cited papers). Some other factors that induce actin reorganization for chemotaxis and phagocytosis, are chemotactic factors, such as formylated oligopeptides (Allen et al., 1997; Caron & Hall, 1998).

Furthermore, suspended cells in solution have a different organization of F-actin, when compared to static or migrating adherent cells. One of the mechanisms involved in this reorganization requires the interaction of cells with extracellular matrix proteins mediated by integrins (Clark et al., 1998). Interestingly, the binding of integrins to extracellular matrix components is regulated from the cytoplasmic side of these molecules, while binding of the specific adhesion ligand triggers, in turn, intracellular events. Important for the cytoskeletal reorganization is the phosphorylation of the focal adhesion kinase (FAK). This protein kinase becomes activated and phosphorylated upon cell adhesion and, in turn, binds and activates a number of intracellular signaling molecules. We will mention here the tyrosine kinase src, and the fact that FAK activates, directly or through src, phosphoinositide 3-kinase (PI3K) (Giancotti & Ruoslahti, 1999).

Once the signal is transmitted within the cell, an array of signaling pathways is set into motion, often redundant, and leading to many types of responses. Moreover, these responses are extremely cell-dependent, and sometimes even inconsistent, as illustrated by the case of cofilin. Some of these pathways are well characterized, while for others the knowledge is still fragmentary.

One of the molecules that “takes center stage” (Leevers et al., 1999) is PI3K, as well as other lipid kinases (Carpenter et al., 1999). This enzyme phosphorylates inositol lipids at the 3’ position, generating either mono- [PI(3)P], di- [PI(3,4)P2], or tri-
phosphoinositides [PI(3,4,5)P₃] (Leevers et al., 1999). In turn, these lipids interact with 3-
phosphoinositides-binding domains present in many signaling molecules. The pleckstrin
homology domain is one of them (Rebecchi & Scarlata, 1998), and it is present in
guanine nucleotide exchange factors (GEFs), that catalyze the conversion of small
GTPases from the inactive, GDP-bound form, to the active, GTP-bound form. Rac is
considered to function downstream of PI3K, although in different systems, it may act
either downstream or upstream of PI3K. There are at least two suggested mechanisms by
which this may happen:

(1) Rac and cdc42 directly associate with PI3K, therefore activating it (Carpenter
et al., 1997). The result is the increased formation of 3’-phosphoinositides, which bind to
gelsolin and related proteins, releasing them from actin filaments and exposing barbed
ends. They also bind to profilin and cofilin, dissociating them from actin monomers. The
net result of these interactions, between different phosphoinositides and actin-binding
proteins, is actin polymerization (Carpenter et al., 1997).

(2) The GEF activity of vav, a pleckstrin-homology domain-containing GEF for
rac, is activated by 3’-phosphoinositides (Han et al., 1998), thus further activating rac.

PDGF is one of the activators of PI3K. Another downstream effector of PDGF
receptor activation is phospholipase Cγ (PLCγ). PLCγ preferentially hydrolyses PI(4,5)P₂,
releasing inositol(1,4,5)trisphosphate (IP₃) and diacylglycerol (DAG). DAG further
activates protein kinase C, while IP₃ releases calcium from intracellular stores (Toker,
1998), with effects upon some of the actin-binding proteins (e.g., gelsolin) (Heldin et al.,
1998). Still another downstream effector of PDGF-receptor is the tyrosine phosphatase
SHP-1, which, by a yet unknown mechanism, induces termination of membrane ruffling stimulated by PDGF receptor occupancy (Cossette et al., 1996).

Overall, little is known about the upstream control of rho proteins, and the downstream pathways are not better characterized (e.g., Aspenstrom, 1999). We will discuss these pathways in more detail within the next section. However, two proteins deserve attention for this section: gelsolin and coflin.

In a report on gelsolin knock-out mice (Gsn^−) (Azuma et al., 1998) showed that fibroblasts from these animals have reduced ruffling activity and reduced migration. Moreover, rac expression is markedly enhanced in Gsn^− mice. Since re-expression of gelsolin in fibroblasts derived from these animals normalizes both rac expression and motility, it is concluded that gelsolin is a likely downstream effector of rac. The proposed sequence of events is as follows: growth factor (EGF, for instance) binding to its receptor → receptor activation → PI3K activation → synthesis of PI(3,4,5)P_3 → vav(?) → rac activation and recruitment to the plasma membrane → activation of phospholipid kinases → synthesis of PI(4,5)P_2 → cycles of gelsolin activation → actin remodeling (Azuma et al., 1998). Although this sequence takes into account many of the known facts about individual steps, it still needs to be confirmed as a whole.

Cofilin regulation is different in phagocytic, versus nonphagocytic, cells. Upon neutrophil stimulation by various signals (chemoattractants, PKC activators, opsonized zymosan, arachidonic acid), the cells develop an intense membrane ruffling activity. Such activity coincides with cofilin translocation to the membrane and its dephosphorylation, thus becoming active in depolymerizing actin filaments (Suzuki et al., 1995; Djafarzadeh & Niggli, 1997; Heyworth et al., 1997). In contrast, in several nonphagocytic cells
(HeLa, COS, PC12, 3T3, etc), activated rac induces: (1) LIM-kinase 1, also known as KIZ, activation. This is a protein kinase with two amino-terminal LIM motifs [a double zinc finger domain at the n-terminus (Stanyon & Bernard, 1999)]. (2) Cofilin phosphorylation by this kinase, thus its inactivation. (3) Filamentous actin stabilization (Arber et al., 1998; Yang et al., 1998). The authors (Yang et al., 1998) suggest that this may be due to a decrease in the rate of actin depolymerization by cofilin.

It is worthy to note that a recent report showed colocalization of several actin-binding and actin-regulating proteins within Listeria actin tails: Arp2/3 and VASP (vasodilator-stimulated phosphoprotein), and also cofilin, capZ and rac, thus suggesting that rac might be involved in the regulation of Arp2/3 (David et al., 1998), perhaps via WASP (Wiscott-Aldrich syndrome protein) (Bi & Zigmond, 1999).

2.2. SMALL GTPASES

2.2.1. General structural and functional characteristics

An important step in understanding the regulation of the actin cytoskeleton was the discovery of the involvement of small GTP binding proteins of the rho family in this process. The proteins from this family, as all small GTPases, are regulated by the binding, hydrolysis, and release of GTP (Fig. 5). GDP is bound in the quiescent state; when activated by exchange factors, GDP is released and replaced by GTP. GTP is then hydrolyzed to GDP in an irreversible step, the inorganic phosphate is released, and the protein again becomes inactive. The $K_d$ for guanine nucleotide binding is several orders
of magnitude smaller than the concentration of the nucleotide within cells, such that the small GTP binding proteins are not controlled by the cellular level of GTP (Bourne et al., 1991). Instead, the interaction with the guanine nucleotides is controlled by associated proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP-dissociation inhibitors (GDIs) (Boguski & McCormick, 1993). When activated, some of the small GTPases associate with the plasma membrane (Lowy & Willumsen, 1993). This association requires posttranslational modifications at the C-terminus of the protein, that include methylation and isoprenylation (Hancock et al., 1990; Kinsella et al., 1991; Seabra, 1998).

The analysis of the thermodynamic cycles of actin and small GTPases of the ras family reveals striking similarities (Fig. 7). Both bind in the inactive state to nucleotide diphosphates (NDP), ADP for actin and GDP for ras. Both are activated by the release of NDP, and binding of the respective nucleotide triphosphate (NTP) with high affinity. Both have a NTPase activity, although the spontaneous hydrolysis NTP is slow. Nevertheless, it can be accelerated by interaction with other proteins (ras) or with itself (actin within filaments). This step is irreversible, forcing the cycle in one direction. The rate-limiting step is the dissociation of the hydrolyzed NDP, which can be accelerated by regulatory factors (exchangers). These characteristics position the proteins of the ras family as good candidates for the control of the timing of assembly and disassembly of complex effector units inside cells (Goldschmidt-Clermont et al., 1992b).
The big, red arrows indicate the irreversible step for each cycle. The small, green arrows indicate the reactions that are accelerated by the exchanger proteins. GAP represents GTPase activating protein.

**Figure 9.** The nucleotide triphosphatase cycle of actin and small GTPases of the Ras family.
2.2.2. Control of actin reorganization by the rho-family proteins

The rho proteins, which include rhoA, rac1, rac2, and cdc42, were found to coordinate the spatial and temporal changes in the actin cytoskeleton that lead to cellular movements (Nobes & Hall, 1995) (Fig. 6). Microinjection experiments showed that rho acts downstream of PDGF and other growth factors, as well as lysophosphatidic acid (LPA), on the pathway leading to the assembly of stress fibers and focal adhesions in Swiss 3T3 fibroblasts (Ridley & Hall, 1992). In contrast, rac1 induces a strong pinocytotic and membrane ruffling activity, accompanied by redistribution of actin fibers at the periphery of the cells (Ridley et al., 1992). At the same time, the increase in stress fibers triggered by PDGF, but not by LPA, is also dependent on rac, which acts upstream of rho (Ridley et al., 1992). Finally, cdc42 promotes the formation of filopodia and it also induces the activation of rac (Nobes & Hall, 1995b).

Since the first reports on the function of GTP binding proteins in fibroblasts, their involvement in actin reorganization has been confirmed in other systems as well: mast cells (Norman et al., 1994), platelets (Hartwig et al., 1995; Hooshmand-Rad et al., 1997), intestinal epithelial cells (Nusrat et al., 1997), cytokinesis in Dictyostelium (Larochelle et al., 1996), border cells in Drosophila (Lee & Montell, 1997), macrophages (Allen et al., 1997; Cox et al., 1997). Moreover, the last years have seen a burst of discoveries of various upstream and downstream effectors for these proteins. Many aspects of the upstream pathways involving the small GTP binding proteins are relevant to the biology of cells, including their involvement in cell proliferation and death. However, our focus is
on how rac promotes actin reorganization, and we will concentrate mainly on those proteins/effectors that were found to mediate rac effects on actin.

Peppelenbosch et al. (Peppelenbosch et al., 1995) suggested that growth factor-induced stress fiber formation in Swiss 3T3 cells is mediated by arachidonic acid release. They observed that inhibitors of leukotriene synthesis inhibit rac-induced stress fiber formation in these cells, and established that leukotrienes act downstream of rac in triggering rho-mediated stress fiber formation. However, there is still no consensus on what may be the intermediate steps between rac and membrane ruffling. Another group, looking at actin polymerization in activated platelets, determined that rac activation induced actin filament uncapping through a sequence of events that included stimulation of phosphoinositide synthesis and gelsolin release from actin filaments (Hartwig et al., 1995). This pathway has been confirmed in epithelial cells as well (Keely et al., 1997), and in the Gsn⁺ mouse (Azuma et al., 1998). However, not in every cell type is gelsolin dissociation from actin filaments phosphoinositide kinase-dependent (Arcaro, 1998). This suggests that more than one mechanism is responsible for rac control on gelsolin, and actin polymerization. Moreover, the mechanism responsible for phosphoinositide kinase activation remains uncertain.

The pathway became even more complex when two groups (Coso et al., 1995; Minden et al., 1995), reported that rac1 and cdc42 control not only cellular morphology, but also gene expression (Minden et al., 1995; Coso et al., 1995). The search of intermediate molecules on this pathway led to the discovery of a novel family of serine/threonine kinases, which are activated by the interaction with GTP binding proteins from the rho family (see, e.g., Manser et al., 1995; Bagrodia et al., 1995). One of
the members of this family, mPAK-3 (p21-activated kinase), contains putative SH\(_3\) binding motifs, and binds \textit{in vitro} to the SH\(_3\) domains of phospholipase C-\(\gamma\) (PLC) and Nck. It was suggested that mPAK-3 is one of the missing links between rac and cdc42, on one hand, and actin cytoskeleton reorganization, on the other hand (Bagrodia \textit{et al.}, 1995; Zhao \textit{et al.}, 1998). However, although more downstream rac and PAK signaling proteins are being discovered, such as the Cool/Pix and Cat proteins, a review from September 1999 still states that "the precise roles that the PAKs play in regulating reorganization of the actin cytoskeleton have remained somewhat elusive, in part because of seemingly conflicting results that have emerged from a variety of studies" (Bagrodia & Cerione, 1999).

One of the most notorious "conflicting" results was that reported by Joneson (Joneson \textit{et al.}, 1996), where the authors compared rac mutants able to bind either PAK, or another rac-interacting protein, POR1 (partner of rac1). This latter protein has no homology with known proteins, contains a putative leucine zipper sequence, and it binds preferentially to GTP-loaded rac. When rac mutants that were able to bind mPAK3 and activate JNK activity, but not POR1, or to bind POR1 but not PAK3, were assayed functionally, the result was that stimulation of JNK through mPAK3, and induction of membrane ruffling through POR1, were independent pathways (Joneson \textit{et al.}, 1996). However, when multiple rac mutants (Fig. 8) were analyzed for their functions, the surprising conclusion emerged, that Rac1 regulates at least four distinct effector systems, and that PAK binding was dispensable for Rac1-induced transformation and lamellipodium formation, as well as activation of JNK (Westwick \textit{et al.}, 1997). Thus, the proteins acting downstream of this GTPase and their respective pathways are still
unclear, since it seems that multiple domains of rac are involved in one function, and individual domains may be interacting with more than one effector, thus prompting variable effects (Fig 8).

2.2.3. Control of the NADPH oxidase

While rac control of membrane ruffling in fibroblasts was being described, two groups reported the involvement of the same protein in an apparently unrelated process (Abo et al., 1991; Knaus et al., 1991). The killing of microorganisms by phagocytic cells (neutrophils, macrophages) requires the production of SO and other ROS (for reviews, see, e.g., Robinson & Babcock, 1998, and Clark, 1999). Responsible for the production of SO is the membrane-bound enzyme, NADPH oxidase, which catalyses the reaction:

\[ \text{NADPH} + 2\text{O}_2 \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+ \]

By dismutation of \( \text{O}_2^- \), SOD further promotes hydrogen peroxide formation:

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

The active enzyme is a complex of the membrane flavocytochrome b558, and several cytosolic factors: p47phox, p67phox and p40phox (Fuchs et al., 1996; Babior, 1999) (Fig. 9). Upon activation, p47phox becomes phosphorylated and all three proteins are recruited at the plasma membrane. However, an absolute requirement for a GTP-binding protein in the activation of the NADPH oxidase was observed (Bokoch & Prossnitz, 1992), and the search for this protein identified rac1 (in macrophages) and rac2 (in neutrophils). In resting neutrophils, rac2 is found in the cytosolic fraction, while in
The yellow and green spheres represent amino acids targeted for this structure/function analysis of rac, and their mutation. GTP is represented as purple spheres. The insert region is not present in ras proteins. Some of the functions, in which specific amino acids were implicated, are also shown.

**Figure 8.** Structural and functional domains of rac, and some of the frequently encountered (natural) mutations.
activated phagocytes it is translocated to the plasma membrane (Quinn et al., 1993). The process is dependent of rac isoprenylation (important for membrane recruitment) and requires that the bound nucleotide is GTP (Bokoch & Knaus, 1994). An interesting difference between phagocytic and nonphagocytic cells is the relative abundance of the different rac isoforms: neutrophils contain mostly rac2, while the other cells of the mammalian body, including macrophages, contain mostly rac1.

Although the function of rac in actin and NADPH oxidase control was described in 1991-1992, it was not until 1998 that a connection was established between the production of SO via a non-phagocytic NADPH oxidase, and other intracellular functions of rac (Kheradmand et al., 1998). Since our work attempts to further characterize such connection, in the next section we will briefly describe what is known about the physiologic functions of SO in eukaryotic cells.

2.3. REACTIVE OXYGEN SPECIES

2.3.1. Nonphagocytic NADPH oxidases: do they exist?

In an inherited disease characterized by recurrent infections and inflammatory reactions with granuloma formation, therefore called chronic granulomatous disease (CGD), phagocytes are unable to produce SO, due to defects in components of the NADPH oxidase. Interestingly, when fibroblasts from patients with CGD were analyzed for their ability to produce SO, it was observed that this was not impaired, and that the
Figure 9. Rac recruits the cytoplasmic components of the NADPH oxidase at the plasma membrane and activates its superoxide producing activity.
enzymatic system producing SO was comparable to the phagocytic NADPH oxidase (Meier et al., 1991). However, it was recognized that the fibroblast and phagocytic oxidases were immunologically and genetically distinct (Meier et al., 1993; Emmendorffer et al., 1993). These findings suggested that more than one NADPH oxidase system exists within the body, which produce SO in non-phagocytic cells (Irani & Goldschmidt-Clermont, 1998).

However, there are major differences between SO production in phagocytic versus non-phagocytic cells: (1) the phagocytic NADPH oxidase evolved to maximize the production of the reactive species required for bacterium killing, while lower amounts are produced in non-phagocytic cells (Clark, 1999; Katusic, 1996); (2) The site where SO is produced by phagocytic cells is either the extracellular space, or the phagolysosome, which is also a space excluded from the cell cytoplasm. In contrast, EC (Rahman et al., 1999), as well as smooth muscle cells (Patterson et al., 1999), produce oxidant species both within the cytoplasmic compartment and extracellularly. The presence of non-phagocytic NADPH oxidase was thus demonstrated in different cell types (Bayraktutan et al., 1998); (Holland et al., 1998) - a notion that is very important in supporting the emerging concept of SO and other ROS as signaling molecules. Moreover, it was suggested that rac1 has a role in the control of this enzyme in nonphagocytic cells, just as rac2 controls the neutrophil NADPH oxidase (Irani et al., 1997).

To further confirm these findings, it was recently reported that in smooth muscle cells an NADH/NADPH oxidase is a major source of superoxide. The two membrane components of the enzyme are p22-phox, the α subunit of the neutrophil NADPH oxidase, and a newly isolated protein, p65-mox (mitogenic oxidase). This latter subunit is
an analog of gp91-phox and is upregulated in conditions known to stimulate superoxide production and growth (Suh et al., 1999; Lassegue et al., 1999).

2.3.2. Biochemistry of ROS

The term “free radical” describes a chemical species that has one or more unpaired electrons, a definition that embraces the atom of hydrogen (one unpaired electron), most transitional metal ions, and the oxygen molecule (O₂) (Halliwell & Gutteridge, 1990). O₂, in its ground state, is itself a radical, having two unpaired electrons that have the same spin quantum number. O₂ readily reacts to form partially reduced species, which are generally short-lived and highly reactive (hence, the term “reactive oxygen species”, not to be confused with the term “free radical”). They include:

1. *Superoxide anion* (O₂⁻), formed through the one-electron reduction of O₂:

\[
O₂ + e^- \rightarrow O₂^-
\]

Besides the known purposeful production of SO during the respiratory burst of phagocytes, this species is a byproduct of many other reactions in aerobic cells: its source is the “leakage” of electrons to O₂, from various components of the cellular electron transport chains, such as those found in mitochondria, chloroplasts, and endoplasmic reticulum. In organic solvents, O₂⁻ is a strong base and nucleophile; however, in aqueous solutions, O₂⁻ is extensively hydrated and much less reactive, acting as a reducing agent (e.g., it will reduce cytochrome c and nitro blue tetrazolium). This charged molecule virtually cannot cross biological membranes (Halliwell & Gutteridge, 1990).
2. Hydrogen peroxide ($H_2O_2$) has no unpaired electrons, and thus is not a radical (Halliwell & Gutteridge, 1990). It is formed by several metabolic reactions, including those catalyzed by superoxide dismutases (SOD), D-aminoacid oxidase, and amine oxidase. It is formed from superoxide, in two steps:

$$O_2^- + H^+ \rightarrow HO_2^*$$

(hydroperoxyl radical)

$$2HO_2^* \rightarrow H_2O_2 + O_2$$

This reactive oxygen species has limited but more durable reactivity, and it can cross biological membranes, a fact that, together with the contribution to the formation of the hydroxyl radical (see below), increase its damaging potential.

3. Hydroxyl radical ($\cdot OH$), that can be formed from either the superoxide anion (Haber-Weiss reaction), or from hydrogen peroxide (Acworth & Bailey, 1995):

$$O_2^- + H_2O_2 \rightarrow O_2 + HO^- + \cdot OH$$

(Haber-Weiss)

$$Me^{n+} + H_2O_2 \rightarrow Me^{(n+1)+} + HO^- + \cdot OH$$

where $Me^{n+}$ is a metal ion, such as iron (II), titanium (III), copper (I), cobalt (II), chromium, vanadium, or nickel. Probably the reaction that occurs most frequently in vivo is the $Fe^{2+}$-decomposition of $H_2O_2$, i.e., the Fenton reaction. $\cdot OH$ is, perhaps, the most toxic oxygen species. It is highly reactive, so it will react at or close to the site of its formation, with most molecules it encounters.
Much work was done in order to understand the oxidative modification of biological molecules via reactive oxygen species. It is generally considered that the modification of proteins is initiated mainly by •OH, although the availability of O₂ and O₂⁻ is important for the course of the process. Cysteine and methionine residues are most sensitive to oxidation, however these are the only oxidative modifications of proteins that can be reversed (Berlett & Stadtman, 1997). The rate constants for these reaction vary from 10³M⁻¹s⁻¹ for O₂⁻ to nearly diffusion-controlled rate for •OH (Kalyanaraman, 1995). Aromatic aminoacids are also important targets of oxidation by ROS. Formation of protein carbonyls is still another effect of oxidative stress (Berlett & Stadtman, 1997).

It is interesting to note that, although it is usually associated with oxidative stress, O₂⁻ generally functions as a reducing agent (redox potential in water \(E_{o}^{\prime} = -0.27\) to \(-0.32\) volts) (Katusic, 1996). It can either be inactivated by superoxide dismutase, in a reaction that produces H₂O₂ (\textit{vide infra}), or it may react with other biomolecules. The most rapid reaction, almost diffusion-limited (rate constant in the range of \(10^9\) M⁻¹s⁻¹ (Huie & Padmaja, 1993)), is with nitric oxide (NO). The reaction yields peroxynitrite (ONOO⁻), which is a potent oxidant. This reaction is important because it limits the availability of both reactants, while being itself extremely reactive. Peroxynitrite reacts with protein thiols, producing S-nitrosylated thiols. It also reacts with tyrosine, yielding a stable product, nitro-tyrosine. These reactions, mostly considered as “fingerprints” of oxidative stress, have profound damaging effects upon the structure and function of the proteins involved (Kalyanaraman, 1995; Rubbo \textit{et al.}, 1995), although there are instances when the physiologic poly-S-nitrosylation has a regulating role (Xu \textit{et al.}, 1998). Still another way by which superoxide regulates protein function is the inactivation of the iron
regulatory protein/cytoplasmic aconitase, by oxidizing its [4Fe-4S]^{2+} cluster to [3Fe-4S]^{1+}, and release of a ferrous ion (Gardner et al., 1995).

Since high amounts of ROS have harmful effects upon cells, a wide variety of mechanisms have evolved to maintain their concentration low, below the toxicity threshold: H_2O_2 in the nanomolar range, and O_2^- in the picomolar range (Hauptman & Cadenas, 1997); (Wolin & Mohazzab, 1997). The most important ROS scavenging mechanisms are catalyzed by the following enzymes: (1) SOD, either cytosolic or extracellular (Cu/Zn-dependent), or mitochondrial (Mn-dependent), which dismutates O_2^- to H_2O_2; (2) catalase, which usually acts in conjunction with SOD, and breaks down H_2O_2 to oxygen and water; (3) glutathione peroxidase, that reduces hydroperoxides and H_2O_2 in a reaction involving glutathione (GSH) and the formation of a disulfide bond between two glutathione molecules (GSSG) (Acworth & Bailey, 1995):

\[
\text{ROOH + 2GSH} \rightarrow \text{GSSG + ROH + H}_2\text{O}
\]

\[
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG + 2H}_2\text{O}
\]

2.3.3. “Oxidative stress” versus “intracellular signaling”

A large body of work developed over the last decade changed our view about ROS. Previously, they were thought of as the damaging oxidants involved in aging (Nohl, 1993), malignant transformation, (Toyokuni, 1996; Dreher & Junod, 1996), reperfusion
injury (Flitter, 1993), nervous tissue degeneration disorders (Borlongan et al., 1996), etc. In other words, the toll eukaryotic organisms had to pay for their aerobic existence.

More recently, a new concept gradually gained acceptance: ROS may play an important role in the homeostasis of the cell. They are found to be involved in several signaling pathways, of which some of the best documented are the regulation of the transcriptional activators nuclear factor kappa-B (NF-κB) and AP1 (Sen & Packer, 1996), and the regulation of apoptosis by p53 (Polyak et al., 1997). NF-κB contains a cysteine in its DNA-binding site, which is essential for DNA binding. This cysteine has to be maintained in a reduced form in order for NF-κB to be active, and it sensitizes NF-κB to the redox status of the cell (Sen & Packer, 1996). AP1 is a transcriptional activator formed of homo- or heterodimers of two proteins: c-jun and c-fos. In vitro, this activity is regulated by the oxidation status of a cysteine in the DNA binding domain (Abate et al., 1990), however, the in vivo mechanism of redox regulation of AP1 is less well understood.

Many other processes were found, more recently, to involve, at some point, control by ROS: cellular proliferation (Burdon, 1995), lymphocyte activation (Goldstone et al., 1995; Goldstone et al., 1996), prostaglandin synthesis (Wolin & Mohazzab, 1997), cellular transformation (Irani et al., 1997), collagenase secretion (Kheradmand et al., 1998). Some growth factors and hormones also induce increase in ROS, which seem to control their effects: insulin (Monteiro & Stern, 1996), angiotensin (Rajagopalan et al., 1996), thrombin (Patterson et al., 1999), PDGF, EGF and other stimuli of tyrosine kinase-receptors (Sundaresan et al., 1996, Suzuki et al., 1997; Marumo et al., 1997). The investigation of the downstream targets for the ubiquitous tumor suppressor protein p53
mutations/deletions of p53 represent a common step in the development of many human cancers), using the powerful “serial analysis of gene expression” technique (SAGE) to identify target genes for this DNA transcription regulatory protein, showed that the most strongly responsive genes code for proteins that regulate the redox state of the cell (Polyak et al., 1997). While this finding opens new avenues in cancer research to elucidate the role of these gene products in inhibiting the development of cancer cells, it further suggests that ROS are directly involved in the unchecked proliferation of cells, through the regulation of either apoptosis or mitosis (Irani et al., 1997).

The concept of intracellular signaling mediated by oxidants is in sharp contrast with the concept of “oxidative stress”, that is mainly a pathological condition, related to situations such as ischemia/reperfusion (Zweier et al., 1989; Crawford et al., 1996), inflammation (Varani & Ward, 1994; Mayers & Johnson, 1998), and cigarette smoking (Moldeus et al., 1986). However, the idea that ROS represent strictly harmful compounds is so strongly anchored, that even in a recent review aiming at analyzing the signal transduction of oxidants, the cells were described as responding to the oxidative stress (Wolin & Mohazzab, 1997). It is our purpose (see next Chapter) to not only show how free radicals are involved in a very important cellular response – actin reorganization in cellular motility – but also to underscore the fact that they are necessary participants to the cellular dynamic processes.
2.4. Why endothelial cells?

The endothelium plays major roles in controlling the traffic of molecules and cells to and from underlying tissues, smooth muscle cells tone, the proliferation of subendothelial layers, generation of extracellular matrix proteins, the formation of endovascular thrombi and thrombolysis. As a consequence, any morphological or functional disruption of the endothelium resulting from a variety of injuries can lead to dramatic effects upon the integrity of the cardiovascular system (Cines et al., 1998).

The functions of the endothelium are achieved in several ways. The composition of the EC plasma membrane may vary from non-adherent for platelets and leukocytes to highly adherent for either of these blood cells, and different blood-borne or tissular factors modulate these properties (Cybulsky et al., 1999; Sendo & Araki, 1999; Fabbri et al., 1999; Tan et al., 1999). The barrier function of EC is of utmost importance for underlying tissues (e.g., Michel & Curry, 1999; Rubin & Staddon, 1999; Feng et al., 1999). However, almost 40 years after Majno and Palade proposed that plasma leakage in acute inflammation caused by histamine, serotonin, or bradykinin results via gaps that form between endothelial cells of postcapillary venules (Majno & Palade, 1961), how exactly molecules cross the endothelial lining is still a matter of debate (McDonald et al., 1999).

EC respond to a variety of signals, such as changes in hemodynamic forces, or blood-borne signals, by synthesis and release of a wide variety of vasoactive and thromboregulatory molecules, as well as growth factors, including: prostacyclin,
thromboxane, free radicals such as NO and SO, endothelins, plasminogen inhibitors, von Willebrand factor, endothelial cell growth factors, etc. (Celermajer, 1997). These factors alter vascular reactivity both within the vessel lumen and the vessel wall. It is therefore not surprising that endothelial dysfunction is associated with most of the known risk factors for cardiovascular disease and may constitute a contributing cause for such disease (Drexler & Hornig, 1999).

The study of endothelial cells (EC) response to different stresses is important from both theoretical and practical reasons. It answers very basic questions related to what triggers different responses, the intracellular signaling pathways mediating these responses, and the nature of the response itself. Likewise, from a physio-pathological point of view, it is important to detect and understand the consequences of different EC reactions upon the functioning of the cardiovascular system. As we mentioned, this thin monolayer of cells coating the blood vessel lumen are crucial in the control of many vascular functions (Majno & Joris, 1996).

EC injury can result from a variety of stresses that lead to EC malfunction. We will focus on one situation that is relevant to human pathology, related to the interventional procedure of coronary angioplasty. This treatment can restore blood flow through vessels previously compromised by atherosclerotic plaques, but it also has the potential to remove the endothelial lining of a large area of the lumen. The recovery of the endothelium after this procedure is usually slow, and sometimes never complete (Van Belle et al., 1998). Endothelial removal has profound impairing effects upon cardiovascular functions, including abolishment of the permeability barrier,
thrombogenesis, increase in vascular tone, and intimal hyperplasia (Allaire & Clowes, 1997). Therefore, scientists have investigated for many years the mechanism for EC migration and restoration of the confluence of the endothelium (e.g., Weidinger et al., 1990; Aepfelbacher et al., 1997). Nevertheless, the knowledge accumulated within the last decades still does not explain why reendothelialization is so difficult to accomplish, while its completing would be an important treatment for patients with coronary artery disease undergoing mechanical revascularization (Van Belle et al., 1998). These are the reasons we chose endothelial cells as the model system in our studies on cellular motility.
CHAPTER 3
HYPOTHESIS

We have accumulated evidence that suggests an involvement of oxygen free radicals, and more specifically of SO, in important cellular responses. NIH 3T3 fibroblasts, which either transiently expressed a constitutively activated form of p21ras (EJ-ras) (Sundaresan et al., 1996), or had been stably transformed with Ha-ras (Irani et al., 1997), produced increased amounts of superoxide. The production of SO was suppressed by co-expression of a dominant negative isoform of rac1. This indicated that rac1 acted downstream of ras on this pathway. Interestingly, the superoxide production was also inhibited by treatment of cells with diphenylene iodonium, a flavoprotein inhibitor, which further suggested that an NADPH oxidase was activated and responsible for the production of SO (Irani et al., 1997). Furthermore, we observed that in Ha-ras-transformed cells the number of cell spanning stress fibers decreased, while the number of actin fibers at the margins of cells, within membrane ruffles, increased (Heldman et al., 1996).

These findings could be reassembled in the following sequence of events:
(1) Ras is activated by extracellular signals, such as growth factor receptor occupancy. (2) Consequently, it activates rac. (3) In turn, rac activates a non-
phagocytic NADPH oxidase. (4) The enzyme produces superoxide. (5) Lamellipodia and membrane ruffles, containing polymerized actin fibers, are formed. The steps (1) through (4) may follow directly each other, or may be separated by a number of additional, unknown, steps. The most intriguing, though, is the step from (4) to (5). The currently accepted view (already described in detail in Chapter 3.2) is that rac induces actin reorganization through its interaction with some yet ill defined effector molecules, and in this context, there is a clear gap between steps (4) and (5). However, further findings from this laboratory have narrowed this gap.

Within tissues, cells are never distant from a source of oxygen (such as capillaries) by more than 200 μm. The need for oxygen results from the use of oxidation as a means to convert energy into molecules, such as ATP, that can be used readily for cellular reactions requiring high activation energy. During development, repair processes, and even normal adult life, the blood supply to certain tissues may vary substantially, leading to relative mismatch in O₂ demand and supply, called ischemia. Reoxygenation after hypoxia is a condition that occurs in vivo during reperfusion of tissues previously exposed to ischemia. It is known to be accompanied by the production of ROS, in particular superoxide (Zweier et al., 1994). In in vitro experiments on HAEC maintained in hypoxic conditions for 4 hrs, leading to a cellular oxygen tension of 10 Torr, re-addition of oxygen induced a reorganization of the actin cytoskeleton, which is similar to the changes observed within cells exposed to growth factors or in EJ-ras transformed cells: increase of the total filamentous actin and translocation of the filaments to
the periphery of the cells (Crawford et al., 1996). When these cells were transduced with replication incompetent adenovirus, containing the superoxide dismutase (SOD) gene, they overexpressed SOD, and the actin response was suppressed. In this context, considering the controlling role of rac in both the activation of the phagocytic NADPH oxidase and actin organization, the next logical step was to ask whether rac-induced formation of free radicals, in particular SO, was involved in the rac control of the actin cytoskeleton. This possibility was also suggested by the fact that an NADPH oxidase-like enzyme has been demonstrated in cells other than the phagocytic lineage, including endothelial cells (Meier et al., 1991; Emmendorffer et al., 1993; Zulueta et al., 1995; Jones et al., 1996; Bayraktutan et al., 1998; Lassegue et al., 1999), as well as by the recent finding that rac1-induced production of free radicals is involved in collagenase-1 expression (Kheradmand et al., 1998). Moreover, conditions known to increase intracellular SO and other free radicals in different cell types were usually accompanied by a pattern of actin reorganization similar to that described in cells microinjected with rac (Ridley et al., 1992; Crawford et al., 1996; Heldman et al., 1996).

Taken together, these findings suggested the following HYPOTHESIS:

**FREE RADICALS, IN PARTICULAR SUPEROXIDE, FUNCTION IN CELLS AS EFFECTORS FOR RAC, NECESSARY BUT NOT ALWAYS SUFFICIENT, ON THE PATHWAY OF ACTIN REORGANIZATION, BY WAY OF ACTIVATION OF A NON-PHAGOCYTIC NADPH OXIDASE.**

This thesis will attempt to test the above hypotheses (Fig. 10).
**Figure 10.** The Question: is rac-induced superoxide formation involved in actin reorganization and cellular motility?
CHAPTER 4
MATERIALS

4.1. CHEMICALS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the best grade. Manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP) was purchased from Alexis Corporation (San Diego, CA). Diphenyleneiodonium sulfate (DPI) and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) were from Toronto Research Chemicals Inc. (North York, Ontario, Canada). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF-DA), Alexa-488 actin (A488A), Alexa-568 actin (A568A), rhodamine-phallloidin, FITC-phalloidin, and Calcein Blue AM (CBAM), were from Molecular Probes (Eugene, OR). Trypsin Neutralizing Solution was from Clonetics (San Diego, CA). The SOD 525 kit for measurement of superoxide dismutase (SOD) activity was purchased from R&D Systems, Inc. (Minneapolis MN).

4.2. CELLS

Two types of EC were under study: (1) Human aortic (HAEC) or human coronary (HCEC) primary endothelial cells (Clonetics, San Diego CA), which were grown in EGM and EGM-2, respectively (Clonetics). These are endothelial growth media
specially formulated to maximize the growth and proliferation of the cells, and are supplemented with different growth factors: bovine brain extract, human EGF and hydrocortisone (for EGM), and human EGF, VEGF, human bFGF, IGF, ascorbic acid, heparin, and hydrocortisone (for EGM-2). The cells were subcultured as recommended by the manufacturer. (2) A mouse aortic EC line (MAEC) kindly provided by Dr. R. Auerbach (Bastaki et al., 1997), which was grown in Dulbecco’s modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA).

Human and mouse endothelial cells were grown to 60-80% confluency on 6-well Falcon plates. The confluency level was found optimal for adenoviral infections (vide infra), and the size of the plates was found optimal for the actin and SOD assays (Chapter 5). For microscopy experiments, the cells were grown on coverslips or on 2- or 4-wells Lab-Tek® chamber slides (Nunc, Naperville, IL), and for flow cytometry they were grown on 12-well Falcon plates.

To test the relevance of our findings with EC to other cells, (Chapter 7) we also used Swiss NIH 3T3 fibroblasts, which were grown in DMEM supplemented with 10% fetal bovine serum. The fibroblasts were grown on coverslips to approximately 40-60% confluency, which was found optimal for transfections, as well as for the microscopic examination. In experiments involving FACS analysis of actin polymerization, the cells were grown in 6-well Falcon plates.
4.3. SPECIAL REAGENTS: THE ADENOVIRAL VECTORS

The recombinant adenovirus Ad-rac\textsuperscript{V12}, containing a cDNA coding for the c-myc-tagged constitutively activated form of rac1 (a gift of A. Hall), was constructed by homologous recombination in 293 cells using the adenovirus-based plasmid JM17, as described (Sundaresan \textit{et al.}, 1996). The E1A gene of the adenovirus was replaced by homologous recombination with the cDNA for the constitutively activated mutant of rac, rac\textsuperscript{V12} (Diekmann \textit{et al.}, 1991), under the control of the cytomegalovirus promoter. Replication incompetent adenoviruses carrying the cDNA for human Cu,Zn-superoxide dismutase (Ad-SOD), and the bacterial \(\beta\)-galactosidase gene (Ad-\(\beta\)Gal), were constructed as described (Crawford \textit{et al.}, 1996). EC transduction was performed by diluting the viral vector in a small amount of culture medium at the desired multiplicity of infection (i.e., the number of infectious viral particles per cell; MOI). Typically, a final volume of 500 \(\mu\)l per well was used in a 6-well plate, and kept for 1.5-2 hr on a rocking platform, at 37\(^\circ\)C, in 5\% CO\(_2\) in air. The cells were then rinsed with complete medium and maintained in the incubator for two more days, to allow full expression of the proteins (Moldovan \textit{et al.}, 1997). For biochemical measurements (see Chapter 5), adenovirus-transduced cells were kept overnight in their respective medium containing 0.1\% FBS, defined arbitrarily as serum starvation condition (Ridley \textit{et al.}, 1992).
CHAPTER 5

SUPEROXIDE IS NECESSARY IN THE CONTROL OF ACTIN CYTOSKELETON REORGANIZATION INDUCED BY RAC

5.1. Methods

5.1.1. Endothelial cell transduction

HAEC, HCEC, or MAEC were grown to 80% confluency and were transduced with the relevant adenoviral preparation, as described in the previous section. As a general observation, human cells could be transduced with MOI up to 2000, prior to reaching a plateau for rac1 expression. In contrast, in mouse cells, an MOI-dependent effect could be detected only up to an MOI of 250. These differences are most likely due to the density of receptors for adenoviruses displayed by human versus mouse EC.

5.1.2. Actin measurement

For the assay of bulk actin concentration, the cells plated on 6-well plates were rinsed with buffer (15 mM HEPES, pH 7.0, 145 mM NaCl, 0.1 mM MgCl2, 10 mM
EGTA), supplemented with a mixture of protease inhibitors [chymostatin, leupeptin, antipain, pepstatin, and 4-(2 aminoethyl)benzenesulphonyl fluoride]. The cells were then lysed with the same buffer, ice-cold, supplemented with 1% Triton-X 100 (lysis buffer), and to which 600 nM (final concentration) of rhodamine-phalloidin were added. The plates were placed on ice and the cells were scraped, then transferred into a 1.5-ml Eppendorf tube. After vortexing for 30 sec., the samples were centrifuged at 50000 x g in a Beckman TL-100 rotor, for 30 min, at 4°C. The supernatants were removed and used for protein assay. The pellets were gently rinsed with 200 µl plain buffer without disrupting them, then 200 µl methanol were added and the samples were kept overnight at 4°C. The phalloidin concentration was measured after transferring the samples into a 96-wells plate by fluorescence detection in a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Framingham MA), using a rhodamine-phalloidin standard curve. All determinations were normalized to the protein concentration.

5.1.3. SOD assay

SOD activity was determined with the SOD 525 kit, following the manufacturer's recommendations. Cells were washed with PBS, and harvested with a cell scraper in 200 µl of ice-cold water supplemented with protease inhibitors. Cells were sonicated 2 x 15 sec, on ice, then the debris were pelleted for 10 min. in a microcentrifuge, at maximum speed, at 4°C. To the supernatants, a mixture of ethanol-chloroform (62.5:37.5, vol/vol) was added, vortexed and microfuged as above. The upper, aqueous phase was used for SOD determination. The change in absorbency was measured at 525 nm, in a Beckman
DU 640B spectrophotometer. The initial, linear rate (typically within the first 3 min.) was used to calculate the SOD 525 units, according to the formula given by the provider:

\[
\text{SOD 525 units} = 0.93 \left( \frac{R_s}{R_c} \right)(1.073 - 0.073 \left( \frac{R_s}{R_c} \right)^2)
\]

where \(R_s\) is the rate of autooxidation of the reagent 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in the presence of the cell lysate, and \(R_c\) is the rate of its autooxidation in the presence of \(H_2O\) (control). All the measurements were normalized to the protein concentration.

5.1.4. Fluorescence microscopy and image analysis

Cells grown on coverslips and transduced with the adenoviral constructs were rinsed with Dulbecco's phosphate buffer saline (PBS) (Gibco BRL, Grand Island, NY), then fixed with 3.7% formaldehyde in PBS for 10 minutes, and permeabilized with 0.2% Triton X-100 in PBS, 10 min. Further steps of the procedure were performed away from light. The coverslips were inverted on a 200 µl rhodamine-phallolidin drop, 30 min., then rinsed with PBS. The slides were incubated next with a mixture of the following first antibodies, for 1 hr: a 1:500 dilution of either rabbit anti-SOD or rabbit IgG (as control) (Accurate, Westburry NY), and a 1:100 mouse anti-c-myc epitope (9E10, SantaCruz Biotechnology, Inc., Santa Cruz, CA). After washing, the coverslips were incubated with a mixture of the secondary antibodies: a 1:60 FITC-goat anti-mouse (SantaCruz) and a 1:100 Cy5-goat anti-rabbit (Jackson Laboratories, Bar Harbor ME). The coverslips were then rinsed with PBS and mounted in Fluoromount-G (Southern Biotechnology...
Associates, Inc., Birmingham AL). The cells were examined with a Noran confocal
microscope (Noran Instruments, Middleton WI) or with a Nikon Eclipse 800 microscope.

5.1.5. Flow cytometric analysis of intracellular antigens

The cells were detached with 0.25 % trypsin for 5 min, then trypsin neutralizing
solution (Clonetics) was added, and the cell suspension was centrifuged at 200 x g (1000
rpm) in a Sorvall RT 7 centrifuge, for 5 min., at room temperature. The cells were then
fixed for 10 min. by resuspension in freshly prepared 1% paraformaldehyde in PBS,
rinsed in PBS supplemented with 1% bovine serum albumin (BSA-PBS), permeabilized
with 0.3% saponin in BSA-PBS, 5 min., and pelleted as above. The incubation with the
first antibody anti-c-myc, diluted in BSA-PBS with 0.3% saponin, was done for 30 min.,
on ice. Cells were washed 2-3 times with the same buffer, incubated on ice with the
secondary antibody, either FITC- or Texas Red-labeled, washed 2-3 times, resuspended
in BSA-PBS, and analyzed immediately with a FACSCalibur flow cytometer (Beckton
Dickinson, Mountain View, CA).

5.1.6. Free radicals detection

The production of free radicals was determined by two different assays.

(1) Lucigenin assay was done as previously reported (Irani et al., 1997). HAEC
were transfected with Ad-racV12 or Ad-βGal, at an MOI of 500. After two days, cells were
serum-starved overnight, then they were scraped and resuspended in Krebs-Hepes buffer.
NADPH (1 mM) was added at time 0 and the cells incubated for the indicated times at 37°C. Cells (10^5) were then added to lucigenin (0.25 mM in the same buffer) and chemiluminescence was measured in a Monolight 2010 luminometer for 60 sec.

(2) **Fluorescence assay** was performed by loading the cells with CM-D CF-DA. Cells were rinsed with Hanks Balanced Salt Solution (HBSS) (Gibco), and incubated with 5 μM CM-D CF-DA in HBSS, from a stock solution in DMSO, for 20-30 min, at 37°C, in 5% CO₂. At the end of the incubation period, the CM-D CF-DA solution was removed, the monolayer was rinsed with HBSS, and either trypsinized as described, and used immediately for FACS analysis, or examined alive with a Nikon Eclipse 800 microscope, at an excitation wavelength of 490 nm. For microscopical examination, cells were grown on 2- or 4-wells Lab-Tek® chamber slides. The upper plastic part of the 4-well chamber slide was removed, while the silicone gasket was left in place. HBSS was added in the less than 1 mm-high wells formed by the gasket, a coverslip was added, while ensuring the removal of remaining air, and the cells were examined alive, on the heated stage (37°C) of the fluorescence microscope. The images were recorded on a SenSys digital camera, with constant exposure settings for all experiments (typically, 1 sec exposure, with an ND16 filter in the light path).

5.1.7. **Western blots**

The cells grown in 6-wells plates were lysed in 200 μl lysis buffer, harvested on ice, sonicated 2 x 15 sec and the debris pelleted in a microcentrifuge at maximum speed, for 10 min. at 4°C. 5-10 μg of protein from the supernatants were run on a 5-20% SDS-
PAGE, for 1 h, at 150 V. The gels were transferred overnight at 5 V to Hybond ECL nitrocellulose membranes (Amersham Corporation, Arlington Heights IL). The first antibody was anti-c-myc epitope (Santa Cruz) and the secondary antibody was HRP-conjugated goat anti-mouse (Hyclone Laboratories, Logan UT). The binding was detected by SuperSignal® (Pierce Chemical Co., Rockford IL), as directed by the manufacturer.

5.1.8. Protein assay

The protein concentration was determined by the bicinconinic acid (BCA) assay (Sigma Chemical Co.), following the manufacturer's directions, and using BSA as standard.

5.1.9. Statistics

All the data were expressed as mean ± SEM. We defined the statistical significance of the data by the t-test for two samples assuming equal variances, using the Excel software. We considered the differences as significant for p < 0.05.
5.2. ACTIN CHANGES AND FREE RADICALS PRODUCTION INDUCED BY RAC

5.2.1. Rac V12 is efficiently overexpressed in cells transduced with an adenoviral vector

To study the role of rac-induced generation of SO in the control of actin remodeling, we devised an experimental system that would allow us to make bulk biochemical measurements. Most of the work reported on the rho family of GTPases was performed using microinjection of either plasmids or proteins, in a relatively small number of cells. This limited findings to mainly qualitative data. However, endothelial cells are susceptible to adenoviral infection, since they harbor within their plasma membrane the receptors required to enter the cells, namely the integrins α5β3 and αVβ5 (Wickham et al., 1993; Sugihara et al., 1998; Nemerow & Stewart, 1999; Chiu et al., 1999). Replication-deficient adenoviruses had been used previously as vectors for the introduction of transgenes in various cells (Wilson, 1996; Kurata et al., 1999), and we have used this method to transduce endothelial cells (Crawford et al., 1996; Moldovan et al., 1997). The foremost advantage of this approach is that nearly 100% of the cells of a monolayer can be transduced, and thus overexpress the protein of interest.

Therefore, several adenoviral vectors were designed, with which we could very efficiently introduce the desired genes into EC. Thus, we could perform quantitative biochemical measurements, in order to correlate the level of activated rac expression to the production of free radicals and to the changes in actin organization. We used the constitutively activated form of rac1, rac V12, in which the mutation of the amino acid in
position 12 from glycine to valine results in the abolishment of the GTPase activity of rac (Diekmann et al., 1991).

The expression of racV12 was detected with an antibody directed against the c-myc tag of the protein, by western blot of human aortic endothelial cells. The transductions were at the indicated MOI, and the assays were performed after three days, on overnight serum-starved cells.

**Figure 11.** Human aortic endothelial cells transduced with Ad-racV12 express constitutively activated rac in a dose-dependent manner, as detected by Western blot.

Western blot analysis of transduced HAEC, using an antibody against the c-myc tag epitope as a probe, showed that the rac protein is strongly expressed, and that the amount of protein increases with the multiplicity of infection (MOI) (Fig. 11). No such rac expression is detected in the cells transduced with a control virus, in which the bacterial β-galactosidase gene was inserted (Ad-βGal). Likewise, mouse endothelial cells
could be transduced by the viral construct and expressed heightened levels of rac\textsuperscript{VI2}, as determined by FACS analysis of the MAEC transduced with increasing concentrations of the adenovirus (not shown). However, in mouse cells we detected a plateau of rac\textsuperscript{VI2} expression at around 250 MOI, therefore this was the maximum used with these cells in the following experiments.

5.2.2. *Overexpression of rac\textsuperscript{VI2} protein in endothelial cells induces dose-dependent changes in F-actin content*  

In HAEC which overexpress rac\textsuperscript{VI2} the changes in F-actin distribution and content were dependent upon the level of rac\textsuperscript{VI2} protein expression (Fig. 12). When the rac\textsuperscript{VI2} content was low in HAEC exposed to reduced serum conditions (0.1% fetal bovine serum), as detected by immunostaining, stress fibers disintegrated and F-actin was mostly present as punctate foci and at the level of peripheral ruffles, where rac\textsuperscript{VI2} was detected. In cells where rac\textsuperscript{VI2} expression was high, strong rhodamine-phalloidin staining was detected throughout the cells, including staining of actin fibers that were less prominent and thick than typical endothelial cells stress fibers (Ridley & Hall, 1992b) (Fig. 12).

In contrast with previous studies, and because of the high efficiency of transfection provided by the adenoviral method, we could assess bulk changes in F-actin concentration that were associated with the reorganization of the cytoskeleton. When compared to the Ad-\betaGal transduced cells, both HAEC and MAEC transduced with Ad-rac\textsuperscript{VI2} were consistently found to contain increased F-actin concentration, which was proportional to the MOI of the recombinant adenovirus added to the cells (Fig. 13).
HAEC were infected with Ad-rac\textsuperscript{V12} at an MOI of 100. The cells were double-stained for rac\textsuperscript{V12} expression (a), and F-actin organization (b). Rac\textsuperscript{V12} was detected by an antibody directed against the c-myc tag and an FITC-labeled secondary antibody (green), and F-actin organization was assessed by staining with rhodamine-phalloidin on the same cells (red).

**Figure 12.** The organization of the actin cytoskeleton in Ad-rac\textsuperscript{V12}-transduced cells depends upon the amount of expressed rac\textsuperscript{V12}.
However, the cells transduced with the lowest MOI displayed a small, non-significant but reproducible decrease of F-actin (Fig. 13, first column), that probably reflected the dissolution of stress fibers (Fig. 12).

Mouse aortic endothelial cells were transduced with Ad-rac\textsuperscript{V12} or Ad-\textbetaGal, at the indicated MOI. F-actin content was assayed by rhodamine-phalloidin binding and normalized to the protein content. The data are expressed as percent of change in the amount of rhodamine-phalloidin in Ad-rac\textsuperscript{V12}, versus Ad-\textbetaGal transduced cells, at the same MOI (to normalize for the "virus-effect" associated with these experiments), ± SEM. * \( p < 0.05 \) when compared to control cells (Ad-\textbetaGal-transduced at matching MOI) and to cells transduced with lower Ad-rac\textsuperscript{V12} MOI.

\textbf{Figure 13.} The concentration of F-actin in Ad-rac\textsuperscript{V12}-transduced cells increases dose-dependently.
5.2.3. Cells overexpressing rac\textsuperscript{VI2} produce increased amounts of free radicals

Rac is the key regulator in the recruitment to the plasma membrane of the cytoplasmic subunits of the phagocytic NADPH oxidase (Fuchs et al., 1996). Therefore, the enzyme is activated and produces the SO necessary for the killing of invading bacteria within phagolysosomes. Several reports, including our own, showed that in non-phagocytic cells SO is also produced by an NADPH oxidase-like mechanism (Emmendorffer et al., 1993; Zulueta et al., 1995; Crawford et al., 1996; Griendling & Alexander, 1997; Bayraktutan et al., 1998; Irani & Goldschmidt-Clermont, 1998). We tested whether rac\textsuperscript{VI2} overexpression in EC could activate NADPH oxidase and induce SO production. We used the fluorescent probe CM-DDCF-DA, a cell permeable fluorescein derivative with a high cellular retention, that fluoresces upon oxidation, mainly induced by H\textsubscript{2}O\textsubscript{2} (Zulueta et al., 1997). H\textsubscript{2}O\textsubscript{2} is produced by the scavenging of SO by SOD, and was used as an indicator of the total ROS production by the transduced cells.

Flow cytometric analysis of Ad-rac\textsuperscript{VI2}-transduced, CM-DDCF-DA-loaded cells showed increased production of free radicals, as compared to Ad-βGal-transduced control cells. This increase was inhibited by addition of the cell permeable antioxidant N-acetyl cysteine (NAC) (Fig. 14a and Table 1). We also used an assay more specific for SO detection, the luciferase chemiluminescence assay (Irani et al., 1997; Li et al., 1998a). HAEC transduced with Ad-rac\textsuperscript{VI2}, compared with control, Ad-βGal-transduced cells, displayed increased production of free radicals, which was proportional to the MOI of the infecting adenovirus (Fig. 14b). To confirm microscopically these biochemical
a. MAEC were transduced with Ad-rac^v12 or Ad-βGal, at an MOI of 250. After two days, overnight serum-starved cells were loaded with CM-DCF-DA (5 mM), without (Rac^v12) or with a 1-hour preincubation with 10 mM NAC (Rac^v12 + NAC). The cells were analysed by flow cytometry for CM-DCF-DA fluorescence. The data are representative of two independent experiments, and are expressed as percent change in CM-DCF-DA median fluorescence versus the respective Ad-βGal-infected controls, ± SEM. b. HAEC were transfected with Ad-rac^v12 or Ad-βGal, at an MOI of 500. After two days, overnight serum-starved cells were harvested, resuspended in Krebs-Hepes buffer, and NADPH (1mM) was added at time 0. The cells were incubated for the indicated times at 37°C, and chemiluminescence was measured for 60 sec. Values represent mean ± SEM from 3 independent measurements, and are expressed as relative light units per 100,000 cells.

**Figure 14.** Free radicals production is increased in aortic endothelial cells that overexpress racv12.
HCEC were infected with Ad-rac^{V12} or Ad-βGal, as indicated. After three days, overnight serum-starved cells were either loaded with CM-DCF-DA (Ad-rac^{V12} and Ad-βGal), or pretreated with MnTMPyP (25 μM) (Ad-rac^{V12} + MnTMPyP), or NAC (10 μM) (Ad-rac^{V12} + NAC), for 1 hr prior to CM-DCF-DA loading. The cells were then examined microscopically for CM-DCF-DA fluorescence. Bar, 50 μm.

**Figure 15.** HCEC that overexpress rac^{V12} produce increased amounts of free radicals, which are efficiently removed by antioxidants.
observations, we loaded either Ad-rac^{VI2}, or Ad-βGal-transduced cells, with CM-DCF-DA. In some instances, the cells were pre-treated 1 hr before CM-DCF-DA-loading with the antioxidants NAC or MnTMPyP. The results (Fig. 15) show increased CM-DCF-DA fluorescence in Ad-rac^{VI2}-transduced cells, as compared with both control cells and antioxidant-treated cells. We conclude that in endothelial cells overexpressing the constitutively activated mutant of rac, an increased production of ROS is triggered, probably as a result of the activation of a NADPH oxidase-like enzyme complex by rac^{VI2}.  

5.3. The effects of antioxidants on rac-induced actin reorganization

5.3.1. NAC and a SOD mimetic reverse the effects of rac^{VI2} overexpression on actin organization

If SO produced by rac^{VI2}-mediated mechanism(s) were contributing to actin cytoskeleton reorganization, then we expected that SO scavengers could reverse this process. We used several antioxidants/ROS scavengers to define the contribution of SO and other ROS produced within endothelial cells, to the actin changes induced as a result of rac^{VI2} overexpression. In one set of experiments we incubated either untreated, or the Ad-rac^{VI2}-transduced HAEC, with NAC. We found that NAC reduced the increase in F-actin resulting from overexpressing rac^{VI2} (Fig. 16a, column 3). Interestingly, it also decreased slightly, but significantly, the amount of actin filaments in control, Ad-βGal-transduced, cells (Fig. 16a, column 2), and in untreated EC (Fig. 16b). Hence, it is likely
that in endothelial cells, the dynamic generation of free radicals contributes to the maintenance of F-actin content, since incubation with an antioxidant also reduces F-actin.

In another set of experiments we incubated Ad-rac<sup>v12</sup>-transduced MAEC and HCEC with the SOD mimetic, MnTMPyP (Faulkner et al., 1994). F-actin was stained by rhodamine-phalloidin and analyzed either by FACS or by fluorescence microscopy. In the presence of this more specific SO scavenger, the amount of actin filaments in rac<sup>v12</sup>-overexpressing cells was significantly reduced as determined by bulk F-actin measurement (Fig. 17a). The organization of actin in these cells was clearly affected by SO scavenging: the ruffles were no longer visible, while we observed a more prominent bundling of F-actin in long, parallel fibers, (Fig. 17b).

5.3.2. SOD co-expressed with rac<sup>v12</sup> reduces the effect of the constitutively active form of rac on F-actin organization

The experiments with the SOD mimetic suggested that SO might be an important mediator of the actin changes induced by rac<sup>v12</sup>. To confirm this hypothesis, we used another specific scavenger of the SO radical, the enzyme Cu,Zn-superoxide dismutase, to decrease the intracellular level of SO, as we had done to study endothelial cells exposed to hypoxia/reoxygenation (Crawford et al., 1996), and to determine the consequent changes in actin cytoskeleton, in the presence or the absence of rac<sup>v12</sup> overexpression. Since neither exogenously added SOD, nor the intracellular SO anion, are membrane permeable, we chose to transfect HAEC with an adenoviral vector which contains the cDNA of human SOD (Ad-SOD) (Crawford et al., 1996). In cells overexpressing SOD,
a. Human aortic endothelial cells were transduced with Ad-racV12 or Ad-βGal, at an MOI of 100. After three days, the cells were treated, where indicated, with 20 mM NAC for one hour prior to F-actin assay by fluorimetry. The results are expressed as percent change in rhodamine-phalloidin fluorescence versus the Ad-βGal control cells, ± SEM. The differences are significant as compared to control (p < 0.05).

b. Non-transduced human aortic endothelial cells were treated for 1 hour with the indicated concentrations of NAC, and F-actin was assayed as described. The differences between treated versus non-treated cells are significant (p < 0.05).

**Figure 16.** NAC decreases the F-actin content in both control and Ad-racV12-transduced endothelial cells
a. MAEC were infected with Ad-rac<sup>V12</sup> or Ad-βGal at the indicated MOI. After three days and overnight serum starvation, cells were incubated for one hour with the SOD mimetic MnTMPyP (25 μM), and F-actin assayed by rhodamine-phalloidin binding and flow cytometry. Results are expressed as percent change in median fluorescence of bound rhodamine-phalloidin, versus control cells, ± SEM. 

b. HCEC were infected with Ad-rac<sup>V12</sup> at an MOI of 500. After three days, overnight serum-starved cells were incubated with MnTMPyP (25 μM) (Ad-rac<sup>V12</sup> + SODm) or vehicle (PBS) (Ad-rac<sup>V12</sup>) for 1 hr, then cells were processed for fluorescence microscopy. Bar, 10 μM.

**Figure 17.** MnTMPyP modifies the effects of Ad-rac<sup>V12</sup> on f-actin content in mouse aortic and human coronary artery endothelial cells.
HAEC were transduced with Ad-SOD at the indicated MOI and the SOD activity was assayed after three days. Results are expressed as SOD 525 units, normalized to the protein content of each sample. Inset: dependency of SOD activity upon viral MOI. b. SOD activity assayed in HAEC transduced concomitantly with Ad-SOD, Ad-rac\textsuperscript{v12} and Ad-\beta Gal, at the indicated MOI.

**Figure 18. Assay of superoxide dismutase activity in human aortic endothelial cells transduced with Ad-SOD.**

the activity of the enzyme is increased, and it is proportional to the MOI (Fig. 18). When we co-transduced HAEC with Ad-rac\textsuperscript{v12}, Ad-SOD, or Ad-\beta Gal, and studied the SOD activity, a significant increase in SOD activity was detected in Ad-SOD-transduced cells, and this effect was unaltered by the co-transfection of the cells by Ad-rac\textsuperscript{v12} or Ad-\beta Gal (Fig. 19). We maintained the mix of MOI constant, by varying the MOI of control Ad-\beta Gal, in order to account for the effect of the replication deficient virus.
HAEC were transduced concomitantly with Ad-rac\textsuperscript{V12}, Ad-SOD, and Ad-βGal, at the indicated MOI. SOD activity was assayed as described. The results are representative of two independent experiments.

**Figure 19. Superoxide dismutase activity in transduced HAEC.**

We then analyzed the actin changes by bulk measurements in a similar co-transduction experiment. When F-actin is measured by rhodamine-phalloidin staining, an increase was detected in rac\textsuperscript{V12} overexpressing cells compared to the level detected in Ad-βGal control cells (Fig. 20, first column). In cells overexpressing SOD alone, F-actin decreased relative to Ad-βGal control cells (Fig. 20, fourth column), consistent with the findings obtained with cells exposed to NAC, and again suggesting that the baseline F-actin content in these cells might be sensitive to the redox state. When both rac\textsuperscript{V12} and SOD were co-expressed, F-actin level was heightened by rac\textsuperscript{V12}, but this increase was reduced in a dose-dependent fashion by the co-overexpression of SOD (Fig. 20, second and third columns).
HAEC were transduced concomitantly with Ad-rac\textsuperscript{VI2} and Ad-SOD, at the indicated MOI. F-actin was assayed fluorimetrically as described. The results are representative of two independent experiments.

\textbf{Figure 20. Superoxide dismutase reverses the effects of constitutively activated rac on the f-actin content}

To confirm these biochemical findings with morphological data, we used a triple staining of HAEC transduced with Ad-rac\textsuperscript{VI2}, Ad-\textbeta\text{-Gal}, and Ad-SOD, to maintain a final MOI of 1000, but where the relative MOI of each adenovirus was varied. We detected F-actin by staining the cells with rhodamine-phalloidin, and the expression of the two proteins, rac\textsuperscript{VI2} and SOD, by immunostaining. Rac\textsuperscript{VI2} protein was detected by a primary monoclonal antibody directed to the c\textit{myc} tag, and a FITC-labeled anti-mouse secondary antibody; and SOD was detected by a rabbit anti-human SOD polyclonal antibody and a
Cy5-labeled anti-rabbit secondary antibody. Confocal microscopic analysis revealed specific patterns of actin organization, which were strongly dependent upon the levels of overexpressed proteins. The control endothelial cells, transduced with Ad-βGal only, displayed a typical actin network rich in stress fibers across the entire cell. No rac^VI2 (c-myc tag) or SOD are immunologically detectable within these cells (Fig. 21-c). In cells containing high amounts of immunologically reactive rac^VI2 (Fig. 21d), we observed a profound reorganization of the actin cytoskeleton (Fig. 21f): the stress fibers were no longer present, and prominent ruffles formed at the cell edges, containing high amounts of F-actin. These changes are readily apparent when a cell expressing large amounts of rac^VI2 is directly compared to adjacent cells with less rac^VI2 overexpression. The appearance of F-actin in SOD-overexpressing cells was not markedly different from control cells, except for the fact that about 40% of these cells acquired an elongated shape, different from the cobblestone appearance of cultured aortic endothelial cells, and the F-actin bundles were thicker and distributed along the long axis of the cell, similar to the rac^VI2-overexpressing cells treated with the SOD mimetic (Fig. 21g-i). When both rac^VI2 and SOD were expressed at high level within the same cell (Fig. 21j-k), the F-actin appearance was intermediary between that present in Ad-rac^VI2- and Ad-SOD-transduced cells (Fig. 21l): the membrane ruffles were still present, but smaller than in rac^VI2 overexpressing cells, and actin stress fibers, slightly thinner than in SOD-expressing cells, were present within the cell body.
Table 21. SOD overexpression in HAEC reduces the effects of rac\textsuperscript{V12} on F-actin organization
5.4. DISCUSSION

The organization of F-actin within endothelial cells in situ and in culture comprises prominent stress fibers, both at the periphery of the cells and crossing the entire cell body, attached at one or both ends to focal adhesions (Wong et al., 1983; Gabbiani et al., 1983). When a discontinuity within the monolayer occurs, a profound reorganization of the actin cytoskeleton takes place before the cells start migrating to cover the gap. The various steps of this process are documented from both in vivo and culture systems: a typical time-course comprises the formation of microspikes within minutes after wounding, the appearance of membrane ruffles within a couple of hours, and of stress fibers within a day from wounding (Vyalov et al., 1996; Aepfelbacher et al., 1997). The spatial and temporal control of the actin cytoskeleton organization in Swiss 3T3 fibroblasts was ascribed to the sequential activation of cdc42, rac and rho (Nobes & Hall, 1995). In our hands, dissolution of stress fibers, the punctate aspect of F-actin within the cell body, and presence of actin fibers at the membrane ruffles, were observed in cells deprived of serum, but expressing low amount of rac\(^{VI2}\), similar to the changes described in Swiss 3T3 fibroblasts upon rac microinjection. We detected an elevated F-actin content in cells that expressed high concentrations of rac\(^{VI2}\) in both human and mouse endothelial cells (Fig. 13), which has been observed in other systems as well (Takaishi et al., 1997). The role of rho in inducing actin stress fibers in endothelial cells was recently characterized (Aepfelbacher et al., 1997). We did not perform a rho activation assay, however it is conceivable that the intrinsic rho contributes to F-actin generation in rac\(^{VI2}\) overexpressing cells. Nevertheless, we cannot ascribe the increased
formation of F-actin solely to rho activity, since rho activation reportedly induces the assembly of preformed actin filaments into stress fibers (Machesky & Hall, 1997), whereas in our cells de novo actin polymerization clearly takes place, which could not be appreciated in the absence of bulk biochemical measurements.

The Ad-racV12-transduced cells produce increased amounts of free radicals (Figs. 14 and 15, and (Irani et al., 1997). By using either a nonspecific yet powerful cell-permeable antioxidant, NAC, or the more specific SO scavengers, MnTMPyP (Faulkner et al., 1994; Weiss et al., 1996) and SOD expression with adenovirus transduction, we decreased the amount of ROS present within these cells. Remarkably, each of these antioxidants reversed the increase in F-actin induced by racV12 overexpression (Figs. 16, 17a, and 20). Moreover, when cells were transduced with Ad-SOD only, or when control or non-transduced cells were treated with NAC, we also observed a slight decrease in the amount of filamentous actin relative to control cells. We consider that these findings support the interpretation that a basal level of SO within the cytoplasm is required for the maintenance of the F-actin level in quiescent endothelial cells, while an increase in the level of free radicals in stimulated cells is necessary for the breakdown of existing stress fibers and repolymerization of actin fibers at the membrane ruffles. It is important to realize that the steady state reached by cells in terms of F-actin is by no means a rigid state: G- and F-actin are in a dynamic equilibrium (Mitchison & Cramer, 1996), and we propose that free radicals are, in fact, accelerating this turnover, while inducing a positive balance on the total amount of F-actin.

Joneson and Bar-Sagi have recently studied the role of SO and free radicals as signaling mediators for mitogenesis and oncogenesis (Joneson & Bar-Sagi, 1998). Using
microinjection of cells, they have increased the cellular concentration of rac or mutants thereof. Using this approach, they have confirmed a previous observation that SO is required for the mitogenic activity and transformation of rac1 overexpressing cells (Sundaresan et al., 1996; Irani et al., 1997). However, they have found that oxidants were not needed to induce the actin cytoskeleton changes. Several important differences between the two studies are noteworthy. Joneson and Bar-Sagi have used fibroblastoid cells, while our studies involved endothelial cells exclusively. Moreover, the approach used to overexpress Rac is different between the two studies. They have used, for most of their experiments, microinjection of cells, while we have used a very efficient adenovirus-mediated cell transduction technique, allowing for expression of rac in >95% of the transduced cells. Therefore, we have been able to perform bulk biochemical assays to quantitate filamentous actin, which the study of Joneson and Bar-Sagi did not permit. Moreover, to inhibit SO-mediated pathways, they have used antagonists such as SOD protein added externally to the cells. While it is difficult to predict how an effect of SOD added outside the cells might be transmitted, as SOD does not seem to be cell permeant, and SO does not cross membranes either, we have overexpressed SOD directly in cells using a replication incompetent adenovirus coding for SOD.

We consider that the differences between these two studies might reflect at least two important characteristics of rac signaling. (1) As it is true with many other signal transduction pathways, the messenger molecules involved in rac signaling are strongly cell type-dependent, as also suggested by many other conflicting reports about rac effects and effectors (see CHAPTER 2). Our findings were similar in EC from two different species and with different genomic programming (primary culture vs. immortalized cell
line), which supports the role of rac-induced formation of ROS in actin reorganization in EC. (2) These results underscore the multifaceted character of rac as a signaling molecule. Multiple rac-ligand mediated molecular interactions are needed to fully transduce rac signals (Westwick et al., 1997). Our results are consistent with the concept that rac controls multiple and probably redundant pathways to induce a specific effect upon the actin cytoskeleton.

However, there are alternative explanations to some of our observations. First, neither of the used inhibitors restores completely the level of F-actin within rac<sup>V12</sup> overexpressing cells, to the control level. Second, the difference in the level of F-actin between control and rac<sup>V12</sup> overexpressing cells is about the same as between control cells, treated with NAC or overexpressing SOD, and rac<sup>V12</sup> overexpressing cells treated with NAC or overexpressing SOD (Figs. 16a and 20). Third, the difference between rac<sup>V12</sup> overexpressing cells not treated and treated with MnTMPyP is about the same at two different MOI (Fig. 17a). These results could be explained by the fact that NAC, MnTMPyP, and SOD reduce the baseline of ROS, that are necessary for actin polymerization, to a lower level. In this circumstances, rac<sup>V12</sup>-induced formation of free radicals is not involved in increased actin polymerization, since the needed amount of ROS is already produced within these cells (see Fig. 16b). This would further imply that alternative pathways are prompted by rac<sup>V12</sup> for the formation of membrane ruffles. Still other interpretations would be that either none of the antioxidants used completely scavenges the amount of ROS produced in the presence of overexpressed rac<sup>V12</sup>, or - most probably - more than one pathways are induced by rac<sup>V12</sup> to bring about the observed increase in F-actin.
In conclusion, we propose that a key mediator for rac downstream effects, such as actin reorganization in endothelial cells, is SO, since antioxidants - and in particular superoxide dismutase - reverse the effects of constitutive activation of rac upon actin. We also propose a major role for oxidants in actin reorganization, possibly through the modulation of interactions between actin and actin-binding proteins. For example, SO could alter the activity of capping proteins near the plasma membrane, promoting the addition of monomeric actin subunits at the barbed end of filaments, while H$_2$O$_2$ could induce actin polymerization and the thickening of stress fibers (Arcaro, 1998; Moldovan et al., 1997; Goldschmidt-Clermont & Moldovan, 1999).

Precisely how SO promotes the uncapping of actin barbed ends is currently under study. Our leading hypothesis also implicates nitric oxide (NO), which activity might be to stabilize capping proteins. Once the balance between SO and NO is tilted in favor of SO, ONOO$^-$ might be generated, with loss of capping activity. Alternative explanations have been considered, including the possibility that SO denatures the actin-monomer binding protein thymosin-$\beta 4$, which would result in more actin monomers bound to profilin, and consequent exchange of the ADP nucleotide bound to actin for ATP. The addition of ATP-bound monomers to the filaments would, in turn, activate the high affinity barbed ends, with consequent reduction of actin critical concentration (Goldschmidt-Clermont et al., 1992a).
<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Effective concentration</th>
<th>Target(s)</th>
<th>Effects</th>
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<tbody>
<tr>
<td>NAC</td>
<td>10 μM</td>
<td>Intracellular oxidized thiols, via increased glutathione</td>
<td>Decrease F-actin</td>
</tr>
<tr>
<td>MnTMPyP</td>
<td>25 μM</td>
<td>Intracellular superoxide</td>
<td>Decrease F-actin; formation of actin cables</td>
</tr>
<tr>
<td>SOD</td>
<td>MOI = 50-500</td>
<td>Intracellular superoxide</td>
<td>Decrease F-actin; formation of actin cables</td>
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<tr>
<td>DPI</td>
<td>10 μM</td>
<td>NADPH oxidase and other flavoproteins</td>
<td>Decrease F-actin</td>
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*Table 1. The effects of the antioxidants used in this study upon F-actin.*
6.1.1. The wounding model

Since in the previous study (CHAPTER 5) we found that rac-induced formation of SO has an important role in the reorganization of the actin cytoskeleton (Moldovan L et al., 1999), we have used an in vitro wounding model, to further characterize the role of ROS in EC migration – a process that requires actin reorganization. MAEC were grown to confluency on 2- or 4-wells Lab-Tek* chamber slides (NUNC, Naperville, IL) or on coverslips, in Dulbecco’s modified Eagle medium (Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA). Freshly confluent monolayers were scraped with a plastic or glass pipette tip, to create a 0.5-1 mm wide wound. The medium was changed immediately, and cells were kept at 37°C, in 5% CO₂, for varying periods of time and under various experimental settings, as specified (Chapter 6).
6.1.2. Free radicals detection and quantification

Cells were loaded with CM-DPane-DA at a final concentration of 5 μM in Hanks balanced salt solution (HBSS) (Gibco), from a 10 mM stock solution in DMSO, for 20-30 min, at 37°C, in 5% CO₂. At the end of the incubation period, the CM-DPane-DA solution was removed, the monolayer was rinsed with HBSS, and the upper plastic part of the 4-well Lab-Tek® chamber slide was removed, while the silicone gasket was left in place. HBSS was added in the 1 mm-high wells formed by the gasket, a coverslip was added, while ensuring the removal of remaining air, and the cells were examined alive, on heated stage (37°C) of the Nikon Eclipse 800 fluorescence microscope, at an excitation wavelength of 490 nm. The images were recorded on a SenSys digital camera, with constant exposure settings within each experiments (typically, 1 sec exposure, with an ND16 filter in the light pass). The integrated fluorescence intensity was measured in 10 rows (50 μm wide), parallel to the wound edge, using the MetaMorph image analysis system. For each time point, two experiments were recorded, and at least four images per experiment were analyzed. To ensure that the subcellular distribution of CM-DPane-DA fluorescence was not due to a volume effect, we loaded the cells concomitantly with CM-DPane-DA and CBAM, an inert cell permeant cytoplasmic marker, and we measured their intensity and distribution at 490 nm and 360 nm excitation wavelengths. The ratio of the digital images was obtained using the “Process-Arithmetic-Divide” menu of MetaMorph, and displayed as a new image, where the intensity of the CM-DPane-DA fluorescent signal was the numerator, and that of the CBAM was the denominator. On these ratio images
we analyzed selected cells with the "Line Scan" menu of MetaMorph, and the result was expressed as a graph where pixel intensity was plotted versus position within the cell (Giuliano & Taylor, 1994).

6.1.3. Study of actin polymerization in MAEC

6.1.3.1. Fluorescence microscopy

To load the cells with fluorescent actin derivatives and study their incorporation into filaments, we adapted a published method (Symons & Mitchison, 1991). The cells were rinsed with loading buffer (LB) (20 mM HEPES, pH 7.5, 138 mM KCl, 4 mM MgCl₂, 3 mM EGTA), then were incubated in LB supplemented with 0.2 mg/ml saponin and 1-3 μM A568A, for 5 min, at room temperature. The cells were carefully rinsed with plain LB, then fixed for 15-30 min in 3.7% formaldehyde in PBS, and mounted in Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL). The preparations were examined at an excitation wavelength of 560 nm. In some experiments after fixation the cells were stained with the nuclear marker Hoechst 33342, and examined at 360 and 560 nm excitation. Inhibitors were added at the indicated concentrations, for one hour, and were present at the same concentrations in LB.
6.1.3.2. Quantification of exposed barbed ends by flow cytometry

For this assay, we used MAEC at 60% confluency, in 12-well plates. A488A was added at a final concentration of 1 μM in LB, for 0, 1, 2, and 3 min, and the cells were then processed for FACS analysis as follows: after rinsing the A488A, the cells were trypsinized, and Trypsin Neutralizing Solution (Clonetics, San Diego, CA) was added after 1 minute. DPI and MnTMPyP were added 1 hr prior to the A488A addition, at 10 μM and 25 μM, respectively. In parallel samples we added 2 μM CyD (Schliwa, 1982), 30 min prior to the A488A addition. 10000 cells were analyzed in a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA), on channel FL1. The number of exposed barbed ends was determined by: (a) measuring the rate of A488A incorporation per second, per cell, in the first 3 min, when the slope was linear; (b) determining the rate of polymerization at barbed ends, by subtracting the rate measured in the presence of CyD from the rate measured in the absence of CyD (Hartwig et al., 1995); (c) dividing this rate by the reported rate of actin molecules addition at the barbed ends at 1 μM actin measured in vitro, i.e., 10 molecules/sec (Hartwig et al., 1995).

6.1.4. Cell migration assay

Intact monolayers of MAEC grown on coverslips were wounded, and within 30 min were prepared for time-lapse microscopy. The coverslips were placed on the top of a closed bath imaging chamber (Warner Instrument Corp., Hamden CT), sealed in place, the chamber was filled with DMEM supplemented with 10% FBS, and put on the heated
stage of the microscope. In experiments using MnTMPyP and DPI, the inhibitors were added to the cells 1 hr prior to wounding, as well as in the incubation chamber, for the whole length of the assay, at the final concentrations of 25 μM and 10 μM, respectively. DIC digital images were acquired with the 40x objective every 90 sec, for at least 5 hrs, using the MetaMorph software. At the end of the assay, individual cells were tracked with the “Track object” menu of MetaMorph, with the selector placed on the nucleus of each tracked cell. In the subsequent analysis of data, we used the mean speed of individual cells (μm/min), computed by the program. To obtain the tracks in Fig. 31 we used the Graph Data submenu, and we determined the module of the resultant vector of the cell path, by measuring the distance between the start and end points for each cell with the “Measure Distances” menu.

6.2. CELLS AT THE WOUND MARGIN PRODUCE MORE FREE RADICALS

For this study, we chose the *in vitro* wounding model, because: (1) it replicates faithfully *in vivo* situations where confluency of the endothelium is disrupted; (2) the cells at the margin of the wound reorient and migrate perpendicular to the direction of the wound, and their motility can be monitored in different experimental conditions, having as intrinsic control the confluent cells away from the wound. Confluent monolayers of MAEC were scratched with a glass tip to create the wound. The formation of intracellular ROS was monitored by preloading the cells with CM-DCF-DA, a derivative of dichlorofluorescin diacetate with improved retention within live cells, and which fluoresces upon exposure to oxidants, in particular H₂O₂. (Zulueta *et al.*, 1997;
Figure 22. Free radicals detection by CM-DCK-DA fluorescence in MAEC from the wound margin and from intact inner rows within the monolayer.
a. Differential contrast image. b. CM-DCF-DA fluorescence. c. Rhodamine-phalloidin staining of F-actin. The images were taken at 1 hr after the wounding of the monolayer. The wound margin is at the top of the micrographs. Bar, 25 μm.

Figure 23. F-actin organization in the free radicals producing MAEC at the wound margin.
While cells before scratching, or distant from the scratched area, displayed low CM-DICF-DA fluorescence, MAEC flanking the wound were consistently and significantly more fluorescent (Fig. 22a, b). Quantification of CM-DICF-DA fluorescence by morphometric analysis of digital images demonstrated increased free radical production in the cell rows closest to the wound margin. The fluorescence increased progressively over time following wounding, and peaked when cells became actively involved in migrating to restore the confluency of the monolayer (Fig. 22c). The ROS-producing cells contained an intact actin cytoskeleton, as detected by rhodamine-phalloidin staining of actin filaments within these cells (Fig. 23). Moreover, time-lapse fluorescence microscopy of the monolayer flanking the wound margins, preloaded with CM-DICF-DA, showed that the cells with the highest fluorescent signal were also the most actively motile, although cell-to-cell variations are likely to occur. Many cells had an intense and transient membrane ruffling activity. Free radicals production was detected not only within the cell body, but also at the edge of the membrane ruffles, the most motile part of the cell (Fig. 24, 1-18, arrowheads).

The increased CM-DICF-DA fluorescence detected within the cells flanking the wound margin could have been due to a volume effect, due to the fact that confluent EC from inner rows are generally more spread (therefore more flattened) than the migrating cells. To rule out a volume effect, we loaded the cells with the cell permeant dye CBAM, whose fluorescence is not influenced by the oxidative status of the cell, nor by other intracellular variables, such as pH (Haughland, 1996). After acquiring digital images corresponding to identical areas for both fluorophores, we executed the arithmetic operation of dividing the pixel intensity of the CM-DICF-DA images by the pixel

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DIC: differential interference contrast image of the wound margin at the beginning of the time-lapse series. The wound is at the left side of the micrograph. The arrow points at a cell that is followed in 1 through 18, which are the respective fluorescent images. The arrowheads delineate the highly motile membrane ruffles. The interval between images is 1-2 min. Bar, 10 μm.

**Figure 24.** Time-lapse fluorescence microscopy of cells at the wound margin, loaded with CM-DCF-DA.
The top micrographs show typical cells from the wound margin (which is on the left side of the micrograph), and the bottom micrographs, confluent cells from the intact monolayer. Cells were loaded with CM-DCF-DA and CBAM, and digital images were recorded with excitation at 490 nm (CM-DCF-DA) and 360 nm (CBAM). The ratio images were obtained as described in Methods, and scans for pixel intensities were obtained along the shown lines. DIC images of the same fields are shown (DIC). The graphs show the pixel scans for the wound margin cell (LS1) and for the cell away from the margin (LS2). The pixel intensity (in arbitrary units) is expressed as a function of the distance (µm) from the left start of the line.

*Figure 25. Volume control for the CM-DCF-DA fluorescence.*
intensity of the CBAM images, resulting in a new image which brightness was proportional to the ratio. Thus, dark regions represent low ratios of CM-DCF-DA over CBAM fluorescence, while bright regions are indicative of specific accumulation of oxidized CM-DCF-DA. A representative image is presented in Fig. 25, where the upper panels show, from left to right, the CM-DCF-DA, CBAM, ratio image, and DIC image, for a cell from the wound margin. The lower panels show matching images for a cell from the interior of the monolayer. The graphs present pixel intensity levels as a function of the position along the scanned line. Two conclusions may be drawn from these data: (1) The intensity of the CM-DCF-DA fluorescence, relative to CBAM fluorescence, is much higher within the cell flanking the wound margin (Fig. 25, compare the pixel intensity levels in LS1 and LS2), consistent with increased production of ROS in these cells; (2) There is specific accumulation of CM-DCF-DA at the edge of migrating EC, while the higher fluorescence detected in the perinuclear area represents a volume effect.

6.3 Actin polymerization in motile MAEC is regulated by reactive oxygen species production

A488A incorporation into filaments was measured by flow cytometry in the presence and the absence of the inhibitor cytochalasin D (CyD) (2 μM). We considered that an increase in A488A fluorescence was secondary to actin polymerization at barbed ends if it could be specifically inhibited by the addition of CyD (Cooper, 1987). We used subconfluent MAEC, which are actively motile and exhibit intense membrane ruffling (McGrath et al., 1998). The cytochalasin-sensitive incorporation of A488A into filaments
within motile MAEC increased significantly during the first 3 minutes of incubation (preliminary experiments showed that, in this assay, after this time point the detected fluorescence starts plateauing).

Many laboratories, including our own, have shown that a major source of SO and derived radicals within EC is the enzyme NADPH oxidase (Crawford et al., 1996; Zulueta et al., 1997; Moldovan et al., 1999). Therefore, we tested the effect of the flavoprotein inhibitor diphenylene iodonium (DPI) (O'Donnell et al., 1993) on A488A incorporation into filaments. We also examined the effect of MnTMPyP, a cell permeant SO scavenger which mimics the effect of the enzyme superoxide dismutase (Faulkner et al., 1994; Gardner et al., 1996).

CyD, at the concentration used in this study, significantly reduced fluorescent monomeric actin incorporation into filaments in control cells (Fig. 26). At the same time, both inhibitors, DPI and MnTMPyP, reduced significantly the incorporation of A488A into cells (Fig. 26). Interestingly, when we compared the incorporation in the presence of CyD (which is due strictly to polymerization at pointed ends, since CyD inhibits polymerization at barbed ends), we observed that in the presence of MnTMPyP this was significantly higher than in control-, or DPI-, CyD-treated cells (Fig. 26). This is an unexpected finding, which suggests that, perhaps, SO processing by the SOD mimetic and the secondary generation of H$_2$O$_2$, might enhance actin polymerization at the pointed ends, while inhibiting polymerization at barbed ends. All antioxidants significantly reduced the elongation of actin filaments at the barbed (fast growing) ends (Fig. 27a). Moreover, we have measured the number of opened barbed ends in EC with and without antioxidants. In control, freely moving sub confluent EC, we measured 3534 ± 1109
A488A polymerized was measured as described at 0, 1, 2, and 3 min after the addition of the labeled monomer. The average amount of A488A incorporated per cell was calculated, and the results expressed as mean fmoles A488A per cell, ± SEM, from three experiments, each in triplicate. Where indicated, DPI (10 mM) and MnTMPyP (25 mM) were added 1 hr, and CyD was added 30 min prior to the assay.

Figure 26. The effects of antioxidants on actin incorporation into filaments.
a. A488A polymerization in subconfluent MAEC, detected by flow cytometry, during the initial 3-minute interval following addition of labeled monomers. Results are the means of pooled data from three independent experiments, each in triplicate, and are expressed as fmoles A488A per cell, calculated by subtracting the A488A polymerized in the presence of CyD from the amount polymerized in the absence of CyD, ± SEM. The incorporation at 1, 2, and 3 minutes in the presence of both DPI (10 μmol/L) and MnTMPyP (25 μmol/L) is significantly different from control cells (p < 0.05). b. Number of exposed barbed ends in the MAEC from a; results are expressed as number of barbed ends per cell, ± SEM. *p < 0.05. There is no statistical difference between DPI and MnTMPyP data.

Figure 27. The effects of antioxidants on actin polymerization in motile MAEC.
opened actin filament barbed ends. This number was decreased to $503 \pm 629$ in the presence of DPI, and $731 \pm 343$, in the presence of MnTMPyP ($p < 0.05$) (Fig. 27b).

To support the flow cytometric data, we used microscopy to study monomeric actin incorporation within cells flanking the wound margins, using as intrinsic control the quiescent cells from distant rows of the confluent monolayer on the same coverslip. We examined MAEC 1 to 4 hrs after wounding. The cells were loaded with CM-DCF-DA, examined alive under the microscope to detect ROS production, then we processed with incorporation of A568A, the cells were fixed, and examined again. A568A was most rapidly incorporated within spreading cells at the margin of the wound (Fig. 28, a-c). The pattern of actin distribution was typical for migrating cells (Small et al., 1998): observed arc-shaped arrays of actin filaments at the base of lamellipodia at the advancing edge, and microspikes parallel to the direction of migration (Fig. 28,a). Cells that exhibited the highest actin staining also stained stronger for free radicals, as detected by CM-DCF-DA fluorescence (Fig. 28, b and c). In contrast, cells away from the wound accumulated little A568A, mostly in a punctuate pattern, with no subcellular compartmentalization, and produced little, if any, free radicals (Fig. 28, d-f).

We also assessed A568A incorporation, in the presence of inhibitors: CyD (2 \mu M), DPI (10 \mu M), and MnTMPyP (25 \mu M). In the absence of antioxidants, A568A incorporation was nearly completely inhibited by CyD, indicating that the fluorescent signal was indeed secondary to the polymerization of A568A (Fig. 29, compare a and d). CyD had no detectable effect on free radical production (not shown). In the presence of DPI, A568A incorporation nearly vanished (Fig. 29, compare panels 5a with 5f). The presence of the superoxide dismutase mimetic MnTMPyP also reduced markedly the
Figure 28. Actin turnover and free radicals production in MAEC at the wound margins and away from the wound.
Figure 29. The effect of inhibitors on A568A incorporation into cells at the wound margin.
polymerization of actin (Fig. 29, h), and many cells acquired a polarized shape, contrasting with the usual cobblestone appearance of cultured EC. In cells with a detectable residual A568A fluorescent signal, stress fibers, instead of lamellipodia, were labeled (Fig 29h, inset). Thus, MnTMPyP affected not only the rate of polymerization of labeled actin, but also the specific site of actin incorporation within EC.

Some actin accumulation was also detected within the cell body, in the perinuclear region, particularly with higher concentration of A568A (3 μM), a previously described finding in microinjected cells (Cao et al., 1993). To rule out that such actin accumulation was taking place within the nucleus (Gonsior et al., 1999), we labeled the nuclei with Hoechst 33342 and performed both standard fluorescence microscopy (Fig. 29c) and confocal analysis of the cells (not shown), in order to define more accurately the localization of the fluorescent actin within the cell body. Fig. 29c is representative and shows that the A568A fluorescence is perinuclear.

6.4. Superoxide scavenging reduces cell motility

If increased production of free radicals is necessary for the dynamic actin turnover within lamellipodia, a process required for the migration of cells into the wound, then SO scavenging should reduce the motility of EC. To test the relationship between increased free radical production and cellular motility, we examined the speed of cells moving into the wound, in the presence or absence of DPI or MnTMPyP, by time-lapse microscopy. We also measured the resultant vector for each individual cell path, as a measure of the efficiency of migration, for five to six hours after wounding.
The parameters measured for control cells were: mean speed, 0.45 ± 0.02 μm/min, which agrees well with the reported speed for subconfluent EC of ~ 0.5 μm/min (McGrath et al., 1998), and mean resultant vector, 97.9 ± 10.9 μm, during 5 hrs. In the presence of DPI these two parameters were significantly reduced to 0.26 ± 0.01 μm/min, and 36.8 ± 3.0 μm, respectively (p < 0.001 for both parameters), and in the presence of MnTMPyP, to 0.28 ± 0.02 μm/min, and 20.3 ± 2.6 μm, respectively (p < 0.001 for both parameters) (Fig. 30). Interestingly, although both inhibitors reduced the speed of the cells by 42.3% for DPI and 37.8% for MnTMPyP, these agents decreased even more the effective distance migrated by the cells, by 62.4% and 79.3%, respectively. This could be explained by the fact that the migration of cells exposed to SO scavengers is more random (Fig. 31, Cell tracks). We ruled out the possibility that this effect was due to a cytotoxic effect of the inhibitors on the cells, since upon their removal the speed of migration was gradually restored to normal values within the next 20 hrs (data not shown).

6.5. DISCUSSION

We report here that EC respond to a stress like mechanical disruption of the confluent monolayer by producing increased amounts of free radicals. For years, the impact of reactive oxygen species has been considered solely as harmful for cells, and in many instances it may be so (Peskin, 1997; van de Vijver et al., 1997; Stadtman & Berlett, 1998). However, more recent evidence has accumulated showing that many physiological processes require the production of minute amounts of various oxidants, such as H₂O₂ (Hong et al., 1997), SO (Irani et al., 1997), or nitric oxide (Monteiro &
a. Speed of migrating cells at the wound edge. b. Linear distance (vector length) covered during the first 5 hrs after wounding. Four experiments were performed for each condition, and 5-10 cells were tracked per each experiment. Data are expressed as means of all cells tracked in individual conditions, ±SEM. * p < 0.001.

**Figure 30.** The effect of antioxidants on cell migration. Quantification of the speeds and mean vector lengths
DIC images of cells at the beginning (Time = 0 min) and the end (Time = 5 hrs) of representative experiments for each condition (Control, 10 μM DPI, and 25 μM MnTMPyP). Cell Tracks correspond to the paths for these cells.

Figure 31. The effect of antioxidants on cell migration. Cell tracks.
Stern, 1996). In our experiments, the increase in ROS production is more prominent within the EC flanking the wound margin. In contrast to the quiescent cells from a confluent monolayer, the cells in this position start migrating within the “empty” space of the wound. This process requires actin cytoskeleton reorganization (Aepfelbacher et al., 1997), and our findings show that fluorescent monomeric actin is more actively incorporated into filaments within cells that are producing heightened amounts of oxidants.

We have previously shown in a model of hypoxia and reoxygenation of human EC that the reorganization of the actin superstructure can be controlled by the oxidative status of the cell (Crawford et al., 1996). Moreover, when we analyzed the mechanism by which the small GTP-binding protein rac1 controls the reorganization of actin cytoskeleton and the formation of membrane ruffles, in human EC, we found that actin polymerization required SO as well. The actin changes induced by overexpression of the constitutively activated mutant of rac1, rac\textsuperscript{V12}, could be reversed by either overexpression of Cu, Zn-superoxide dismutase, or by incubation with MnTMPyP, and with the antioxidant n-acetylcysteine (Moldovan et al., 1999).

With this study, we attest that actin reorganization within cells exposed to a stress like confluency disruption takes place in the presence of upregulated intracellular ROS production, as detected by free radical-induced CM-DCF-DA fluorescence (Figs. 22 - 26). A key question is whether the presence of ROS is causative for, independent of, or secondary to, the actin changes. Experimental limitations do not allow for a definition of the temporal relationship between free radicals production and the transition from “quiescent” actin cytoskeleton, mainly characterized by the presence of stress fibers, to
the migratory phenotype, with the formation of a distinctive lamellipodium. However, our findings suggest that removal of ROS by two different mechanisms, either inhibition of the enzyme that produces SO (by DPI), or scavenging already produced SO (by MnTMPyP), has significant effects upon actin reorganization and cell migration.

The inhibition of both free radicals production and actin incorporation into filaments in the presence of DPI (Figs. 26-29) suggests that: (1) an endothelial NAD(P)H oxidase-like enzyme is the leading source of the free radicals detected by CM-DCF-DA (Zulueta et al., 1995; Crawford et al., 1996; Mohazzab et al., 1996; Holland et al., 1998; Moldovan et al., 1999); and (2) SO has a role in the polymerization of specific actin structures. The detection of CM-DCF-DA fluorescence at the tips of the membrane ruffles (Fig 24) is consistent with the activation of a non-phagocytic NADPH oxidase via rac1 bound to GTP, since rac1 was also detected in the membrane ruffles (Ridley et al., 1992, Moldovan et al., 1999).

DPI, which is a flavoprotein inhibitor, is not absolutely specific for NADPH oxidase. Nitric oxide (NO) synthase is another major flavoprotein in EC. We have ruled out the possibility that DPI affected the production of free radicals by EC through inhibition of NO synthase, since a classic inhibitor of NO synthase (No-nitro-L-arginine methyl ester, L-NAME) did not affect endothelial cell production of ROS in our assay (data not shown). This finding is consistent with the fact that EC-derived NO has been shown to inhibit smooth cell migration (Gorog & Kovacs, 1998). However, the role of NO in cellular motility is dependent upon the specific intracellular signaling pathways, since in different systems, including endothelial and epithelial cells, it is required for locomotion (Noiri et al., 1996; Noiri et al., 1997). It has been suggested that one of the
effects of increased SO production is the consumption of NO via formation of ONOO\(^-\) (Wolin, 1996; Munzel et al., 1997). Therefore, if NO were necessary for EC migration in our system, then the increase in SO would lead, via NO consumption, to inhibition of migration, and antioxidants would, on the contrary, promote migration, opposite to what we observed. Nevertheless, the issue of NO involvement in EC migration still needs further experiments to be elucidated, because neither of the chemicals we used in these experiments is absolutely specific.

The effect of MnTMPyP showed that removal of SO prevented efficient migration, reduced motility to a lesser extent, and inhibited actin polymerization due to the limitation of available barbed ends (Figs. 26 - 27). However, the detection of stained stress fibers (Fig. 29g, inset), suggested that the effect of MnTMPyP is more complex than the effect of DPI. We consider this particular finding significant, because: (1) fluorescent stress fibers were detected only in the presence of MnTMPyP; (2) this result is consistent with our previous observation that, when SOD was overexpressed in human aortic EC via adenovirus transduction, a similar pattern of cell polarization and parallel actin fibers accumulation occurred (Moldovan et al., 1999). Dismutation of SO results in the formation of H\(_2\)O\(_2\) (Faulkner et al., 1994), and it is known that treatment of cells with H\(_2\)O\(_2\) induces increase in actin polymerization in various cells, including EC (Hinshaw et al., 1986), (Hinshaw et al., 1991; Omann et al., 1994), perhaps through a process that involves the small GTP-binding protein Rho, and the actin-binding protein cofilin (Carlier & Pantaloni, 1997).

A key result of our report is the requirement of ROS for the exposure of uncapped barbed ends (Fig. 27). This finding suggested a possible mechanism by which ROS
would promote actin polymerization (Moldovan et al., 1999). Specifically, ROS may either promote actin filaments severing, barbed ends uncapping, or both, and independently of the mechanism for this effect, the net result is the increase in barbed ends exposure, which dramatically increases the speed of actin polymerization (Carlier & Pantaloni, 1997). Moreover, the increase in polymerization from pointed ends in the presence of MnTMPyP suggests that H$_2$O$_2$ has the effect of either uncapping pointed ends and promote polymerization, or reducing depolymerization at the pointed end. This observation could explain the increase of actin polymerization observed in cells treated with H$_2$O$_2$ (Hinshaw et al., 1986; Hinshaw et al., 1991).

The physiological production of ROS by EC at the margins of the wound is also necessary for migration to take place, as removal of SO either by inhibition of the enzyme that generates it (DPI), or by dismutation (MnTMPyP), reduces the motility of the exposed cells (Figs. 30 - 31). It is instructive to relate this finding with that of neutrophils from patients with chronic granulomatous disease (Clark, 1999). Although the phagocytic NADPH oxidase from these patients is non-functional due to various mutations, the migratory ability of these cells is intact. However, there are major differences between SO production in phagocytic versus non-phagocytic cells: (1) the phagocytic NADPH oxidase evolved to maximize the production of the reactive species required for bacteria killing, while much lower amounts are produced in non-phagocytic cells (Clark, 1999; Katusic, 1996); (2) The site where SO is produced is either the extracellular space, or the phagolysosome, which is also a space excluded from the cell cytoplasm. In contrast, in our experiments the probe we used, i.e., CM-DCF-DA, specifically detected the intracellular production of oxidants. This is in agreement with
other recent reports that showed that EC (Rahman et al., 1999), as well as smooth muscle
cells (Patterson et al., 1999), produce oxidant species within the cytoplasmic
compartment.

6.6. CONCLUSIONS

The role ascribed to SO and derived oxidants in biology is clearly expanding. By
analogy with NO, whose activity at low concentrations is to transduce signals within
vessels and neurons (Lowenstein et al., 1994), while high concentrations produce damage
to cells and microorganisms (Dugas et al., 1995), SO – and probably other oxidants as
well – function as messengers at low concentrations, while larger amounts are required
for harmful activity (Irani & Goldschmidt-Clermont, 1998). We propose that, although
there may be instances when actin reorganization occurs in the absence of any specific
production of ROS, in most instances the targeted production of ROS, in a coordinated
manner, modulates actin cytoskeleton reorganization. The localized production of
oxidants could selectively affect the function of either actin monomer sequestering
proteins, capping proteins, or severing proteins, reversibly or irreversibly, in a process
that may be analogous to the activation of NF-κB by oxidant-triggered degradation of
IkB (Sundaresan et al., 1996), which, in turn, may affect the parameters of the actin
polymerization/depolymerization processes.

An important question would then be: what is the possible mechanism by which
ROS could control actin/actin-binding proteins interaction? There are at least two ways:
by means of direct reaction with any of these proteins, or indirectly, affecting other
processes which, in turn, influence actin turnover. As we pointed in the Literature Review chapter, many of the proteins involved in actin control, as well as actin itself, are redox sensitive. We will enumerate some of the potential redox-control points:

1. Thymosin β4 has a methionine in position 6. Its oxidation decreases, but does not abolish, actin-sequestering properties (Hannappel & Wartenberg, 1993). If this oxidation would occur *in vivo*, it would support the increased availability of actin monomers in the presence of oxidants.

2. Gelsolin is a downstream effector of rac. An important difference was recently described between the plasmatic and cytosolic proteins: the plasma form has an S-S in domain 2, which is absent from the cytoplasmic form of gelsolin. This can be induced by mild oxidation (with oxidized glutathione) (Wen et al., 1996). Interestingly, an important proportion of the studies of actin-gelsolin interaction was done with the plasma-derived protein, since it is easier to be purified, however, it is not clear how much their properties differ.

3. LIM-kinase, the cofilin regulating enzyme, is a downstream rac effector. It has Zn-binding domains, containing -SH groups, which are generally sensitive to the redox conditions of the environment, thus prone to regulation via ROS (Stanyon & Bernard, 1999).

4. As mentioned, actin itself is clearly a redox-sensitive protein. It is only active in reducing buffers, containing milimolar amounts of di-thiothreitol. Treatment of actin with oxidants induces thiol oxidation and conformational shifts (Omann *et al*., 1994; DalleDonne *et al*., 1995), but the concentrations used and the assay systems are not always physiological. However, neutrophil stimulation induces S-thiolation of actin from
glutathione (Chai et al., 1994). Also, oxidation of some of actin thiols induces formation of more elastic actin networks (Tang et al., 1999). Could these be indices that ROS may act directly upon actin?

There are many indirect ways in which ROS may control actin reorganization, as well. For instance, coflin regulation consists in cycles of phosphorylation – dephosphorylation. The phosphatase that catalyses dephosphorylation is not yet known, but these enzymes are inhibited in the presence of oxidants (Krejsa & Schieven, 1998). This would mean that, at least in some systems, a pro-oxidant environment would lead to increased coflin phosphorylation, thus inactivation, leading to increased actin polymerization. Another interesting possibility would involve the endoplasmic reticulum calcium release channel (ryanodine receptor)(Xu et al., 1998; Aghdasi et al., 1997). It is activated in the presence of thiol oxidants, and the effect is a sustained increase in the intracellular calcium concentration. Since gelsolin is activated by calcium, the end result would be increased severing activity and exposure of barbed end, as observed in our experiments.

Of course, these are all hypotheses that will need very thorough testing in order to understand the way(s) ROS can control actin cytoskeleton reorganization.
CHAPTER 7

LESSONS FROM PLANT RAC PROTEINS

7.1. RATIONALE

The rapid production of reactive oxygen species (ROS), a process referred to as the oxidative burst, plays a critical role for the response against pathogens both in plants (Lamb C & Dixon RA, 1997), and animals (Robinson & Babcock, 1998). It has been hypothesized that biochemical mechanisms underlying the oxidative burst in phagocytes and pathogen transduced plant cells have been conserved during evolution. In fact, several studies have suggested that the enzyme system responsible for the generation of ROS in plants is similar to the mammalian NADPH oxidase (Desikan et al., 1996), and the selective inhibitor of the mammalian NADPH oxidase, DPI, blocked pathogen triggered oxidative burst in plants as well (Lamb & Dixon, 1997). Moreover, immunological methods have been used to demonstrate that antibodies raised against various components of the mammalian NADPH oxidase complex cross-reacted with plant proteins in the predicted size ranges. More recently, homologs of mammalian gene encoding gp91phox, the key component of the NADPH oxidase, have been isolated from Arabidopsis and rice (Torres et al., 1998).
The discovery that rac proteins are key activators of the phagocytic NADPH oxidase (Abo et al., 1991; Knaus et al., 1991), prompted the search for related proteins in plants. Thus, several genes encoding Rho/Rac-like proteins have been isolated from number of plant species (Li et al., 1998b). The plant Rho/Rac sequences are most similar to mammalian Rac proteins (up to 65% identity). However, not much is known about the specific functions of these genes in plants. In this study, we identified four novel members of the Zea mays Rho/Rac gene family (ZmRac-A, -B, -C and -D). Given the high level of conservation between Zea mays rac proteins, and their mammalian counterparts, we used the heterologous expression approach in mammalian cells to gain insights into the functions of ZmRac proteins. As the sequence homology between plant and mammalial rac proteins is approximately 65%, we anticipated that certain interactions of plant rac with mammalian proteins (NADPH oxidase) may be conserved, while other (PAK, POR) might not be.

7.2. METHODS

7.2.1. Site-directed mutagenesis

The mammalian rac proteins containing a G12V mutation behave as constitutively activated (CA) isoforms. Alternatively, the T17N mutation confers a dominant negative (DN) phenotype. Based on the homology between mammalian and plant rac proteins, we generated similar mutations (G to V and T to N at the relevant locus, after aligning the
sequences of the plant rac protein with the mammalian protein), with the assumption that the mutants thus obtained would have similar phenotypes. To this purpose, we used the transformer mutagenesis kit (Clontech Inc.). Subsequently, the eight ZmRac DNAs (4 CA and 4 DN) were subcloned into the mammalian expression vector pZeoSV2 (Invitrogen).

7.2.2. Reactive oxygen species detection

We used two techniques for the detection of free radicals.

(1) First, we performed electron paramagnetic resonance (EPR) spectroscopy, which is the most specific technique for ROS detection (although not the most sensitive). The spin trap selected for these experiments was DEPMPO. The resulting spectrum corresponding to the adduct of this spin trap and SO is readily identifiable as an octet in a magnetic field in the range of 3275-3400 Gauss. Mammalian NIH 3T3 fibroblasts, grown in Dulbecco’s modified Eagle medium, were transiently transfected with mammalian or Zea mays rac cDNAs, using the Lipofectin kit (Boehringer Mannheim), according to the manufacturer’s recommendations. After transfection (20 hours), the cells were serum-starved overnight (in the culture medium containing 0.5% serum). For EPR, the medium was removed and the cells were washed with PBS treated with the chelating agent Chelex 100 Resin (Bio-Rad, Hercules, CA) to remove metal ions in the medium that could generate artifactual signals. The cells were collected using plastic scrapers in 1 ml PBS and spun down at 1200 rpm in an Eppendorf microcentrifuge, then resuspended in 250 μl PBS. 25-50 μl of the cell suspension was used for the EPR assay. The spin trap DEPMPO was added to a final concentration 100 mM at time zero (Frejaville et al., 1995), to a total
volume of 200 μl, (adjusted with PBS). The samples were assayed for their EPR signal at 2, 10, and 30 minutes after the addition of DEPMPO. As controls, we used Chelex-treated PBS, untransfected NIH 3T3 fibroblasts, and empty plasmid-transfected cells.

(2) We confirmed the EPR results by microscopy, on CM-DCF-DA-loaded cells. NIH 3T3 fibroblasts were transiently transfected as described above, with half of the plasmid labeled with rhodamine, to identify the transfected cells, and incubated overnight in 0.1% serum-DMEM. Thereafter, the cells were washed twice with HBSS, then incubated with 5 μM CM-DCF-DA in the same solution, for 20 minutes, at 37°C, in 5% CO₂ (Zulueta et al., 1997). The cells were then rinsed with HBSS and examined alive with a Nikon Eclipse 800 fluorescence microscope, at an excitation wavelength of 580 nm to visualize the rhodamine-labeled plasmid, and 490 nm to visualize oxidized CM-DCF-DA.

7.2.3. Actin assays

To assess the organization of F-actin in fibroblasts transiently-transfected with the ZmRac-encoding plasmids (50% labeled with rhodamine), the cells were fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, stained with 0.66 μM FITC-phalloidin for 30 min, rinsed with PBS, mounted in Fluoromount, and examined with the fluorescence microscope, at an excitation wavelength of 490 nm.

For the actin turnover assay, cells were transfected as above were loaded with A488A as previously described (Symons & Mitchison, 1991). The cells were then gently
rinsed with LB, treated with 0.25% Trypsin-EDTA for 1 minute, and Trypsin Inhibiting Solution was added. The resuspended cells were analyzed immediately by flow cytometry. For microscopic analysis, we followed the same procedure, except that after rinsing the fluorescent actin solution, the cells were fixed with 3.7% formaldehyde in PBS, for 30 min, then rinsed again, mounted in Fluoromount, and examined with the fluorescence microscope, at an excitation wavelength of 490 nm to visualize Alexa 488-Actin, and 560 nm to visualize the rhodamine-labeled plasmid.

7.3. **The effect of plant rac mutants on superoxide production in transfected mammalian fibroblasts**

To characterize the function of plant rac proteins, we transfected their respective cDNAs in mammalian cells. Considering that maize and mammalian rac proteins are highly conserved, we sought to find out whether ZmRac proteins can regulate the production of SO and other ROS in mammalian cells. Site-directed mutagenesis was used to generate CA and DN versions of ZmRac proteins (A, B, C, and D). Analogy with known activating mutation (Glycine 12 to Valine) and dominant negative mutation (Threonine 17 to Asparagine) was used to engineer the plant mutants.

We compared the effect of constitutively activated human racV12 to that of several ZmRac proteins on SO production in NIH 3T3 cells. We have previously shown that NIH 3T3 cells transiently expressing racV12 produced large amounts of SO, using EPR with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), while the expression of the
dominant negative mutant, rac$^{117}$, inhibited the production of ROS in these cells (Irani et al., 1997).

We used now the more specific spin trap DEPMPO for SO assay (Frejaville et al., 1995), and we have found that NIH 3T3 cells transiently transfected with the dominant positive ZmRac isoforms markedly increased their ROS production. The levels of SO production were higher in cells expressing CA ZmRac-D and CA ZmRac-A, than CA ZmRac-C and CA ZmRac-B. Moreover, in cells transfected with the matching dominant-negative isoforms, there was no detectable SO production (Fig. 32). We have also used fluorescence microscopy to confirm that transfection of ZmRac genes could induce SO production. Swiss 3T3 cells transfected with a mixture of unlabeled (50%) and rhodamine-labeled (50%) plasmids, were exposed to CM-DCF-DA. Cells expressing the activated ZmRac proteins were fluorescent and the intensity of fluorescence depended upon the ZmRac isoform that was being expressed (Fig. 33). The efficiency of transfection was not detectably different amongst the plant and human Rac-encoding plasmids, as assessed by rhodamine fluorescence. The efficiency of human rac$^{V12}$ and various ZmRac-CA proteins, in inducing ROS production in this assay, was in the following decreasing order, as detected by both methods (EPR and microscopy): ZmRac-D ≥ rac1$^{V12}$ ≥ ZmRac-A ≥ ZmRac-C ≥ ZmRac-B ≥ empty plasmid. The expression of DN isoforms of ZmRac showed no increase in ROS production compared to cells transfected with the empty vector, confirming the EPR data. Thus, ZmRac-CA mutants are capable of inducing the production of ROS in mammalian cells (Figs 32 and 33).
EPR spectra of NIH 3T3 cells transiently transfected with constitutively activated (CA) or dominant negative (DN) isoforms of human Rac 1 or ZmRac A, B, C, and D (as indicated). The spectra were recorded with the spin trap DEPMPO, for the time indicated.

**Figure 32: EPR spectroscopy.**
NIH 3T3 fibroblasts were transfected with rhodamine-labeled plasmid (red), encoding either constitutively activated (CA) or dominant negative (DN) forms of rac, as mentioned. After three days, overnight serum starved cells were loaded with CM-DCF-DA (green) and examined alive. The images are digital overlays of both fluorescent and DIC images.

**Figure 33.** Free radicals detection in fibroblasts trasfected with individual rac isoforms.
7.4. THE EFFECT OF PLANT RAC MUTANTS UPON ACTIN REORGANIZATION IN FIBROBLASTS.

Microinjection of rac\textsuperscript{V12} into fibroblasts induces membrane ruffling activity, a process that requires the reorganization of the actin cytoskeleton (Hall, 1998). Therefore, we sought to find out whether activated ZmRac could induce a similar response in Swiss 3T3 cells. Cells were transfected with ZmRac or human mutants, and stained with FITC-phalloidin, in order to study actin organization. Rac\textsuperscript{V12} induced membrane ruffling, as expected, however the constitutively activated isoforms of ZmRac-B,-C, and-D, had less effect on ruffling, and ZmRac-A-CA had no detectable effect on membrane ruffling (Fig. 34).

Ruffles formation results from both \textit{de novo} polymerization of actin filaments and reorganization of existing filaments at the cell edge, resulting in liquid phase pinocytosis (Condeelis, 1998; Mitchison \& Cramer, 1996; Small \textit{et al.}, 1998). We measured A488A incorporation into cells transfected with the plasmids encoding rac\textsuperscript{V12} or its maize homologues using a flow cytometry assay (Moldovan \textit{et al.} Circ. Res. 2000, \textit{in press}). We found that rac\textsuperscript{V12} and CA ZmRac-B,-C and-D induced fluorescently-labeled G-actin polymerization, while CA ZmRac-A had no detectable effect on actin incorporation (Fig. 35). The dominant negative isoforms of ZmRac proteins, as well as and rac\textsuperscript{N17}, had no significant effect neither on actin uptake (Fig. 35), nor on ruffle formation (data not shown). Lack of effect of CA ZmRac-A on actin incorporation was consistent with its inability to induce membrane ruffling, a process that requires actin reorganization. We also assessed microscopically the level of actin incorporation in fibroblasts transfected...
NIH 3T3 fibroblasts were transfected with: a,b: Rac^{V12}; c,d: ZmRac A (DP); e,f: ZmRac C (DP); g,h: empty vector. a,c,e,g: Overlay of the green (FITC-phalloidin) and red (rhodamin-plasmid) fluorescence images. B,d,f,h: DIC of the same cells Bar, 50 μm.

Figure 34. Actin organization in fibroblasts transfected with different rac isoforms.
FACS analysis of A568A incorporation into fibroblasts transfected with Rac isoforms, as indicated. The results are expressed in median fluorescence arbitrary units, ± SD. *p < 0.05, as compared to empty vector control. #p < 0.05, as compared to the respective dominant positive Rac.

Figure 35. A488A incorporation into plant-rac isoforms-transfected fibroblasts
with CA ZmRac-D and DN ZmRac-D, as compared to the empty vector control. In CA ZmRac-D-transfected cells, the A488A was actively incorporated, and in amounts that were proportional to the amount of plasmid detected within these cells. In contrast, very little fluorescent actin incorporation was detected within fibroblasts transfected with either DN ZmRac-D, or the empty vector, and there was no correlation with the presence or absence of the plasmid.

7.5 Discussion

Heterologous systems represent a powerful and rapid method to study proteins originating from organisms in which transgenes are difficult to engineer, such as plants. Moreover, when the protein under study is involved in several pathways, as it is the case for rac, this approach may be relevant to the characterization of the specific domains of the molecule involved in each function. In this respect, this study was appropriate to help us understand that, in order for the actin reorganization to occur, the production of ROS is necessary, but not sufficient (see CA ZmRac A).

The amino acid sequences of maize rho/rac-related GTPases were compared with a number of proteins of the rho family from various organisms. This comparison revealed that the maize proteins belong to a subfamily of Ras-related protein superfamily, of the rac/cdc42 group and are more distantly related to the rho proteins (Li et al., 1998b). The deduced amino acid sequence of ZmRac proteins revealed the presence of four sequence motifs, G1, G3, G4 and G5, that are conserved in most small GTP binding proteins (Borg et al., 1997), and are associated with nucleotide binding and GTP hydrolysis. The ZmRac
proteins also contain the characteristic G2 effector region, fairly conserved within each subfamily, but less conserved across subfamilies. The CXXL motif at the C-terminus, that is required for prenylation of the C-terminal Cys residue, a post-translational modification of the C-terminus of rac proteins necessary for membrane localization, is also present in ZmRac-A, -B, -C and -D. The C-terminus region of rac proteins is the most variable region. There is approximately 60% identity and 70% similarity between human and maize Rac sequences at the amino acid level. More specifically, the G2 motif (amino acids 26-45), a sequence essential for the induction of actin polymerization, as well as for interaction with the target enzymes PAK and NADPH oxidase, is well conserved between plants and mammals. Other effector regions have been identified at amino acids 143-175 (Diekmann et al., 1994) and residues 124-135, the insert region of the human rac. This region is considered necessary for the activation of NADPH oxidase in phagocytes (Joseph & Pick, 1995; Nisimoto et al., 1997), SO production, and promoting of cell cycle progression (Joneson & Bar-Sagi, 1998). The domain between residues 143-175 and the insert region are not found in ZmRac proteins, in spite of the ability of these rac isoforms to enhance the production of SO.

Our results suggest that CA ZmRac-D can be used as a strong activator of the oxidative burst and, perhaps, to promote the defense response of plants against infectious agents. Moreover, the structure of the Rac gene has been highly conserved throughout evolution, such that a maize Rac gene product is capable of regulating the generation of SO in mammalian cells. This effect of rac seems remarkably conserved, and suggests that the rac binding domain of the SO generating enzyme complex must be also highly conserved. Our results suggest that the G2 region (amino acids 26-45), which is highly
conserved between plants and animals, could be essential for ROS production, at least in non-phagocytic cells. Other functions of rac proteins, such as the regulation of the actin cytoskeleton, appear more selective, since the reorganization of the actin cytoskeleton (regulated by rac1 in mammalian cells) was not as consistent as the production of SO. We did not find that the insert region (amino acids 124-135) was needed neither for ROS generation, nor for actin regulation, since this region is not conserved in plant rac proteins. However, it might be important to generate membrane ruffles, as the plant rac were less efficient at producing ruffles, in spite of their ability to induce actin polymerization.

7.5. CONCLUSIONS

Our results show that dominant positive variants of ZmRac proteins can induce SO production in mammalian cells, while corresponding dominant negative versions have no effect. The DN effect of rac is mediated by the sequestering of exchange factors by the GDP-locked rac DN mutants, preventing the interaction of endogenous rac with its exchange factor. Such protein-protein interaction does not seem to have been conserved from plants to mammals. Furthermore, the expression of dominant positive isoforms of ZmRac-B, -C, and -D also induce actin polymerization in mammalian cells. These results imply structural and functional conservation of the Rho/Rac gene families in plants and mammals. Ongoing research aims at further understanding of the molecular mechanisms involved in human versus Zea mays rac control on NADPH oxidase and actin reorganization.
CHAPTER 8
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

8.1. REACTIVE OXYGEN SPECIES REPRESENT MEDIATORS FOR RAC IN THE CONTROL OF
ACTIN REORGANIZATION AND CELLULAR MOTILITY

Several important conclusions can be drawn from our work:

1. The constitutively activated form of rac1 protein, rac^{V12}, overexpressed in
human and mouse endothelial cells via an adenoviral vector, induces the formation of
ROS in these cells, as shown by the lucigenin assay for SO and the increased
fluorescence of the oxidation-sensitive probe CM-DCF-DA.

2. This increase in ROS could be inhibited by different antioxidants: NAC, the
SOD mimetic MnTMPyP, DPI (all membrane permeant and added in the extracellular
medium) and SOD (overexpressed via an adenoviral vector). The effects of MnTMPyP
and SOD show that SO is the primary oxidant species produced, and the effect of DPI
shows that the source of this SO is likely to be an NADPH oxidase, present in EC and
probably regulated by rac.

3. Rac^{V12} overexpression leads to the formation of membrane ruffles, and in EC it
also induces a consistent increase in F-actin.
4. This increase and reorganization of F-actin can be reversed by antioxidants (NAC, MnTMPyP, DPI, SOD), which is strongly suggestive of the fact that the presence of ROS, specifically SO, is necessary for the formation of F-actin.

5. This conclusion is also supported by the fact that NAC and SOD also decrease the amount of F-actin in control cells, that do not overexpress rac^{V12} protein, which implies that SO/ROS are normally needed for the maintenance of the F-actin content in EC.

6. The cells, that display increased production of ROS, also show the highest rate of monomeric actin polymerization, as observed by fluorescently-labeled actin incorporation in permeabilized cells, a process that is antagonized by the addition of antioxidants.

7. Migrating EC from an in vitro wound margins likewise produce increased amounts of ROS, a process that is inhibited by MnTMPyP and DPI.

8. Actin incorporation takes place mostly at barbed ands, as shown by the rate of polymerization in the presence of cytochalasin D, and the number of free, uncapped barbed ends is decreased by the removal of ROS by MnTMPyP and DPI. This important finding suggests that the mechanism by which SO/ROS could increase actin reorganization in stimulated EC, namely the uncapping of the fast-growing (barbed) ends of actin filaments.

9. The inhibition by antioxidants of the actin reorganization, a process required for cellular motility, reduces both the speed of migrating EC, and the efficiency of migration, thus further emphasizing the importance of the reactive oxygen species in the physiology of EC.
10. We propose that, in contrast with the concept of "oxidative stress", physiological amounts of free radicals, specifically superoxide and derived oxygen species, are important for the intracellular signal transduction pathways triggered by rac activation and leading to the reorganization of the actin cytoskeleton.

8.2. UNANSWERED QUESTIONS

The most important conclusion of our work is that ROS are important for actin reorganization. Obviously, the next question is: "What is their mechanism of action?" In other words, what is the mechanism by which free radicals promote actin remodeling (increased polymerization, increased turnover, and thus increased adaptability of the actin network, that is necessary for cellular migration).

Some preliminary results showed that \textit{in vitro} actin polymerization in the presence of \text{H}_2\text{O}_2 is affected by the presence of profilin in the buffer: the inhibition of polymerization observed in the presence of \text{H}_2\text{O}_2, probably due to the denaturation of actin by oxidants, is reversed if profilin is also present. The important lesson this experiment teaches us is that SO/ROS most probably affect the interaction between actin and some of its binding proteins. We have presented in the INTRODUCTION the way oxidation-reduction of some protein moieties may affect protein conformation, and the type of subtle conformational changes that are involved in all actin-actin binding protein interactions and effects.

We have also introduced some of the best known actin-binding proteins, and showed that many of them have the potential of being regulated by redox conditions, e.g.,
gelsolin, the cofilin-LIM kinase system, and thymosin β4. Thus, a general question that should be addressed by in vitro experiments, is: do SO and H₂O₂ affect the interaction between actin and actin-binding proteins, and consequently actin polymerization/depolymerization rates, in a way that supports the hypothesis that reactive oxygen species play a role in actin reorganization necessary for cellular migration? What is the effect of SO on actin polymerization in the presence and absence of various regulating proteins? Is their interaction with actin regulated in a different way by SO and by H₂O₂?

In vitro experiments should be matched by a concurrent in vivo search for the fate of actin binding proteins in the presence of ROS, such as their intracellular localization or their oxidative status in different redox conditions.

Another set of questions should be related to EC migration. We showed that rac⁶¹² overexpression in these cells induces formation of ROS and actin remodeling, but are free radicals produced by endogenous rac activation involved in EC migration? Which is the specific species involved? What is the role of SO versus H₂O₂ in actin reorganization, and how does the effect depend on SO/H₂O₂ concentration? How is the migration of EC influenced by the overexpression of rac⁶¹²? In addition, how do these findings relate to the wide use of antioxidants in different diseases, including atherosclerosis?

These are only a few of the multiple research avenues opened by the finding that free radicals are necessary for the normal functioning of the cells. We trust that our work has contributed to opening these avenues, by linking the production of oxidants to physiological cellular responses, such as the reorganization of the actin cytoskeleton.
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