# Growth factor-induced signal transduction in adherent mammalian cells is sensitive to gravity

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ABSTRACT **Epidermal growth factor (EGF) activates a well-characterized signal transduction cascade in a wide variety of cells. This activation leads to increased cell proliferation in most cell types. Among the early effects evoked by EGF are receptor clustering, cell rounding, and early gene expression. The influence of gravity on EGF-induced EGF receptor clustering and gene expression as well as on actin polymerization and cell rounding have been investigated in adherent A431 epithelial cells with the use of sounding rockets to create microgravity conditions. EGF-induced *c-fos* and *c-jun* expression decreased in microgravity. This was caused by alteration of the EGF receptor and protein kinase C-mediated signal transduction pathways. In contrast, neither the binding of EGF to the receptor nor the receptor clustering were changed under microgravity conditions. Because cell morphology was also modulated under microgravity conditions, and the growth factor-induced signal transduction cascades have been demonstrated to be linked to the actin microfilament system, it is tempting to suggest that the actin microfilament system constitutes the gravity-sensitive cell component.—Boonstra, J. Growth factor-induced signal transduction in adherent mammalian cells is sensitive to gravity. *FASEB J.* 13 (Suppl.), S35–S42 (1999)**

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A NUMBER OF STUDIES have indicated that gravity affects mammalian cell growth and differentiation (1, 2). Experiments in space revealed that gravity influences many processes in humans, such as bone development, heart function, and temporary immunocompetence (3–6). These latter findings strongly suggested that, in addition to effects of gravity at the macro level, processes at the micro level are also impaired by gravity. These suggestions were exemplified by the observations that in human HeLa cells an increased gravity value resulted in an alteration of gene expression. In addition, activation of isolated human lymphocytes was depressed under microgravity conditions (7, 8). These observations suggested that the observed effects of gravity on lymphocytes and other mammalian cells originate from gravity-dependent modulations of the molecular signaling cascades from plasma membrane to nucleus, which lead to the onset of gene expression.

The well-characterized cellular response of human epidermal A431 carcinoma cells to epidermal growth factor (EGF)[2] to some extent resembles the cascade of molecular events involved in the activation of human lymphocytes. This was therefore considered an appropriate model system to study the effects of gravity on molecular processes that are fundamental in regulation of cell function, including lymphocyte activation and bone formation. The advantages of the model system concern the unlimited availability of the well-known A431 cell, the faculty to keep them in culture, the existence of many probes and antibodies required for such research, and last but not least a thorough knowledge of the molecular machinery involved in their activation.

## GROWTH FACTOR-INDUCED SIGNAL TRANSDUCTION

Polypeptide growth factors, among them EGF as one of the best-known factors to date, play a fundamental role in regulation of cell proliferation and differentiation. Many details of the molecular machineries that underly growth factor action have been described in detail as discussed in many recent review articles (9–12).

One of the first identified signal transduction pathways that is activated by the EGF receptor concerns the phosphatidylinositol bisphosphate ($PIP_2$) turnover catalyzed by phospholipase Cγ (PLCγ). The products formed by PLCγ are two second messengers, inositol trisphosphate ($IP_3$) and diacylglycerol (DG). DG operates within the cell membrane

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[2] Abbreviations: EGF, epidermal growth factor; $PIP_2$, phosphatidylinositol bisphosphate; PLCγ, phospholipase Cγ; $IP_3$, inositol trisphosphate; DG, diacylglycerol; PKC, protein kinase C; GAPs, GTPase activating proteins; PMA, phorbol myristate acetate; CB, cytochalasin B.

and acts as an activator of protein kinase C (PKC) (13), whereas $IP_3$ is released into the cytoplasm and raises the intracellular $Ca^{2+}$ concentration by inducing its release from intracellular stores (14). $Ca^{2+}$ in its turn is involved in activation of the $Ca^{2+}$ and calmodulin-dependent CAM-kinase, a serine/threonine kinase (15).

Another important signal transduction pathway activated by the EGF receptor concerns the ras pathway. Ras proteins are membrane-bound guanine nucleotide binding proteins of low molecular weight. Binding GTP activates ras and subsequent hydrolysis of the bound GTP to GDP causes inactivation. GTP binding can be catalyzed by guanine nucleotide exchange factors, whereas GTPase activity can be accelerated by GTPase activating proteins (GAPs). The formation of the active GTP.ras form is stimulated by activated tyrosine kinases (16, 17). The formation of GDP.ras is stimulated by the GAP of ras (rasGAP) and rasGAP has been demonstrated to be associated with and phosphorylated by the EGF receptor and other tyrosine kinases (18, 19). In addition, it has been reported that the modulation of a guanine nucleotide exchange factor might be a target for the EGF-induced activation of ras (20, 21).

Ras proteins have been demonstrated to act as key regulators in mitogenesis because these proteins act as intermediates between receptor tyrosine kinases at the plasma membrane and an intracellular cascade of serine/threonine kinases (22, 23). Thus, for example, activated ras will cause activation of Raf-1 (24). Recently, however, it was shown that activation of Raf may occur independently of ras. In this case the translocation of Raf to the plasma membrane is sufficient for the activation. It was suggested that Ras functions as a membrane anchor for Raf, rather than as an activator (25). Raf-1 is a serine/threonine kinase that phosphorylates and activates another serine/threonine kinase MEK (MAP/ERK kinase), which in its turn stimulates MAP kinase (26). MAP kinase acts on numerous effector molecules, including serine/threonine kinases and transcription factors, which altogether determine the cellular response (26, 27).

## EFFECT OF MICROGRAVITY ON EGF-INDUCED GENE TRANSCRIPTION

As has been outlined above, the EGF-induced signal transduction cascade in A431 cells is considered an attractive model to study the possible effects of microgravity on a molecular level. Because the EGF-induced signal transduction cascades comprise a large number of different components, including lipids, proteins, and ions as described briefly above, it was decided to study the effect of microgravity on EGF-induced expression of the oncogenes *c-fos* and *c-jun*. The expression of these genes marks the end-point in the EGF-induced signal transduction cascade. The possible effects of altered gravity conditions were initially studied by determining the EGF-induced rise in c-fos mRNA levels under simulated hypogravity and hypergravity conditions. *c-Fos* expression was determined by the sensitive so-called RNAse protection assay as described in detail previously (28). It was demonstrated that the EGF-induced *c-fos* expression was slightly depressed under simulated hypogravity conditions as measured in a fast-rotating clinostat, whereas the expression was increased under hypergravity conditions as measured in the centrifuge. Subsequently these experiments were repeated in a sounding rocket, which allowed a microgravity ($G<10^{-4}$) period of approximately 7 min. The sounding rockets used for our studies were launched from the Esrange base in Kiruna, northern Sweden. Users of this sounding rocket facility were provided with ground laboratories containing standard laboratory equipment. The payload of the rocket constituted the experimental modules that performed the space experiments fully automatically. The same experiments were performed simultaneously on the ground, providing the 1-G ground control. Microgravity was reached under free-fall conditions approximately 1 min after launch of the rocket. Immediately after reaching microgravity, at the onset of the experiments, samples were taken to determine whether the high G values occurring during launch influenced the parameters of interest. After the rocket reached its highest point, it started to descend back to earth. The experiments were usually ended before the rocket reached the earth atmosphere. As stated above, all parameters were determined simultaneously under microgravity conditions and on the ground. The first studies demonstrated clearly that EGF-induced *c-fos* and *c-jun* expression in microgravity was reduced. On the other hand, the expression of the constitutively expressed $\beta_2$-microglobulin gene, which is not modulated by EGF, remains unaffected (29). Quantitative analysis shows that *c-fos* and *c-jun* expression in microgravity is reduced by approximately 50%, as compared to the normal gravity control samples (29).

## EFFECT OF MICROGRAVITY ON EGF BINDING AND RECEPTOR ACTIVATION

One likely explanation for the observed inhibition of gene expression may well be that microgravity leads to decreased binding of EGF to the cell surface-located EGF receptor. Therefore EGF binding was studied using $^{125}$I-EGF during two sounding rocket

flights. Under normal conditions, EGF binding to mammalian cells has been analyzed in detail by use of Scatchard analysis (30–32). However, microgravity did not influence the binding properties of the receptors (33). These observations were in agreement with other investigations demonstrating that microgravity conditions did not alter receptor ligand binding (34, 35). Therefore we conclude that microgravity inhibits EGF-induced signal transduction downstream of the initial activation, that is, the binding of EGF to its receptor.

It has been well established that the EGF receptor tyrosine kinase is activated by EGF binding because the receptors dimerize (36–38). Usually the EGF receptor dimerization is determined by chemically cross-linking the dimers, followed by gel electrophoresis (38). However, this approach is not very well suited for application in a sounding rocket, due to the requirements of relatively large numbers of cells. Therefore, we used an alternative method to this end, the so-called label fracture method (39).

The label fracture method, as developed by Pinto da Silva and Kan (40) is a method particularly suited to visualize the lateral distribution of plasma membrane-located proteins, provided that the proteins can be labeled by antibodies in combination with colloidal gold particles at their external domain. This method has been used to establish the effect of EGF on the lateral distribution of the EGF receptor in A431 cells (39) and it was demonstrated that EGF caused a significant clustering of the receptors within 5 min of addition. More recently, this method has been used to quantify the lateral EGF receptor distribution in relation to the receptor density (41). We have applied this method to establish the effect of microgravity on the EGF-induced receptor clustering as an indication for EGF receptor activation. Therefore A431 cells were brought into microgravity for 5 min by use of sounding rockets during which period the cells were treated with EGF, as described above. The cells were then fixed and after recovery the samples were treated for label fracture analysis according to standard protocols (41). Comparison of the receptor distributions of control and EGF-treated cells under normal gravity and microgravity conditions demonstrated clearly that the receptor distributions in the presence and absence of EGF were not influenced by gravity (41). These observations clearly demonstrate that neither EGF binding to the receptor nor EGF-induced receptor redistribution, and hence receptor activation, are influenced by microgravity conditions. Thus microgravity influences EGF-induced signal transduction downstream of EGF binding and EGF receptor redistribution, but upstream of early gene expression.

## MICROGRAVITY-SENSITIVE SIGNAL TRANSDUCTION PATHWAYS

The observations described so far indicate that specific signaling pathways are affected by microgravity. This could mean that microgravity affects a limited number of gravity-sensitive cellular targets. The expression of *c-fos* and *c-jun* genes is rapidly induced by growth factors but can also be triggered by a variety of agents that mimic the partial activation of signal transduction pathways but that bypass the EGF receptor. Examples of such agents are the phorbol ester phorbol 12-myristate 13-acetate (PMA), which activates PKC, the calcium ionophore A23187, which mimics the EGF-induced increase in the intracellular free calcium concentration, and forskolin, which raises the intracellular cyclic AMP concentration.

During two sounding rocket flights, A431 cells were activated with EGF, PMA, A23187, and forskolin, respectively. Quantitative analysis of the *c-fos* and *c-jun* expression demonstrated that both EGF- and PMA-induced expressions were strongly inhibited under microgravity conditions, whereas the forskolin- and A23187-induced gene expressions were not affected. Because EGF and PMA are known to be activators of PKC, these data indicated that the cellular response to PKC-mediated signal transduction is a molecular target for microgravity (42). These indications are supported by the observations that the synthesis of interleukin-2 and interleukin-1, respectively, by a human leukemic lymphocyte line and a human leukemic monocyte line after phorbol ester stimulation were reduced in cultures under microgravity conditions (43). Others reported, however, an enhancing effect of microgravity on PKC activity (44).

## EFFECT OF MICROGRAVITY ON CELL MORPHOLOGY

The data described above indicate an important role of PKC in the gravity sensitivity of mammalian cells and these initial studies have been supported by other studies as well (reviewed by Claassen and Spooner, 45). However, initial experiments performed in the clinostat and in sounding rockets indicate that, in addition to the PKC, the cytoskeleton may be involved in the gravity sensitivity. This suggestion is based on the observations that EGF-induced cell rounding was enhanced under simulated microgravity conditions in the clinostat, whereas this process was inhibited under hypergravity conditions in the centrifuge (46). Cell rounding is an actin-mediated process (47) and therefore these observations suggest that the actin microfilament system may be sensitive to altered gravity conditions.

## ROLE OF THE CYTOSKELETON IN SIGNAL TRANSDUCTION

An important aspect of the signal transduction network concerns the efficiency with which the various components are able to influence each other. For example, EGF-induced *c-fos* expression has been determined already within 6 min after addition of EGF to the cells (42), demonstrating that EGF-induced signal transduction is very efficient. In this respect it is tempting to suggest that the cells contain a system that is involved in the organization of a proper localization of the various components involved in signal transduction. The cell system that seems to be suited for such a task is the cytoskeleton. Binding of the signal transduction components to the cytoskeleton would allow a very precise defined localization inside the cell. In combination with the plasma membrane, which may function as a target for cytoplasmic signal transduction proteins as well, the defined localization would probably facilitate various interactions between proteins to allow the efficient signal transduction.

With respect to the feasibility of the cytoskeleton to mediate signal transduction inside the cell, the actin microfilament system is the most attractive component of the cytoskeleton. Actin is a highly conserved protein, consisting of a single chain of 375 amino acids with a molecular mass of 42 kDa. The polymerization of actin *in vivo* is controlled by a diverse family of proteins, designated the actin binding proteins. These include G-actin binding proteins, capping proteins, severing proteins, cross-linking proteins, and membrane attachment proteins (48). Of particular interest with respect to growth factor-induced signal transduction, is the association of the actin microfilament system with the plasma membrane through specific actin binding proteins. These interactions are present in three different systems, i.e. adherens junctions, focal adhesions, and membrane skeleton. Each of these systems represent a different signal transduction machinery with its own specific function (for review see Boonstra et al., 9).

In addition, another important association of the actin microfilament system with signal transduction systems has been described. This includes the involvement of small G-proteins, especially of the Rho family, in the regulation of actin metabolism. The Rho family of G-proteins consists of the Rac, Rho, and Cdc42 subfamilies (49–52). The Rac subfamily includes Rac1 and Rac2, Rac1 being involved in the regulation of lamellipodia and membrane ruffling (53). The Rho subfamily consists of RhoA, RhoB, and RhoC. RhoA participates in the formation of actin stress fibers, as well as in mediating redistribution of cytoskeletal components (49, 53). The Cdc42 subfamily consists of Cdc42Hs, G25K, and RhoG (50) and participates in the formation of filopodia (54). Addition of growth factors to cells usually results in the formation of membrane ruffles and the disappearance of stress fibers, ultimately resulting in cell rounding (55, 56). These observations clearly demonstrate the intimate relationship between growth factor-induced signal transduction and regulation of the cytoskeleton.

As discussed above, it is tempting to suggest that the actin microfilament system acts as a matrix for growth factor-induced signal transduction. This would imply that the components involved in these signal transduction cascades are in one way or another associated with the microfilament system.

As early as the 1980s, evidence accumulated that growth factor receptors, among them the EGF receptor, were associated with the cytoskeleton. Thus it was shown by biochemical and ultrastructural methods that 20–25% of the EGF receptor population of A431 cells was insoluble to Triton X-100 (32, 57, 58) and thus associated with the cytoskeleton. In contrast, a mutated EGF receptor in which the cytoplasmic domain of the receptor was deleted, did not bind to the cytoskeleton (59).

Subsequently, the association between the EGF receptor and the cytoskeleton was further analyzed in detail. Selective extraction of A431 cells into the three major cytoskeletal fractions, i.e. the microtubules, the microfilaments, and the intermediate filaments, and EGF binding studies on these fractions revealed that the EGF receptors were associated with the actin microfilament system (60). Because a wide variety of actin binding proteins is known to represent substrates of the EGF receptor, it was not clear whether the receptor binds directly or indirectly to actin. Therefore, both the EGF receptor and actin were purified to homogeneity and subsequent cosedimentation assays demonstrated unequivocally that the receptor itself is an actin binding protein with no other proteins involved (61).

In addition to the EGF receptor, various components involved in EGF-induced signal transduction also appeared to be associated with the cytoskeleton. Thus it was shown that cytoskeletons isolated from A431 cells contained high activities of PI kinase, PIP kinase, PLC, and DG kinase (62). As for the EGF receptor, these proteins were found to be associated with the actin microfilament system. It is interesting to note that the cytoskeleton-associated kinase activities were significantly increased on treatment of intact cells with EGF (62). Recently, it was demonstrated that EGF caused an activation and translocation of c-Src to the cytoskeleton, depending on the actin binding domain of the EGF receptor (63).

These findings indeed suggest that the actin microfilament system might function as a matrix to align the signal transduction components. In this

respect, it is of interest to mention that the EGF-induced actin polymerization is related to membrane ruffling (55, 56), and that the early signal transduction events occur predominantly in these newly formed membrane ruffles. Thus, in an extensive study on the localization of F-actin, EGF receptor, PLCγ1, and tyrosine-phosphorylated proteins in A431 cells treated with EGF for 2 and 5 min, respectively, it was shown that immediately after the formation of the membrane ruffles, after addition of EGF to the cells, a strong co-localization was observed between F-actin on the one hand and EGF receptors, PLCγ1, and tyrosine-phosphorylated proteins on the other. This co-localization was most apparent after 2 min of EGF addition. In particular, the appearance of tyrosine-phosphorylated proteins after addition of EGF was confined to the membrane ruffles, and these tyrosine-phosphorylated proteins appeared throughout the cells only after longer incubation periods in the presence of EGF (64). These findings indicate that the membrane ruffles represent the signal transduction organelles of the cells after addition of EGF, and actin plays an essential role in the establishment of these membrane ruffles.

In view of these findings, remodeling of the actin microfilament system may have implications for the subcellular localization and activity of molecules involved in signal transduction (65). This idea is supported by observations that inhibitors of actin polymerization modulate signal transduction (64–68). Recently, we have demonstrated that dihydro-cytochalasin B (CB), an inhibitor of actin polymerization (69), inhibits EGF-induced actin polymerization (70). However, co-treatment of the cells with EGF and CB resulted in enhanced EGF receptor tyrosine kinase activity as well as in super-induction of the *c-fos* gene. These data suggest that EGF-induced actin polymerization may be important for negative feedback regulation of signal transduction initiated by the EGF receptor. The phosphorylation of $Thr^{654}$ residue of the EGF receptor by PKC is one of the best-characterized negative feedback control mechanisms for signal transduction by the EGF receptor. A synthetic peptide corresponding to the regions flanking $Thr^{654}$ of the EGF receptor was used to analyze EGF-stimulated PKC activity by phosphorylation. Co-treatment of cells with CB and EGF resulted in a complete loss of EGF-induced phosphorylation of the peptide (70). These observations strongly suggest that polymerized actin is obligatory for negative feedback regulation of the EGF receptor tyrosine kinase through the PKC pathway.

## EFFECT OF MICROGRAVITY ON THE ACTIN MICROFILAMENT SYSTEM

As described above, the observed effects of microgravity on cell morphology suggested that the actin microfilament system was sensitive to gravity conditions. Therefore, some preliminary experiments were performed during two sounding rocket flights in which the cells were treated or not with EGF according to standard protocols. After fixation and recovery of the cells, the actin filament system was labeled specifically with fluorescent-labeled phalloidin, a toxin known for its high affinity for filamentous actin. Analysis of the cells by fluorescence microscopy and by fluorometry according standard procedures (60) revealed clearly that the F-actin content of the cells was increased under microgravity conditions (33). Addition of EGF caused a further increase in the amount of F-actin, but no difference in activation was observed under normal and microgravity conditions. These preliminary observations, however, clearly indicate that the actin microfilament system may represent a gravity-sensitive cell component.

The question that arises is whether the microgravity-sensitive PKC pathway is related to the microgravity-sensitive component of the cytoskeleton. Some observations suggest that this relationship does exist. It has been known for a long time that PKC is involved in down-modulation of the EGF receptor (71) and that the actin microfilament system is required for PKC translocation to the plasma membrane (72). We have demonstrated that de-polymerization of the actin microfilaments resulted in a hyperactivation of the EGF receptor tyrosine kinase activity, probably due to the lack of the PKC-mediated feedback (70). Under microgravity conditions we have observed an increase in F-actin content of the cells and this increase in F-actin content may cause an increased association of PKC to the membrane. It is of interest in this context, that an increase in PDBu, a phorbol ester that is used to activate PKC, binding was observed in human leukocytes under microgravity conditions (35), as well as an increase in other PKC-mediated processes (44). Thus, the increased level of membrane-associated PKC may cause an increase in down-regulation of EGF receptor tyrosine kinase activity as has been observed under microgravity conditions. This hypothesis explains the observations that EGF-induced signal transduction is decreased under microgravity conditions at a level downstream of receptor organization. However, in this concept the reduction of *c-fos* expression under microgravity conditions in the presence of phorbol esters is rather puzzling. Higher PDBu binding, an increased down-modulation of EGF receptor activity, and an increased binding of PKC to the membrane skeleton suggests an increase rather than a decrease in PKC activity. It should be realized, however, that the signal transduction cascade from PKC leading to *c-fos* expression is not fully understood. It could well be that specific isoforms of

PKC are involved in this signal transduction pathway and that these isoforms are different from the ones leading to EGF receptor down-modulation. And, as suggested recently, microgravity may influence the intracellular distribution of the various isoforms of PKC differently (35).

These findings provide, however, no clue as to the nature of the gravity-sensitive component of the cells. Most observations seem to indicate that the actin microfilament system represents the gravity-sensitive component because the actin microfilament system is modulated as soon as cells are exposed to microgravity conditions (see above). The actin microfilament system is, however, a rather complicated system in which many cellular proteins are involved, such as actin-binding proteins, capping proteins, severing proteins, cross-linking proteins, and many others (53). In addition, a wide variety of cell signaling proteins and molecules are associated with the actin microfilament system or have a role in actin microfilament dynamics, such as the proteins of the Rho family (49, 50, 54), the molecules and proteins involved in phosphatidylinositol metabolism (73–75), and many others (60–64). This complexity makes it very difficult to identify the real gravity-sensitive component of the cells. However, in addition to a chemical role in cellular functioning, the actin microfilament system also has a physical role and it is therefore tempting to suggest that a physical force, such as gravity, will have a significant effect on the actin microfilament system. Recent physical studies of actin networks *in vitro* have influenced our understanding of cytoplasmic sol-gel transitions (76, 77). The degree of entanglement, filament stiffness, and the capacity of microgel formation within actin gels may be governed by specifically actin-binding proteins. Therefore physical stress/response coupling may be an important parameter during cytoskeleton-mediated signal transduction, especially under microgravity conditions. At this time it is still unclear whether the possible effects of gravity on the microfilament system are due to effects on actin polymerization/depolymerization directly or to effects on the actin binding proteins or the Rho family members involved in the actin metabolism. Involvement of the latter group of proteins seems unlikely, however, because thus far no indications to this end have been obtained. In this respect it seems attractive to study actin microfilament systems *in vitro* under microgravity conditions to obtain a better understanding of the gravity effects on cells *in vivo.* FJ

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