Role of ß2-chimaerin in the behaviour of murine mammary carcinoma cells in response to extracellular matrix components

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Abstract. Chimaerins are high affinity receptors for phorbol esters and diacylglycerol (DAG), unrelated to the protein kinase C isozymes. These receptors have deep implications in tumour biology since they regulate the activity of Rac1, a small GTP-binding protein of the Ras superfamily. It has been demonstrated that chimaerins have GTPase activating protein (GAP) activity, leading to the acceleration of GTP hydrolysis from Rac1 and therefore facilitating the transition to its inactive state. Rac regulates various cellular events, including gene transcription, cell cycle, adhesion and migration. It has also been described that Rac is implicated in the intracellular response to the binding of specific extracellular matrix proteins to integrin receptors. In this work, we analysed cell morphology, actin cytoskeleton reorganisation and metalloprotease (MMP) secretion in response to matrix proteins in mouse mammary carcinoma cells transfected with the ß2-chimaerin GAP domain. Overexpression of ß2-chimaerin induced important cytoskeletal rearrangements in response to matrix stimuli. Transfectant cells also showed activation of MMP-9 activity after stimulation with collagen IV and epidermal growth factor. The restitution of normal Rac levels by ß2-chimaerin activity induced an increase in the sensitivity of tumour cells to extracellular factors, suggesting a regression of the malignant phenotype.

Introduction

It is well established that Rho-family proteins play important regulatory roles in oncogenic transformation and in the metastatic cascade. The family has several members, including Rho A, B, C, D and E, Rac 1, 2 and 3, and Cdc42. Rac GTPases bind guanine nucleotides and cycle between an inactive state (bound to GDP) and an active state (bound to GTP). They also possess intrinsic GTPase activity (1-3). The switch between active and inactive states of Rac is highly regulated by guanine exchange factors (GEFs) that activate the GTPases by increasing the release rate of the bound nucleotide, GTPase-activating proteins (GAPs) that stimulate the endogenous activity of GTPase, and GDP-dissociation inhibitors (GDIs) that prevent the replacement of GDP for GTP (4). Early studies determined that Rho/Rac GTPases regulate cell morphology and actin reorganisation. Rho is activated by extracellular ligands such as lisophosphatidic acid and regulates the formation of stress fibers. Rac is activated by different growth factors such as platelet-derived growth factor, epidermal growth factor (EGF) or insulin, leading to the formation of lamellipodia and membrane ruffles (5).

Studies from several laboratories have established the existence of novel, 'non kinase' receptors for the phorbol esters, including the chimaerins (6). Like protein kinase C (PKC) isozymes, these proteins bind with high affinity to phorbol esters and diacylglycerol. Chimaerins may have important implications in carcinogenesis because they regulate the activity of Rac1 (7). In fact, it has been shown that chimaerins have Rac-GAP activity, leading to acceleration of GTP hydrolysis from Rac1 and its subsequent inactivation (8). Several chimaerin isoforms have been isolated to date (α1- or n-, α2-, ß1- and ß2-chimaerin), which in all cases have a carboxy-terminal GAP domain responsible for Rac inactivation (9).

In a previous report, we established that ß2-chimaerin is a key regulator of invasion and metastasis. Using F3II murine mammary carcinoma cells, we showed that ectopic expression of the ß2-chimaerin GAP domain (ß-GAP) causes alterations in actin polymerisation in response to EGF, decreased growth rate and migratory capacity, as well as a marked reduction in tumour invasive capacity and metastatic dissemination (10). Thus, ß2-chimaerin inhibits key steps of the metastatic cascade.

In the present work, we analysed the *in vitro* behaviour of mammary carcinoma cells transfected with ß-GAP. We focused on the reorganisation of the actin cytoskeleton and the secretion of tumour proteases in response to extracellular matrix (ECM) proteins.

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Material and methods

Tumour cell lines and culture conditions. The sarcomatoid mammary carcinoma cell line F3II is a highly-invasive and metastatic variant, established from a clone of a spontaneous,

hormone-independent BALB/c mouse mammary tumour (11). Generation of the cell line GB1, which expresses the catalytic domain of ß2-chimaerin (ß-GAP), was reported previously (10). Briefly, a mammalian expression vector for ß-GAP (pCR3ε) was transfected into semiconfluent F3II mammary carcinoma cells using lipofectamine (Life Technologies) according to the manufacturer's instructions. After 30 days of culture, colonies resistant to G418 were selected by limiting dilution. G418-resistant clones were then expanded, and the expression of ß-GAP domain was determined by quantitative RT-PCR (10).

Cells were cultured in MEM 41500 (Life Technologies), supplemented with heat-inactivated 10% FBS, 2 mM glutamine, and 80 µg/ml gentamicin at 37˚C in a 5% CO_2 atmosphere. GB1 cells were maintained in 200 µg/ml geneticin. Stock cell cultures were routinely subcultured twice a week by trypsinisation, using standard procedures. Quantification of cell number was made by hemocytometer counting. In all cases, viability was >90%, as assayed by trypan blue exclusion technique.

Rac1 activity assay and Western blotting. Cells were plated at a density of $5x10^5$ cells/well in 6-well tissue culture plates and maintained in MEM supplemented with 10% FBS for 24 h. After 24 h in serum-free medium cells were stimulated with 100 ng/ml EGF (Gibco, BRL) for 15 min, or 10 µg/ml fibronectin (Gibco, BRL) or collagen IV (Gibco, BRL) for 4 h. Monolayers were extensively washed with phosphate-buffered saline (PBS) and lysed in GPLB-150 buffer (20 mM Tris pH 7.4, 150 nM NaCl, 5 mM $MgCl_2$, 0,5% NP40, 10% glycerol) supplemented with a protease inhibitor cocktail (Sigma Chemical Co.). Lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4˚C. An aliquot was removed for determination of total Rac1. Rac-GTP levels were determined with a PBD 'pull-down' assay, as described before (8). Briefly, lysates were incubated for 1 h at 4˚C with glutathione S-transferase-PAK-binding domain (GST-PBD) fusion protein coupled to Glutathione-Sepharose 4B beads (Amersham Biosciences). Bound complexes were washed three times in GPLB-150 lysis buffer, and the beads were boiled for 5 min in 4X sample buffer and resolved by 12% SDS-PAGE. Samples were then transferred to PVDF membranes (Hybond P, Amersham Biosciences), and analysed by Western blot using a monoclonal Rac1 antibody (Sigma Chemical Co.). Bands were detected by enhanced chemo-luminescence (ECL).

Preparation of ECM substrates. Culture plates were coated with fibronectin or collagen IV (Gibco, BRL). ECM proteins were added to culture plates at a concentration of 10 µg/ml in PBS and incubated for 1 h at 37˚C. The solution was aspirated, and plates were exposed to UV radiation for 20 min. The wells were extensively washed with PBS and used the same day.

Preparation of conditioned media and zymography. Secreted tumour derived matrix metalloprotease-9 (MMP-9) activity was analysed in conditioned media. Semiconfluent tumour cells were trypsinised and seeded onto uncoated plates or substrate-coated 6-well plates at a concentration of $3.5x10^5$ cells/well.

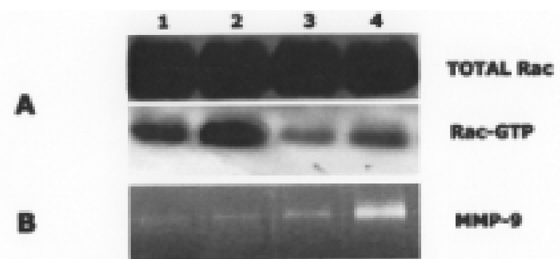

Figure 1. (A) Pull-down Rac activity assay. (B) Zymographyc analysis of conditioned media. Control (lines 1 and 2) and ß-GAP (lines 3 and 4) cells. Control and transfected cells were stimulated with 100 ng/ml EGF (lines 2 and 4 respectively).

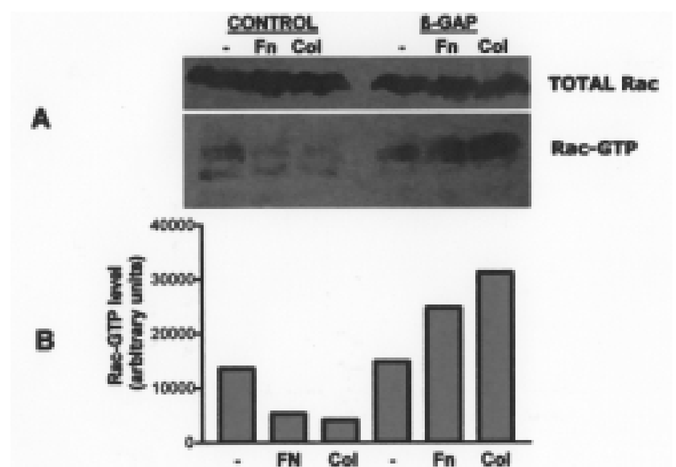

Figure 2. (A) Rac activity assay were performed from control (lines 1-3) and ß-GAP (lines 4-6) cells. Starved cells were stimulated during 4 h with 10 ng/ml of Fibronectin (lines 2 and 5) or Collagen IV (lines 3 and 6), Lines 1 and 4 show non-stimulated cells. (B) Densitometric analysis of the pull-down Rac activity assay.

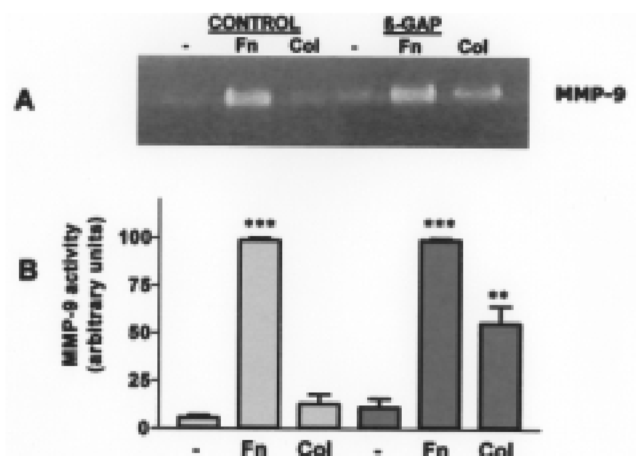

Figure 3. (A) Conditioned media obtained from semiconfluent monolayers incubated with 10 µg/ml fibronectin or collagen IV for 24 h were subjected to zymographic analysis. Lines 1-3 correspond to control cells and lines 4-6 to ß-GAP cells. Whereas 2 and 5 show fibronectin stimulation, 3 and 6 show collagen stimulation, while 1 and 4 show non-stimulated cells. (B) Band intensity was quantified by densitometric analysis and plotted in the y axis as densitometric arbitrary units. While, 100 were assigned the higher intensity value (line 2), the other values were standardised in relation to this one. Three independent experiments were performed and subject to statistical analysis (one-way ANOVA contrasted with Tukey-Kramer Multiple Comparisons Test). Fibronectin stimulation showed extremely significant differences, $p<0.001$, (***) respect to non-stimulated controls in both cell lines. Collagen IV stimulation showed very significant difference, $p<0.01$, (**) in ß-GAP cells respect to non stimulated control.

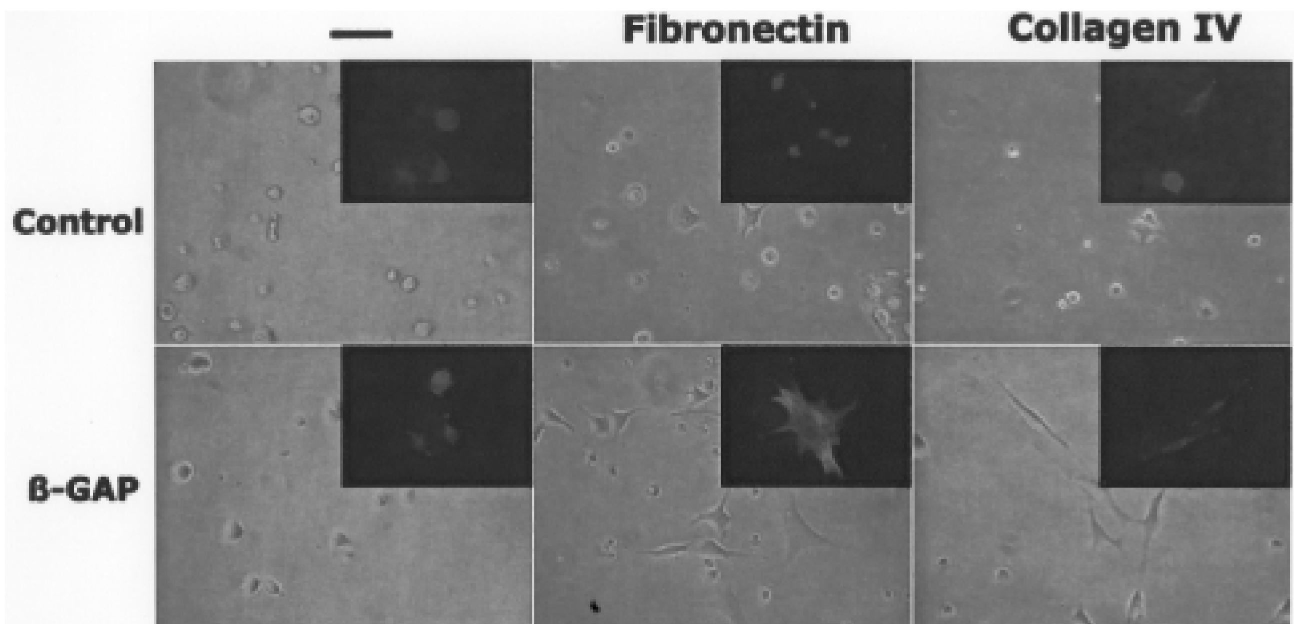

Figure 4. Control (1-3) and ßGAP (4-6) cells were seeded on fibronectin (2 and 5), or collagen IV (3 and 6) or on non-coated surface (1 and 4). After 4 h, cells were photographed with a contrast phase microscope; insets show fluorescence images of actin staining with rhodamine labeled-phaloidine.

Cells were cultured for 24 h in MEM supplemented with 10% FBS. Monolayers were extensively washed with PBS to eliminate serum traces. Serum-free MEM plus fibronectin (1 ml) or collagen IV (10 µg/ml) or 100 ng/ml of EGF were added and incubated for 24 h. Conditioned media were individually harvested and concentrated approximately 4X by vacuum centrifugation (Speed Vac AES 1010, Savant).

Conditioned media were analysed for gelatin degrading activity by electrophoresis on SDS-polyacrylamide gels containing 0.1% gelatin (Sigma Chemical Co.). Gels were washed in renaturalisation buffer: 2% Triton X-100 and incubated for 48 h at 37˚C in incubation buffer: 0.25 M Tris-HCl pH 7.4, 1 M NaCl, 25 mM $CaCl_2$. White zones of lysis indicating gelatin degradative activity were revealed by staining with Coomassie Brilliant Blue. The intensity of the bands was quantified by densitometric analysis (Kodak Digital Science 1D).

Cell morphology and actin staining. Cells were seeded at a density of $1.5x10^4$ cells on glass coverslips pre-coated with either fibronectin or collagen IV. Control cells were plated on untreated glass coverslips. Cells were incubated for 4 h in serum-free medium. To document cellular morphology, adherent cells were directly photographed in a phase contrast microscope (Olympus).

For actin staining, cells were washed with PBS and fixed in 3.7% paraformaldehyde 0.5% NP40 in PBS. Fixed cells were permeabilised with 0.5% NP40 in PBS, blocked with 5% BSA, and then incubated for 1 h with rhodamine-labeled phalloidin (Molecular Probes). Images were recorded in a fluorescence microscope (Nikon).

Results

EGF modulates MMP-9 secretion in ß-GAP transfectant cells. We investigated the effect of EGF, a well-known Rac activator (10), on Rac activation levels in either control and ß-GAP transfectant F3II mammary carcinoma cells. Expression of ß-GAP produced a significant reduction in the basal levels of active Rac (Fig. 1A). After EGF stimuli both transfectants and control cells showed an increase in Rac-GTP levels respect to their respective non-stimulated controls.

In order to determine the role of ß-GAP in the regulation of MMP-9 secretion, we studied the effect of EGF on its secretion. The zymographyc analysis of conditioned media revealed that basal secretion levels of MMP-9 were similar in both control and ß-GAP expressing cells. However, upon EGF stimulation ß-GAP transfectants showed a marked increase in MMP-9 secretion (Fig. 1B). MMP-9 secretion levels were not altered in control F3II cells after EGF treatment.

Collagen IV but not fibronectin enhances MMP-9 secretion in ß-GAP transfectants cells. ECM proteins, like fibronectin and collagen IV, are important ligands for integrin-dependent intracellular signaling. First, we evaluated the modulation of Rac activity by ECM proteins in ß-GAP transfectants and control cells. Cell monolayers were treated with fibronectin or collagen IV, and Rac-GTP levels were evaluated using the 'pull down' assay. Interestingly, while neither ECM protein significantly affected Rac-GTP levels in control cells, they markedly enhance Rac-GTP levels in ß-GAP transfectants. The effect was significantly higher for collagen IV (Fig. 2).

In the next series of experiments, we investigated the effect of soluble ECM proteins on MMP-9 secretion. We observed that ß-GAP cells showed an important increase in MMP-9 secretion in response to the addition of collagen IV (Fig. 3). On the other hand, control F3II cells showed no differences in MMP-9 secretion under the same experimental conditions. Treatment with soluble fibronectin, however, induced a significant increase in MMP-9 secretion levels in both cells lines (Fig. 3). These data support the concept that soluble fibronectin induces MMP-9 secretion by a Rac-

independent mechanism, while soluble collagen IV modulates MMP-9 mediated proteolysis in a Rac-dependent manner in mammary cancer cells. We have also evaluated the effect of fibronectin and collagen IV coatings on MMP-9 secretion in monolayers of both cell lines. Under these conditions, no relevant effects were observed (data not shown).

Collagen IV and fibronectin induce actin cytoskeleton reorganisation in ß-GAP cells. Rac plays an essential role in actin cytoskeleton reorganisation and cell morphology. To evaluate whether ß-GAP expression affects cell morphology and actin cytoskeleton reorganisation in response to ECM proteins, control and transfectants cells were seeded for 4 h on either fibronectin or collagen IV coatings in serum-free conditions. Changes in actin polymerisation were examined using rhodamine-labeled phalloidin staining. ß-GAP cells seeded on a fibronectin coating showed a marked increase in cell spreading, as well as in intracellular fibrilar actin (Fig. 4). Collagen IV coatings induced a spindle morphology in ß-GAP cells, and lower levels of cytoplasmatic actin polymerisation than fibronectin (Fig. 4). On the other hand, F3II control cells showed a rounded morphology on both ECM substrates and no significant morphological changes or induction of actin polymerisation were observed.

Discussion

Interactions between luminal epithelial cells and the surrounding microenvironment govern the normal development and function of the mammary gland. Alterations of these interactions can induce abnormal intracellular signaling pathways that affect the development and progression of breast tumours (12). In normal cells, Rac-dependent signaling pathways regulate cell morphology through the reorganisation of actin cytoskeleton, gene expression, cell proliferation and survival. Although, these cell functions are very important in tumourigenesis, it is not completely clear how Rac can contribute to the deregulation of these processes. In this regard, no mutations have been detected in Rac genes neither isolated as oncogenes, as has been demonstrated for Ras. Nevertheless, it is known that GEF proteins that activate Rac (Tiam) are oncogenic (1).

Previous studies carried out in our laboratory have demonstrated that in an aggressive mouse mammary carcinoma model, the overexpression of ß2-chimaerin produce the reversion of different steps involved in tumour invasion and metastasis, stressing the impact that Rac-mediated pathways have in tumour biology (10). Based on our previous findings and taking into account that the behaviour of mammary epithelium is highly conditioned by ECM, we decided to study key processes related with normal and malignant breast development, such as reorganisation of the actin cytoskeleton and the secretion of tumour proteases in response to ECM proteins.

Rho-GTPases may be activated either by growth factors or ECM (13). The interaction of ECM proteins with integrins triggers a signaling cascade that produce, among other effects, Rac activation (14-16). Anchorage-dependent proliferation in non-transformed cells would be regulated by a substrate-dependent mechanism, which will include signaling cascades where Rac would mediate the pathway that communicates the extracellular environment with the nuclei, in order to produce a specific cell response.

When we analysed the effect of ECM coatings on actin cytoskeleton in F3II tumour cells, we observed that collagen IV and fibronectin induced a dramatic morphological response in ß2-chimaerin transfectants in the absence of serum, suggesting a Rac-related mechanism. Interestingly, similar results were obtained in fibroblasts seeded on a fibronectin matrix (17).

Anchorage-dependent cell survival is determined by external signals, mainly from serum soluble factors and cell interactions with ECM. Our results are coincidental with other works (18,19), which prove that fibronectin induces survival signals or entry into the cell cycle in normal fibroblasts. On the other hand, transformed cells exhibited certain differential characteristics of growth independence. Furthermore, it has been shown that Rac promotes anchorage-independent cell survival during malignant transformation (19). Based on our results, we can speculate that over-expression of ß2-chimaerin GAP domain induces actin cytoskeleton reorganisation in response to fibronectin. It is important to remark that control tumour cells, in which ß2-chimaerin expression levels were much lower, were not able to organise a morphological response in the presence of ECM.

Loss of integrity of the basement membrane is a histological marker of the transition of a tumour towards an invasive phenotype. Tumour cells produce, secrete and activate different types of proteases that cleave specifically ECM molecules. Thus, we analysed the proteolytic profile of the tumour cell line transfected with chimaerin. Growth factors and ECM molecules tightly control MMP expression. In turn, owing to their proteolytic activity, MMPs regulate ECM assembly, edit excess ECM components, remodel the microenvironment and release bioactive ECM fragments and growth factors, all of which are implicated in morphogenesis of epithelial tissues (20). In fact, EGF is able to activate transcriptional factors that regulate the activity of gene promoters of proteases, through pathways that include Rac and JNK (21). Indeed, differentiation of the mammary gland during lactation involves changes in the predominant cell type from non-epithelial to epithelial, in the expression of different combinations of ECM components and integrin receptors, and in the enzymes responsible in ECM remodeling, the MMPs, which also cooperate with hormonal stimuli to induce morphological and functional changes required at various developmental stages (12).

Since F3II cells are responsive to EGF, we observed that overexpression of ß2-chimaerin GAP domain allows to the transfected cell line to respond with an increase in MMP-9 secretion after EGF stimulation. This behaviour is not observed in the control tumour cell line. This selective response of ß-GAP cells could be explained as a reversion of a 'saturation effect' provoked by high basal activation levels of Rac in wild-type F3II cells.

When F3II cells are seeded on ECM coatings, no modulation in the secretion profile of MMP-9 is observed. However, when the factors are incorporated in a soluble way to the culture media, an increment is observed only in response to collagen IV in tumour cells that overexpress ß2-

chimaerin GAP domain. Interestingly, fibronectin induces an increase of MMP-9 in both control and transfected cells, suggesting a Rac-independent regulation. Therefore, cell polarity seems to play a role in the modulation of protease secretion in response to ECM factors.

In an elegant work, Schedin *et al* (22) demonstrated a role for fibronectin fragments in suppressing mammary gland development through induction of MMP activity, which contributes to cell death, loss of alveolar-like development and promotion of ductal-like development. Further evidence that fibronectin has a role in suppressing mammary epithelial cell growth *in vivo* is suggested by correlations between fibronectin levels and tumourigenicity. Loss of fibronectin protein is associated with mammary tumour progression and reversion of transformed phenotype has been associated with an increase in fibronectin expression (22).

Inversely to the canonical analysis, the fact that a tumour cell line transfected with ß2-chimaerin show higher levels of proteases when exposed to ECM components may be considered as a favorable fact, since it reflects the capacity of response that is lacking in aggressive, control tumour cells. To understand the lack of response of the control cell line, we should take into account that tumour cell processes that normally are exquisitely regulated become independent of regulatory signals.

Chimaerin overexpression would diminish the levels of Rac associated with the malignant phenotype. As a consequence, the signaling pathways in which Rac participates would be, to a certain degree, recomposed. Such recomposition of the normal levels of Rac generates a new balance in cell signaling, which would lead to a 'noise reduction' inside transformed cells. Thus, malignant cells would be in a condition to receive and respond to signals imposed by the microenvironment reverting to, at least in part, the autonomous behaviour that characterises the cancerous tissue.

Chimaerin overexpression produces an increase in the sensibility of the tumour cell line to given external stimuli, opening a possible therapeutic avenue for those tumours in which Rac overexpression is the overriding altered signal.

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