Heregulin promotes expression and subcellular redistribution of ADP-ribosylation factor 3

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Abstract To identify genes whose expression is modulated by heregulin-β1 (HRG), a regulatory polypeptide for mammary epithelial cells, we performed differential display screening of MCF-7 cell mRNA. One cDNA clone upregulated by HRG was identical to human ADP-ribosylation factor 3 (ARF3), a guanine nucleotide-binding protein functioning in vesicular trafficking, phospholipase D activation and intracellular transport. HRG treatment increased expression of ARF3 mRNA and protein. Also, HRG triggered a rapid redistribution of ARF3, first to cell membranes and then to the nuclear compartment, where ARF3 colocalized with acetylated histone H3 in discrete regions. In addition, the ARF3 protein was developmentally regulated in the mammary gland with the highest levels in virgin and post-weaning glands. Together, these findings suggest for the first time that stimulation of ARF3 expression, subcellular redistribution and interaction with acetylated histone H3 may play a role in the action of HRG in mammary epithelial cells.

Key words: ADP-ribosylation factor 3; Heregulin; Breast cancer cell

1. Introduction

Growth factors and their receptors play an important role in the regulation of epithelial cell growth. Heregulin-β1 (HRG), a combinatorial ligand for the human epidermal growth factor receptors (HERs) HER3 and HER4, is a secretory polypeptide that affects growth stimulation, differentiation, invasiveness and motility of breast cancer cells [1–7]. In mammary epithelial cells, HRG predominantly uses HER3/HER2 to exact its biological effects [8]. Additionally, HRG is known to be expressed in the mammary mesenchyme adjacent to lobuloalveolar structures and is maximally expressed during pregnancy [7]. HRG also plays a role in the morphogenesis and ductal migration of mammary epithelial cells [2,5], promotes the responsiveness of these cells to lactogenic hormones in vitro [6] and induces the differentiation of mammary epithelium into secretory lobuloalveoli [7]. These observations suggest that HRG is a regulatory polypeptide having distinct biological effects, such as growth stimulation, differentiation, invasiveness and migration, in mammary epithelial cells.

ADP-ribosylation factors (ARFs) are members of the Ras super-family of 20 kDa guanine nucleotide-binding proteins that were initially discovered by their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A [9]. ARFs regulate exocytic and endocytic vesicular trafficking, phospholipase D activation, endoplasmic reticulum (ER) and Golgi morphology and function, ER–Golgi transport and nuclear membrane fusion [10–13]. Although ARFs follow a very similar protein folding with that of Ras, they distinctly differ in two structural aspects that are absent in Ras: an extra β-strand and an N-terminal α-helix [14]. In mammals, the ARF family consists of six ARFs and about 10 ARF-like proteins. Both ARFs and ARF-like proteins are highly conserved molecules [15,16]. The expression of ARFs has been shown to be tissue-specific and developmentally influenced. For example, ARF3 mRNA increases from 2 to 17 days post-natally in rat, while mRNAs for other family members decline or remain unchanged [13]. Among the ARF family members, ARF-like protein 4 is the only reported member with nuclear/nucleolar localization [17].

Like other GTPases, inactive ARFs exist as a cytosolic GDP-bound form and become activated and often membrane-associated when bound with GTP. Since ARFs possess a low intrinsic GTPase activity, GTPase-activating proteins are required for ARF function [18]. GTP hydrolysis by ARF is a prerequisite for vesicle uncoating [18]. The cyclic nature of activation and de-activation of ARFs is thought to correspond with its interaction and release from intracellular membranes [19]. In spite of the widely believed involvement of ARF3 in secretory pathways, very little is known about its role in the action of growth factors.

Because the diverse functions of HRG are likely dependent on induced changes in the expression of specific cellular gene products, we used the differential display of mRNA to identify HRG-regulated genes in breast cancer cells. Our results suggest that ARF3 expression and its nuclear translocation are positively regulated by HRG.

2. Materials and methods

2.1. Cell cultures and reagents

Human breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium and F12 (1:1) supplemented with 10% fetal calf serum. Antibodies used were against ARF3 (Transduction Laboratories), acetylated histone H3 (UBI) and the HA (MBL). Actin-binding phalloidin, fluorescently conjugated second antibodies and

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Abbreviations: ARF3, ADP-ribosylation factor 3; HER, human epidermal growth factor receptor; HRG, heregulin-β1

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the DNA-intercalating fluorescent dye ToPro3 were from Molecular Probes.

2.2. Cell extracts and immunoprecipitation

For the preparation of cell extracts, cells were washed three times with phosphate-buffered saline (PBS) and lysed in RIPA buffer supplemented with 100 mM NaF, 200 mM $NaVO_5$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin on ice for 15 min. The lysates were then centrifuged in an Eppendorf centrifuge at 4°C for 30 min. Cell lysates containing equal amounts of protein were immunoprecipitated with the desired antibody and analyzed by SDS–PAGE [20–23].

2.3. Differential display analysis

Differential display was performed using MCF-7 with and without HRG treatment using the Delta Differential Display kit and as previously described [21,22].

2.4. Northern blot hybridization

Total cytoplasmic RNA was isolated using the Trizol reagent and 20 μg of RNA analyzed via Northern blot hybridization using a 600 bp cDNA of human ARF3 open reading frame. rRNA (28S and 18S) was used to assess the integrity of the RNA. The blots were routinely reprobed with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for RNA loading and transfer control. In some experiments, cells were treated with 50 μg/ml cycloheximide (a translational inhibitor) or 10 μg/ml actinomycin D (a transcriptional inhibitor) in the presence or absence of HRG treatment (1 nM). Individual bands were quantified by scanning densitometry and graphed as the mean ± standard deviation (S.D.) of three experiments.

2.5. Immunofluorescence and confocal imaging

MCF-7 cells were plated on glass cover slips in six-well culture plates and transfected with an HA-tagged ARF construct using Fugene 6 (Roche). Subconfluent cells were serum-starved for 24 h, then separate wells were treated with HRG (1 nM) for 0, 30 or 60 min or overnight for 16 h and the cells were rinsed in PBS and fixed in 4% phosphate-buffered formaldehyde for 20 min. Following fixation, cells were processed for immunofluorescence staining as previously described [22]. Fluorescent labeling was visualized using a Zeiss LSM 510 microscope and a 40× objective. Quantitation of two channel pixel intensity along a fixed line (Fig. 3A–C) was performed using Zeiss LSM 510 image analysis software.

3. Results and discussion

3.1. Identification of ARF3 as a growth factor-regulated gene

To identify genes whose expression in human breast cancer cells may be modulated by HRG, we prepared total RNA from MCF-7 breast cancer cells treated with or without HRG. Complementary DNAs were synthesized using a reverse transcriptase in the presence of [α-^{32}P]dCTP and subjected to a polymerase chain reaction. A total of 90 reactions were performed using nine 3′-degenerate oligo-dT primers and ten 5′-random primers for each treatment. Analysis of PAGE gels showed bands were of equal intensity. Using these bands as internal controls, we looked for bands having differences in intensity in the HRG-treated lane. This analysis resulted in the identification of a 186 bp band differentially expressed in HRG-treated cells (Fig. 1A). The differentially expressed gene product was then amplified, cloned and sequenced. Five

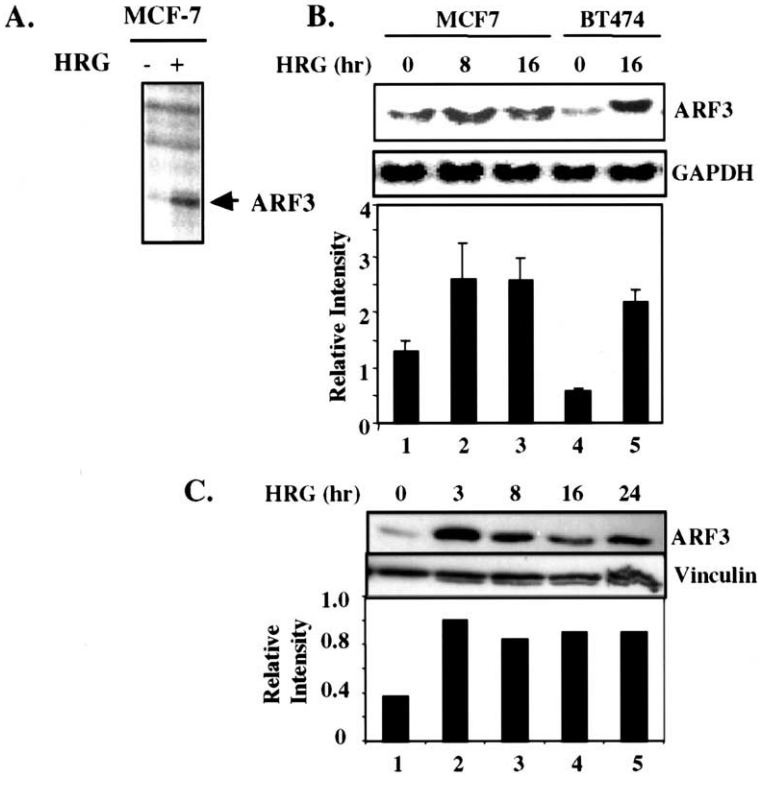

Fig. 1. Identification of ARF3 as a differentially expressed gene in HRG-stimulated cells. A: Representative differential display band patterns of control and HRG (1 nM for 12 h)-treated MCF-7 cells. The arrow indicates the band of interest, which is upregulated by HRG treatment. B: MCF-7 and BT-474 cells were treated with 1 nM HRG for 8 or 16 h. Total RNA was isolated, and the ARF3 mRNA level was detected using Northern blot analysis. Subsequently, the blot was reprobed using a GAPDH cDNA probe. The mean (±S.D.) expression of ARF3 mRNA from three separate experiments was determined by densitometry and is shown in the bottom panel. C: MCF-7 cells were treated with 1 nM HRG for 16 h. Total lysates were run on SDS–PAGE gels and blotted with an anti-ARF3 monoclonal antibody. Anti-vinculin antibody was used as an internal control. Quantitation of the ratio of ARF3 to vinculin is shown in the bottom panel.

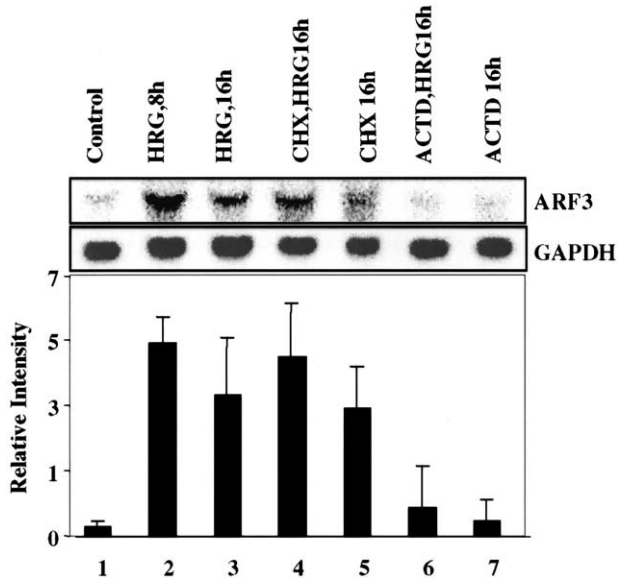

Fig. 2. HRG induces ARF3 mRNA expression in the absence of de novo protein synthesis. MCF-7 cells were treated with cycloheximide (50 μg/ml) or actinomycin D (10 μg/ml) in the presence or absence of HRG (1 nM). Total RNA was isolated and the level of ARF3 mRNA was detected using Northern blotting. The mean (±S.D.) expression of ARF3 mRNA from three separate experiments was determined by densitometry and is shown in the bottom panel.

clones were sequenced and all five sequences were 100% identical to human ARF3 from 2721 to 2906 (ARF3 GenBank accession number BC017565). To determine whether ARF3 mRNA can be modulated by HRG in tumor cells, we performed Northern blot hybridization using the 0.6 kb ARF3 cDNA as a probe. HRG treatment of MCF-7 and BT-474 cells was accompanied by a significant increase in the ARF3 mRNA level (Fig. 1B). HRG treatment also increased the level of ARF3 protein in MCF-7 cells (Fig. 1C). Taken together, these results suggested that HRG upregulates the expression of ARF3.

3.2. HRG regulates ARF3 at the pretranslational level

The observed HRG-mediated increase in ARF3 mRNA expression may have been due to increased synthesis of newly transcribed mRNA, enhanced stability of ARF3 mRNA, or both. Pretreatment of cells with actinomycin D, an inhibitor of transcription, abolished the HRG-mediated induction of ARF3 mRNA expression, suggesting the need for continuous RNA synthesis in the observed increased expression of ARF3 mRNA in HRG-treated cells (Fig. 2A). Treatment with cycloheximide, a translational inhibitor, had only a marginal effect on ARF3 mRNA levels (Fig. 2A). We next examined the status of ARF3 promoter chromatin by CHIP analysis using antibodies specific for acetylated forms of H3 and H4. There was a significant enhancement in the association of acetylated histone H3 with the ARF3 promoter region in HRG-treated MCF-7 cells, but not in control serum-starved cells as detected by chromatin immunoprecipitation (data not shown). This finding provided evidence for open chromatin conformation at the ARF3 promoter following HRG treatment. These observations suggested a role of histone H3 in the regulation of ARF3 promoter following HRG treatment and suggested that HRG regulated ARF3 expression at a pretranslational level.

3.3. HRG rapidly redistributes ARF3 in breast cancer cells

Since HRG is known to regulate vesicle trafficking [24], and ARF3 is involved in exocytosis [25], we next sought to determine the influence of HRG on the subcellular distribution of ARF3. To visualize the effect of HRG on ARF3 localization, MCF-7 cells were transiently transfected with a HA-tagged ARF3 and treated with HRG for various lengths of time, then HA-ARF3 was localized by immunofluorescent labeling and confocal microscopy. In control MCF-7 cells, HA-ARF3 was normally localized within the cell cytoplasm, with some accumulation around the nuclear membrane (Fig. 3A). After 60 min of HRG treatment, there was a dramatic relocalization of HA-ARF3 to the nucleus (Fig. 3B). However, after 16 h of HRG treatment, ARF3 was no longer seen in the nucleus, but was rather localized in the cytoplasm and at the cell periphery. These observations suggested that HRG-generated signals could trigger an early redistribution of ARF3 to the nucleus. ARF3 has been shown to bind the mitotic kinesin-like protein 1 in a GTP-dependent fashion [26]. Our observations suggested a role for ARFs in protein shuttling into the nucleus, which could happen as a result of signaling with a cellular growth factor such as HRG. However, no nuclear localization and/or function for ARF3 has previously been reported.

3.4. ARF3 colocalizes with acetylated histone H3

Since HRG induced rapid ARF3 movement into the nucleus, we wanted to determine if ARF3 might be involved with the chromatin remodeling induced by HRG. MCF-7 cells were again transfected with HA-ARF3, serum-starved for 24 h and then treated with 1 nM HRG for either 30 or 60 min. Immunofluorescence and confocal microscopy again showed the rapid relocalization of ARF3, first to the cell membrane (Fig. 3E) and then to the nucleus (Fig. 3F). Acetylated histone H3 was virtually absent in serum-starved cells (Fig. 3D), but also rapidly increased with HRG treatment. Interestingly, ARF3 and acetylated histone H3 colocalized to specific spots around the nucleoli after 60 min of HRG treatment (Fig. 3F). This is the first report of specific nuclear localization of ARF3 following growth factor signaling and the first report of colocalization of ARF3 with acetylated histone H3. Thus ARF3 may be an effector protein for growth factor-induced changes in gene expression.

3.5. ARF3 expression in mammary epithelial cells

Growth factors and their receptors play a critical role in epithelial cancer promotion and progression. We next explored whether there is any relationship between the levels of ARF3 and tumorigenic potential of breast cancer cell lines. Results in Fig. 4A show that ARF3 is an abundantly expressed protein in most of the cell lines used here. There was no correlation between the level of ARF3 and overexpression of the HER2 receptor, as HER2 overexpressing SKBR3 and BT-474 cells exhibited varied levels of ARF3 and low HER2 expressing MCF-7 cells had a higher ARF3 content than SKBR3 cells. We next determined the expression profile of ARF3 in various murine organs and the different stages of mammary gland development. Higher levels of ARF3 protein were detected in ovary and uterus, and protein levels were extremely low in testis, brain, lung, and adrenal and thyroid glands (Fig. 4B). Interestingly, in the mammary gland, the ARF3 protein expression was developmentally regulated with the highest levels in virgin and post-weaning glands

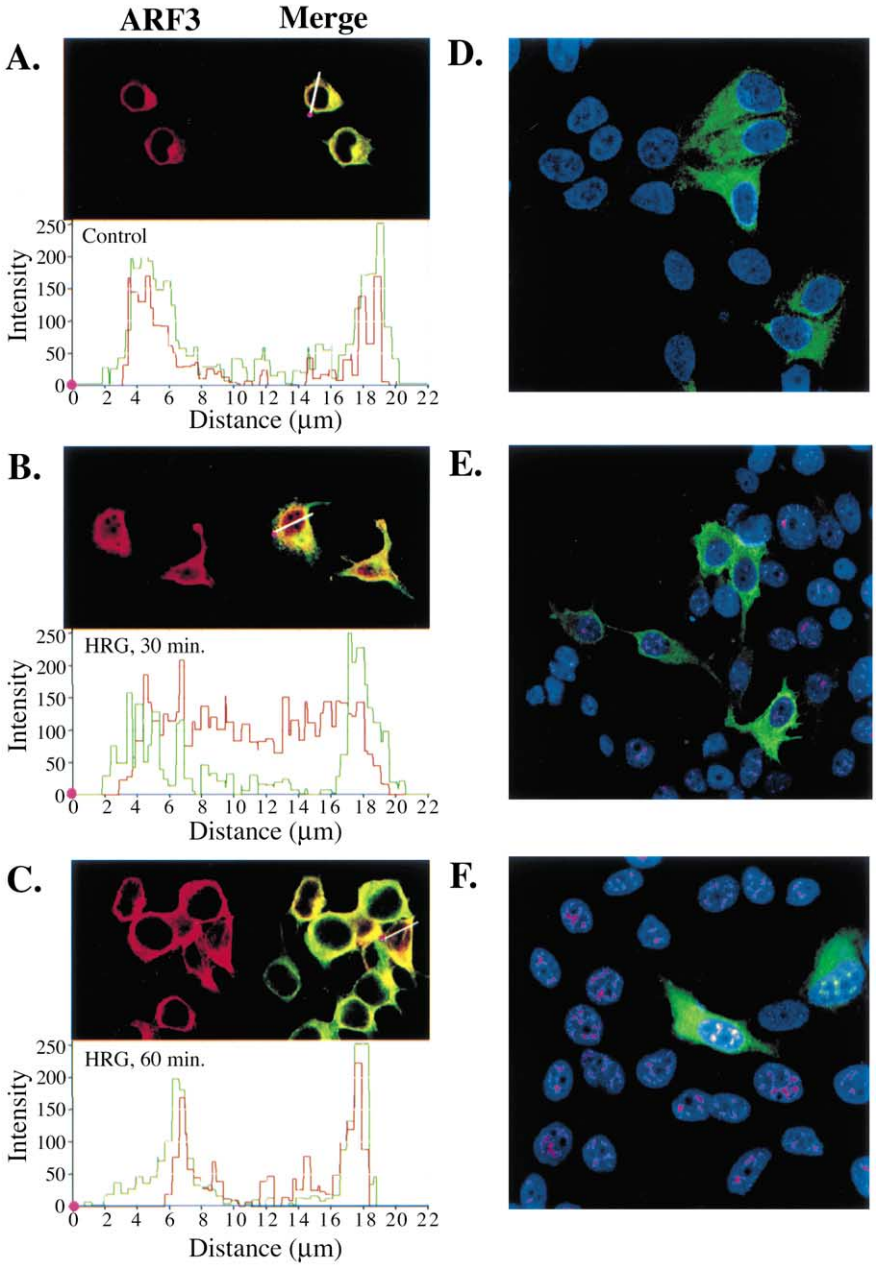

Fig. 3. HRG redistribution of ARF3 in breast cancer cells. A–C: MCF-7 cells were transfected with HA-tagged ARF3, serum-starved for 24 h, then treated with HRG for 0 (A), 1 (B) or 16 h (C) and the localization of HA-ARF3 (red) and actin (green) was visualized using immunostaining and confocal microscopy (40× magnification). Pixel intensity and immunofluorescent colocalization along the indicated lines were quantitated and are shown on the graphs below. D–F: MCF-7 cells were transfected with HA-ARF3, serum-starved for 24 h and then treated with 1 nM HRG for 0 (D), 30 (E) or 60 (F) min. Immunofluorescence and confocal microscopy showed the rapid relocalization of ARF3 (green), first to the cell membrane (E) and then to the nucleus (F). Acetylated histone H3 (red) and ARF3 colocalized (yellow) to specific spots around the nucleoli after 60 min of HRG treatment (F). Blue, nuclear counter stain.

and its levels were significantly reduced during lactation and early post-weaning stages of mammary gland development (Fig. 4C). Since ARF3 expression was highest during the late post-weaning stage characterized by remodeling and also in virgin mammary gland, these observations suggest a physiological role of ARF3 in the mammary gland.

In summary, we have presented new evidence that growth factor stimulation of breast cancer cells significantly increases the expression of ARF3. Since the level of baseline ARF3 expression did not correlate well with the HER2 overexpression in different cell lines, ligand-activated cellular pathways, rather than receptor levels, may be important in the regulation of ARF3 expression. In addition, we have also provided new evidence in support of growth factor-triggered rapid nuclear redistribution of ARF3, implying a putative role of ARF3 in modulating nuclear functions and/or gene expression in growth factor-activated cells. Consistent with such a possibility, we noticed a temporal relationship between the kinetics of the nuclear presence of ARF3 and its colocalization with the acetylated histone H3, an index of increased transcription, in HRG-treated cells (data not shown). In summary, our findings have clearly demonstrated for the first time a potential

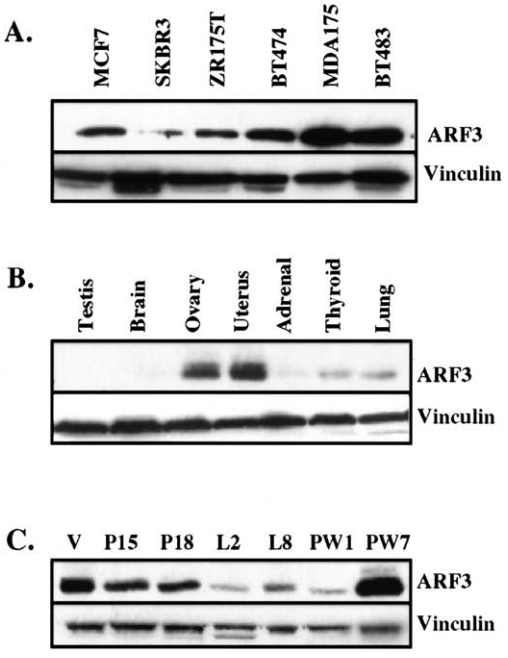

Fig. 4. ARF3 expression in mammary epithelial cells. A: ARF3 protein expression in breast cancer cells as determined by Western blot. B: ARF3 protein expression in multiple mouse organs. C: ARF3 protein expression during mammary gland development. V, virgin day 21; P, pregnancy days 15 and 18; L, lactation days 2 and 8; PW, post-weaning days 1 and 7. For each experiment, vinculin protein was used as a loading control.

role of ARF3 in the action of a physiologically relevant growth factor, HRG, in the mammary gland. In addition, we provide new evidence of a role for growth factor-initiated signals in the redistribution of ARF3 to the nucleus in response to an extracellular signal.

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