

## Growth Factor Regulation of a 26S Proteasomal Subunit in Breast Cancer

Christopher J. Barnes, Feng Li, Amjad H. Talukder, and Rakesh Kumar

**Abstract** **Purpose:** We sought to gain insight into the mechanisms of heregulin- $\beta$ 1 (HRG) action on breast epithelial cells by identifying and characterizing HRG-regulated proteins.  
**Experimental Design:** Differential display mRNA screening of human breast cancer cells grown in the presence or absence of HRG was used to identify HRG-regulated genes. Biochemical and functional studies were undertaken to examine the impact of HRG and the therapeutic antibody herceptin on protein expression, localization, and function.  
**Results:** We identified the ATPase subunit 4 (S4) of the 26S proteasome as a HRG-regulated target. Both S4 mRNA and protein levels were increased by HRG; however, this HRG-stimulated increase was blocked by the therapeutic antibody herceptin. S4 expression was significantly increased in primary human breast tumors and in estrogen receptor – negative tumors. Coimmunoprecipitation, immunofluorescence, and ATPase activity assays suggested that HRG also induced S4 activity and formation of a functional proteasome complex.  
**Conclusions:** This is the first demonstration of growth factor – regulated expression, localization, and activity of the S4 subunit of the 26S proteasome in human breast cancer cells. These findings now provide a potential mechanistic rationale for the use of proteasome inhibitors in breast cancers with active HRG signaling.

Growth factors and their receptors play an important role in the regulation of epithelial cell growth, differentiation, and function. Heregulin- $\beta$ 1 (HRG), a combinatorial ligand for the human epidermal growth factor receptors 3 and 4 (HER3 and HER4), is a secretory polypeptide that affects growth stimulation, differentiation, invasiveness, and motility of breast cancer cells (1–6). In mammary epithelial cells, HRG predominantly binds to HER3/HER2 heterodimers to enact its biological effects (7). Additionally, HRG is expressed in the mammary mesenchyme adjacent to lobuloalveolar structures and is maximally expressed during pregnancy (5). HRG also plays a role in the morphogenesis and ductal migration of mammary epithelial cells (1, 4), promotes the responsiveness of these cells to lactogenic hormones *in vitro* (5), and induces the differentiation of mammary epithelium into secretory lobuloalveoli (6). These observations suggest that HRG is a regulatory polypeptide having distinct biological effects, such as growth stimulation, differentiation, and increased invasiveness and migration in mammary epithelial cells. However, the underlying

mechanisms of HRG cytoplasmic signaling leading to gene expression are not well established.

Recent evidence implicates degradation of intracellular signaling proteins and transcriptional regulators through ubiquitination and cleavage by the multicatalytic 26S proteasome as an important means of regulating cell signaling. Also, proteasome-mediated degradation of cell cycle regulatory proteins allows cells to progress through the cell cycle (8). It has been suggested that ubiquitin-mediated proteolytic cleavage is integral to mechanisms underlying carcinogenesis and metastasis, including the aforementioned cell cycle regulation, apoptosis, and angiogenesis (9, 10). Along these lines, several proteasome inhibitors are currently in preclinical testing.

The 26S proteasome is the major nonlysosomal protease in eukaryotic cells and is responsible for the degradation of all short-lived proteins and 70% to 90% of all long-lived proteins (11). It consists of two components, each of two major subcomplexes. There are two central stacked 20S multimeric rings, capped on either end by the multimeric 19S rings. Components of the 19S complexes regulate substrate binding, unfolding, and entry into the 20S subunits, whereas the 20S complexes possess regulated protease activity. The 20S and 19S complexes are normally separate inside of the cell, but when they come together structural alterations occur within the 20S complex that facilitates substrate entry into the 20S axial pores and subsequent protease-mediated degradation. Interestingly, mutations in the S4 subunit of the 19S complex, which is located physically adjacent to the 20S axial pore, caused persistent closure of the protease pore, blocked substrate entry, and inhibited proteasome activity (12). These data suggest that S4 may be a key regulatory component of the proteasome complex.

**Authors' Affiliation:** Department of Molecular & Cellular Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas  
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**Requests for reprints:** Rakesh Kumar, Department of Molecular & Cellular Oncology, University of Texas M.D. Anderson Cancer Center, Box 108, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-745-3558; Fax: 713-745-3792; E-mail: rkumar@mdanderson.org.

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To understand the mechanisms of HRG action on breast epithelial cells, we have used differential display of mRNA to identify HRG-regulated genes. Our results suggest that HRG regulates the expression and activity of the S4 gene, a subunit of the 19S complex of the 26S proteasome.

## Materials and Methods

**Cell cultures and reagents.** MCF-7, BT-474, and ZR75R human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA) and were maintained in DMEM and F12 (1:1) supplemented with 10% FCS. Monoclonal antibodies were against the T7 epitope (Novagen, Madison, WI), the 20S proteasome complex (HC3 and HC8 clone, Affiniti Research, Mamhead, United Kingdom), the HER2 receptor (Ab1, NeoMarkers, Fremont, CA), and anti-ubiquitin (NeoMarkers). Alexa 488- and Alexa 546-labeled goat anti-mouse secondary antibodies, Alexa 488-labeled goat anti-rabbit secondary antibody, and the DNA-intercalating fluorescent dye ToPro3 were purchased from Molecular Probes (Eugene, OR). 4D5 was from Genentech (South San Francisco, CA). All other reagents, including heregulin, cycloheximide, and actinomycin D, were from Sigma (St. Louis, MO).

**Cell extracts and immunoprecipitation.** For the preparation of cell extracts, cells were washed thrice with PBS and lysed in radioimmunoprecipitation assay buffer supplemented with 100 mmol/L NaF, 200 mmol/L NaVO<sub>5</sub>, 1 mmol/L phenylmethylsulfonyl fluoride, 10 g/mL leupeptin, and 10 g/mL aprotinin on ice for 15 minutes. The lysates were then centrifuged in an Eppendorf centrifuge at 4°C for 30 minutes. Cell lysates for immunoprecipitation were prepared using Nonidet P-40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.5% Nonidet P-40, 1× protease inhibitor mixture, and 1 mmol/L sodium vanadate]. Immunoprecipitation was done for 2 hours at 4°C using 1 µg of antibody per milligram of protein, with equal amounts of protein in parallel samples. Immunocomplexes were then recovered by adding 50 µL of a 50% protein A Sepharose bead suspension and incubating at 4°C for 2 hours. The beads were then sequentially pelleted and washed with NaCl free lysis buffer five times. Samples were then resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with the appropriate antibodies, using an enhanced chemiluminescence method (13).

**Differential display analysis.** Differential display was done using MCF-7 cells grown in serum-free media with and without HRG treatment using the Delta Differential Display kit and as previously described (14).

**Northern blot hybridization.** Total cytoplasmic RNA was isolated using the TRIzol reagent and 20 µg RNA were analyzed via Northern blot hybridization using a 1.6 kb full-length S4 cDNA. rRNA (28S and 18S) was used to assess the integrity of the RNA. The blots were routinely reprobbed with human glyceraldehyde-3-phosphate dehydrogenase 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 nmol/L). Individual bands were quantified by scanning densitometry and graphed as the mean ± SD of three experiments.

**Tissue sample and Western blotting.** Mouse tissue samples were collected, snap-frozen in liquid nitrogen, and stored at -80°C until processed for immunoblotting. Human breast tissue samples were obtained from a tissue bank maintained by the University of Texas M.D. Anderson Cancer Center Breast Cancer Core Pathology Laboratory. These specimens have been used in previous studies (15). Tumor estrogen receptor α (ER) status for Fig. 2C was determined by the Surgical Pathology Core Facility at University of Texas M. D. Anderson Cancer Center. Specimens from patients who had undergone surgery for breast cancer were snap-frozen in liquid nitrogen and stored at -80°C (16). Thawed tissue samples were homogenized in Triton X-100 lysis buffer [20 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 0.1% deoxycholate (v/w), 2 mmol/L

EDTA, 2 mmol/L NaVO<sub>5</sub>, and protease inhibitor mixture], and equal amounts of protein were analyzed by Western blotting. The protein vinculin was used routinely as a loading control.

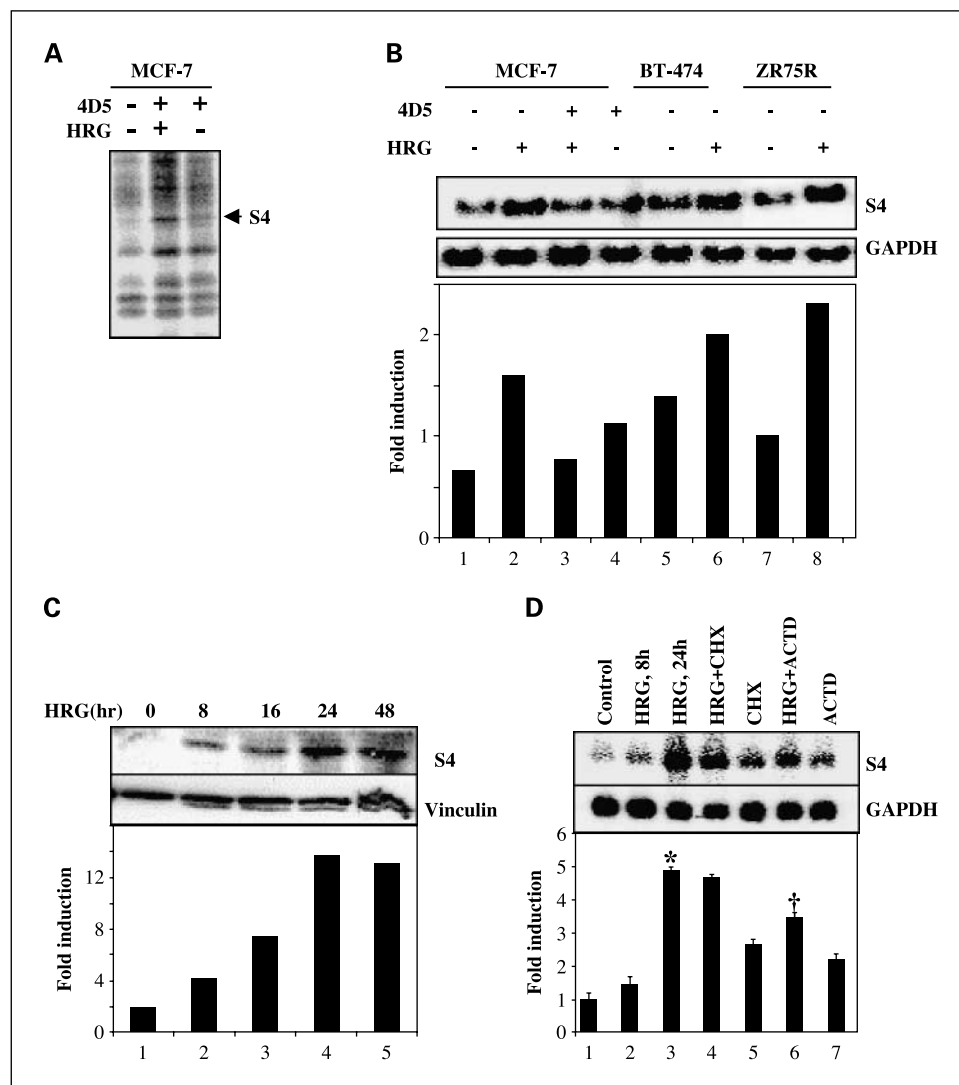
**ATPase activity assay.** Changes in ATPase activity of S4 after HRG treatment were measured using the EnzCheck Phosphate Assay kit (Molecular Probes) essentially as described, with ubiquitinated proteins immunoprecipitated from MCF-7 cells as an enzymatic substrate. Briefly, subconfluent MCF-7 cells were transiently transfected with T7-S4, grown in media supplemented with 2% serum for 48 hours, and then treated with either HRG (1 nmol/L) for 0, 2, or 6 hours or treated with heregulin for 30 minutes before 6-hour HRG treatment. Cells were lysed in radioimmunoprecipitation assay buffer, and T7-S4 was immunoprecipitated from each whole cell lysate using an anti-T7 antibody and a rabbit anti-mouse antibody conjugated to Sepharose beads. Beads were washed five times in lysis buffer, resuspended in 100 µL lysis buffer, and then used immediately in the free phosphate detection assay. Parallel reactions with and without addition of 30 µg resuspended, ubiquitinated proteins were run for each treatment group. Absorbance values at 360 nm were recorded every 5 minutes, and inorganic phosphate concentrations were calculated based on a standard curve. The ratios of reactions with and without ubiquitinated proteins were plotted as the change from baseline in cumulative inorganic phosphate concentration with time. Each reaction was done at least thrice and one representative series is shown.

**Immunofluorescence and confocal imaging.** MCF-7 or SKBR3 cells were grown on glass coverslips in six-well culture plates; upon reaching ~50% confluence, cells were transiently transfected with T7-tagged S4 and then serum starved for 24 hours. Separate wells for staining of the 20S subunit were not transfected. Subconfluent cells were serum-starved for 24 hours, then treated with HRG (1 nmol/L) for 0, 30, or 60 minutes, or for 16 hours. Cells were then cells were rinsed in PBS and fixed in 4% phosphate-buffered paraformaldehyde for 10 minutes. Following fixation, cells were processed for routine immunofluorescence for detection of T7-S4, the 20S protein complex, and/or HER2; cells were then counterstained with the nuclear dye ToPro3. Fluorescent labeling was visualized using a Zeiss LSM microscope with a ×40 objective and PC-based LSM510 version 2.01 software.

**Statistical analyses.** All statistical analyses were done using the PRISM statistical package (GraphPad Software, San Diego, CA). Data in Fig. 1D were analyzed using ANOVA for overall differences followed by the SNK test for differences between individual groups. Differences in Fig. 3B were analyzed by linear regression. The data in Fig. 4C, D, and F were analyzed using the Student's *t* test. Significant differences were accepted if *P* < 0.05.

## Results

**Identification of S4 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 either vehicle or HRG. Complementary DNAs were synthesized using a reverse transcriptase in the presence of [<sup>32</sup>P]dCTP and amplified by PCR. A total of 90 reactions were done using nine 3'-degenerate oligo-dT primers and ten 5' random primers for each treatment. Reaction products were separated by PAGE. Some products showed equal intensity with or without HRG. Using these bands as internal controls, gels were analyzed for differentially expressed PCR products. This analysis resulted in the identification of a 426 bp band differentially expressed in HRG-treated cells (Fig. 1A). This differentially expressed gene product was then amplified, cloned, and sequenced. Five clones were sequenced and all five sequences were 100% identical to the human 26S protease S4 regulatory subunit mRNA from nucleotide 875 through 1,295 (Genbank accession no. L02426).



**Fig. 1.** *A*, differential display analysis of total RNA from MCF-7 cells that were grown in serum-free media, treated with HRG (1 nmol/L), or treated with HRG plus the HER2 blocking antibody herceptin (30 nmol/L). *B*, various breast cancer cells were grown in serum-free media or treated as labeled. Total cellular RNA was isolated and analyzed by Northern blot. *C*, MCF-7 cells were grown in serum-free media then treated with HRG for varying times. Proteins were analyzed by Western blot as labeled. The S4-to-vinculin expression ratios were quantified by densitometry and the ratio for each group is shown below. *D*, MCF-7 cells were grown in serum-free media, then treated as labeled. Total cell RNA was analyzed by Northern blot. The ratio of S4 to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) RNA is shown below. CHX, cycloheximide; ACTD, actinomycin D. \*, significantly different from control group; †, significantly different from the 24-hour HRG group,  $P < 0.05$ .

We next sought to determine whether S4 mRNA levels could be modulated in human tumor cell lines using Northern blot hybridization and a 1.6 kb full-length S4 cDNA as a probe. HRG treatment of MCF-7, BT-474, and ZR75R breast cancer cells induced a significant increase in S4 mRNA levels. This effect was blocked by pretreatment of cells with the anti-HER2 receptor blocked antibody herceptin (Fig. 1B). It is established that HER2 is required for the cellular effects of HRG on breast cancer cells. HRG treatment also increased S4 protein levels in MCF-7 cells in a time-dependent manner (Fig. 1C).

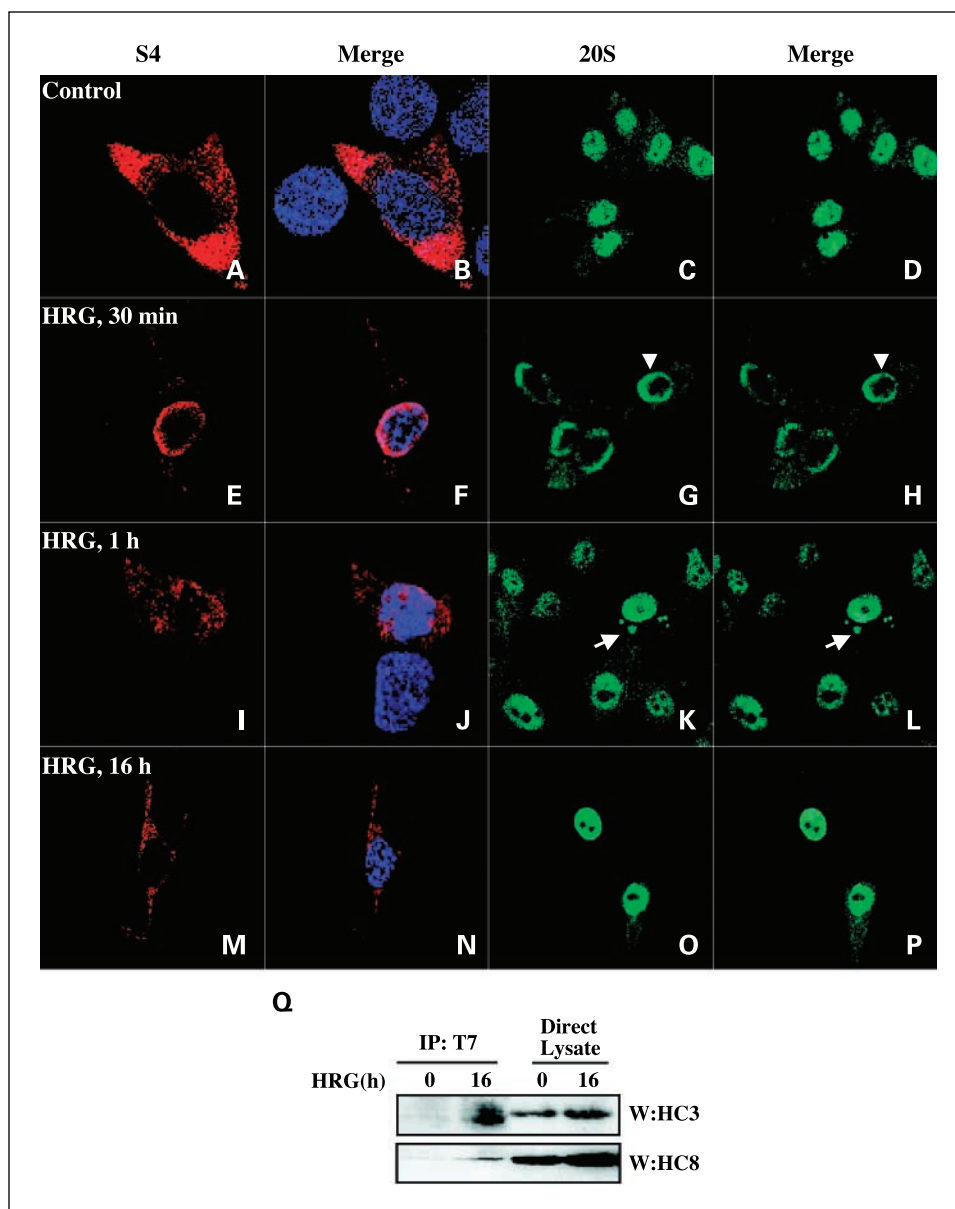
**Heregulin regulates S4 at the pretranslational level.** To determine if HRG regulates S4 expression at the transcriptional or the translational level of gene expression, serum-starved MCF-7 cells were treated with HRG alone (Fig. 1D, lanes 2 and 3) or were cotreated with either the transcriptional inhibitor cycloheximide or the translational inhibitor actinomycin D. Results indicate that HRG likely regulates S4 expression at the pretranslational level. Taken together, these results suggest that HRG up-regulates the expression of S4 and that the HER2 blocking antibody herceptin can inhibit the HRG-induced increase in S4 expression in breast cancer cells.

**Heregulin-mediated S4 localization with the 20S proteasomal subunit.** To visualize the effect of HRG on S4 localization, MCF-

7 cells were transiently transfected with T7-tagged S4, then treated with HRG for various lengths of time, and T7-S4 was localized by immunofluorescent labeling and confocal microscopy. In control MCF-7 cells, T7-S4 was normally diffusely localized within the cell cytoplasm (Fig. 2A and B). After 30 minutes of HRG treatment, there was a dramatic relocalization of T7-S4 to the perinuclear region and the area inside of the nuclear membrane, with a dramatic loss of cytoplasmic staining (Fig. 2E and F). After 60 minutes of HRG treatment, T7-S4 was localized more diffusely through the nucleus and the cytoplasm (Fig. 2I and J). By 16 hours after HRG treatment, the T7-S4 was again absent from the nucleus and localized diffusely in the cytoplasm staining (Fig. 2M and N).

The 19S proteasomal subunit and 20S proteasomal subunit are not normally colocalized but come together following stimulation (10). We wished to determine if S4, a component of the 19S complex, was colocalized with the 20S protein complex. Polyclonal antibodies against 20S did not prove useful in immunofluorescence staining, so a monoclonal antibody that was not compatible with the T7 immunostaining was used to localize the 20S complex in a parallel experiment. As is shown in Fig. 2C and D, the 20S complex is primarily localized to the nucleus but is also present in the cytoplasm. After 30 minutes of

**Fig. 2.** A, MCF-7 cells were transiently transfected with T7-tagged S4 (two left-hand columns: A-B, E-F, I-J, M-N) or not transfected (two right-hand columns: C-D, G-H, K-L, O-P), serum starved for 24 hours, then treated with heregulin (1 nmol/L) for the indicated time periods. Localization of T7-S4 (red, left-hand columns), the 20S proteasome complex (green, right-hand columns), and nuclear DNA (blue) were visualized by immunostaining and confocal microscopy ( $\times 40$  magnification). Colocalization with nuclear blue staining turns red fluorescence pink and green fluorescence white. Rapid but transient relocalization of T7-S4 (E-F) and 20S (G-H) to the perinuclear region and within the nucleus (arrowheads) is evident after 30 minutes of heregulin treatment. The 20S complex was also infrequently localized to cytoplasmic inclusions after 60 minutes (K-L). Q, immunoprecipitation of transiently transfected T7-S4 from MCF-7 cells. Western blot analysis was done for the HC3 and HC8 components of the 20S proteasomal complex.

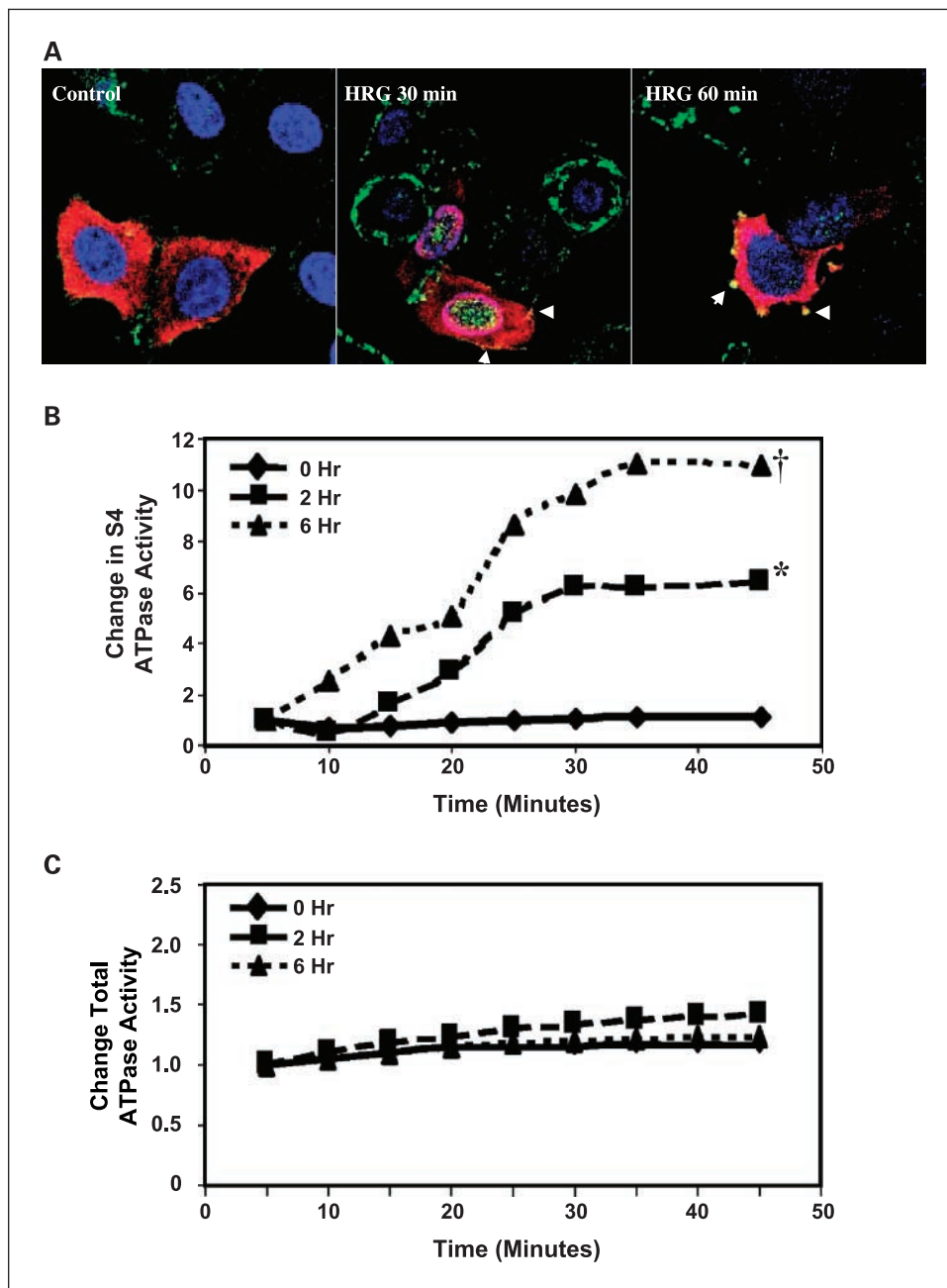


HRG treatment, the 20S complex also became concentrated in a perinuclear ring (Fig. 2G and H). This relocalization was transient, with a similar diffuse nuclear distribution visible after 60 minutes of HRG (Fig. 2K and L). Cytoplasmic inclusions in which the 20S subunit was concentrated were also occasionally seen (arrow). By 16 hours after HRG treatment, 20S localization was similar to that seen in untreated cells (Fig. 2O and P). We next sought to confirm this *in vivo* HRG-stimulated association of transfected T7-S4 with components of the 20S subunit through immunoprecipitation of the transfected protein and Western blot for endogenous 20S components. MCF-7 cells were transiently transfected with T7-S4, serum starved, and then stimulated with HRG. Association of the 20S subunit with T7-S4 was observed with using two separate specific antibodies (Fig. 2Q).

**Association of S4 with the human epidermal growth factor receptor 2.** The insulin-like growth factor receptor and tumor necrosis factor receptor are associated with the 26S proteasome following ligand binding (17, 18). We wished to determine if the

S4 proteasomal subunit, which functions in the recruitment of proteins to the proteasome, was associated with HER2 following treatment with HRG. SKBR3 cells were transfected with T7-S4 for this experiment because of their abundant expression of HER2 (19). We found that S4 and HER2 were indeed associated at the cell periphery following 30 and 60 minutes of HRG treatment (Fig. 3A). Nuclear staining for HER2 was considered nonspecific as this pattern was not evident when cells were independently treated with HRG and immunofluorescently stained with a monoclonal antibody against HER2 (data not shown).

**S4 ATPase activity.** S4 is an established catalytic protein with ATPase activity. To determine if HRG-stimulated association with the 20S subunit resulted in changes in S4 function, we conducted an *in vitro* ATPase activity assay using immunoprecipitated S4 and immunoprecipitated endogenous ubiquitinated proteins as enzymatic substrates. Results showed a significant, time-dependent increase in S4 ATPase activity with HRG stimulation (Fig. 3B). Total cellular ATPase activity was only slightly increased (Fig. 3C), indicating that the change in S4



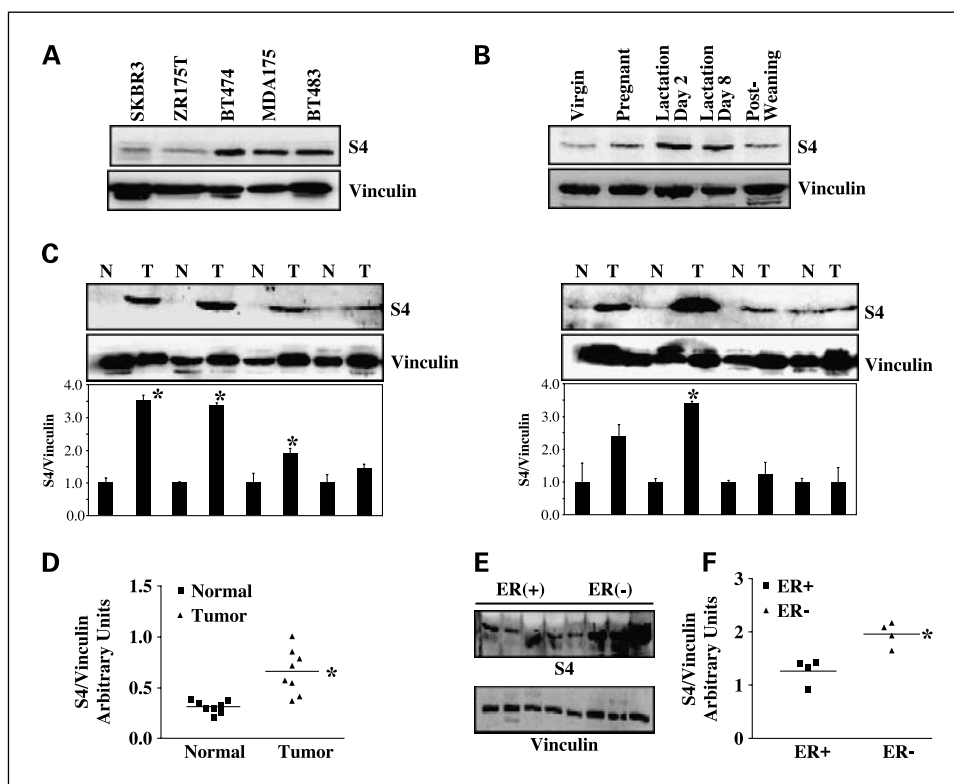
**Fig. 3.** A, SKBR3 cells were transiently transfected with T7-tagged S4, serum starved for 24 hours, and were then treated with heregulin (1 nmol/L) for the indicated time periods. Localization of T7-S4 (red), HER2 (green), and nuclear DNA (blue) was visualized by immunostaining and confocal microscopy ( $\times 40$  magnification). Colocalization S4 red fluorescence pink and HER2 green fluorescence appears yellow (arrowheads). B, the enzymatic activity of the S4 proteasomal subunit was measured following the indicated times of 1 nmol/L heregulin treatment using immunoprecipitated S4 and an inorganic phosphate assay with immunoprecipitated, ubiquitinated proteins as substrates. Data are displayed as fold change from baseline with data normalized for ATPase activity in the absence of ubiquitin. \*, slope is significantly different from 0- and 6-hour HRG groups; †, slope is significantly different from 0- and 2-hour HRG groups,  $P < 0.05$ . C, total cellular ATPase activity under the same conditions described for (A).

activity was likely a specific, HRG-stimulated response. Thus, S4 expression, localization, and ATPase activity are regulated following cell stimulation with HRG.

**Breast epithelial expression of S4.** Because HRG is an established regulatory polypeptide that influences the growth and proliferation of mammary epithelial cells and because our screen for HRG-regulated proteins was done in the MCF-7 human breast cancer cell line, we next examined S4 protein expression in various breast cancer cell lines. Results showed varying, but ubiquitous, levels of expression (Fig. 4A). We next wished to characterize developmental and cancer-related expression of S4. Mammary gland development and pregnancy are known to modulate breast epithelial proliferation, growth, and differentiation. Changes in S4 expression were

determined during mouse mammary gland development (Fig. 4B). Results indicate that S4 expression increases during pregnancy and through lactation and is then down-regulated during the postweaning period. To determine whether S4 might be up-regulated in human breast cancer, we did Western blot analysis of S4 protein expression in eight human breast cancers, together with paired normal breast epithelium (Fig. 4C). In four of the eight paired tissue samples, S4 protein expression was significantly up-regulated in the tumor tissue. However, when the ratio of S4 protein to vinculin (loading control) protein expression was compared between all normal and all tumor tissue from these samples (Fig. 4D), S4 was significantly higher overall in tumor compared with normal breast samples.

**Fig. 4.** S4 and vinculin protein expression in various human breast cancer cell lines (A), mouse mammary gland development (B), and paired samples of normal human breast epithelium and breast tumors (C). For (C), S4 and vinculin expression were quantified by densitometry and the ratio for each sample is shown below. D, scatter graph with median group values (horizontal lines) of the mean ratios of densitometry values for S4 and vinculin for each normal and tumor sample. \*, significantly different from normal samples,  $P < 0.05$ . E, S4 and vinculin protein expression in pathologically classified ER- and ER+ human breast tumors. F, scatter graph with median group values (horizontal lines) of the mean ratios of densitometry values for S4 and vinculin for each tumor sample. \*, significantly different,  $P < 0.05$ .



Human breast cancers are routinely risk-stratified according to histologic nuclear ER expression, with decreased or absent nuclear ER expression highly correlated with tumor aggressiveness and risk of metastasis (20). We determined S4 protein expression in pathologist-classified ER+ or ER- tumors (Fig. 4E). When S4 expression was normalized to vinculin protein expression and compared between ER+ and ER- tumors (Fig. 4F), results indicated that increased tumor S4 expression was significantly correlated with a loss of nuclear ER expression. Thus, S4 is a regulated protein in mammary epithelium that is overexpressed in human breast cancer and, in particular, in ER- tumors that exhibit an aggressive phenotype.

## Discussion

Ubiquitin-mediated proteasomal degradation via the 26S proteasome multimeric protein complex is the main mechanism of protein degradation and turnover for the majority of cellular proteins. As such, 26S proteasomal function plays a critical role in cell responses to extracellular signals, environmental changes, cell cycle progression, and normal cell function. Among the subunits of the 19S regulatory complex, the S4 subunit has been singled out as being

critical for facilitating substrate entry into the 20S protease complex.

We report here new data on the active regulation of S4 expression and function following cell stimulation by HRG, a polypeptide ligand for HER family growth factor receptors. Although 26S-mediated degradation of growth factor receptors, nuclear receptors, and transcription factors, among others, has been previously reported (11), this is the first report of specific subunit regulation with growth factor signaling. Given the essential role of the S4 subunit in 26S proteasomal activity (12, 21) and our data on elevated S4 expression in human breast cancer, the S4 subunit emerges as a critical, regulated component of proteasomal function. These data may prove useful in understanding the cellular consequences of growth factor signaling. In addition, proteasomal inhibition has been shown to induce cell senescence (22). The S4 subunit may provide a unique target of novel cancer therapies directed at regulation of the 26S proteasome.

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