The 27-kDa Heat Shock Protein Facilitates Basic Fibroblast Growth Factor Release from Endothelial Cells*

(Received for publication, December 9, 1996, and in revised form, January 6, 1997)

Randolph S. Piotrowicz‡, Jody L. Martin§, Wolfgang H. Dillman§, and Eugene G. Levin‡¶

From the ‡Departments of Vascular Biology and Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 and the §Department of Medicine, University of California, San Diego, La Jolla, California 92037-0618

Basic fibroblast growth factor is an important mitogenic and angiogenic factor that stimulates endothelial cell growth and migration. This hormone is not secreted via the classical vesicular pathway, and the identification of intracellular proteins that facilitate its release remains lacking. Transfection and expression of the 27-kDa human heat shock protein in bovine arterial endothelial cells doubles the rate of estrogen-induced basic fibroblast growth factor secretion, preferentially inducing the release of high molecular weight forms of the hormone. The secreted basic fibroblast growth factor is mitogenic to breast adenocarcinoma cells cultured in the conditioned medium obtained from the transfected endothelial cells. In contrast, decreasing the level of the endogenous heat shock protein homolog with an antisense vector markedly decreases basic fibroblast growth factor release. Anti-heat shock protein or anti-basic fibroblast growth factor antibodies co-precipitate both proteins from endothelial cell extracts, demonstrating a direct association between the two proteins. This interaction is likely to be an important step in the mechanism of basic fibroblast growth factor secretion.

The growth of tumors beyond several millimeters in diameter requires the recruitment of capillaries into the tumor and the establishment of a tumor blood supply (1). Controlling or influencing this process is the paracrine communication between tumor, stromal and endothelial cells (2, 3). Key regulatory factors in breast tumor development include insulin-like growth factors, basic fibroblast growth factor (bFGF1/FGF-2), and β-estradiol, the latter potentiating the mitogenic effects of the former peptide hormones on the tumor cells (4, 5). Breast tumor bFGF is predominantly of paracrine origin (6, 7); thus the former peptide hormones on the tumor cells (4, 5). Breast tumor bFGF is predominantly of paracrine origin (6, 7); thus factors facilitating the release of bFGF from endothelial cell and stromal cells facilitate tumor growth. In addition, since bFGF regulates endothelial cell growth and migration, factors that induce the release of bFGF would also facilitate tumor angiogenesis (8).

The mechanism by which bFGF is released from cells remains to be elucidated. bFGF is actually four different translation products of a single message species exhibiting apparent molecular masses of 18, 22, 22.5, and 24 kDa (9–11). The translation of the 18-kDa species begins at a classical AUG start site whereas the higher molecular weight species (HMW bFGF) are initiated at CUG codons 5′ to the AUG start site (11). Neither the 18-kDa nor the HMW bFGF species possess a signal sequence and therefore are not secreted via the classical vesicular pathway involving the endoplasmic reticulum (ER) and Golgi apparatus. Indeed, agents that block trafficking through the Golgi-ER pathway do not inhibit the release of bFGF from cells, and novel pathways for bFGF release have been suggested (12, 13). The identification of proteins involved in the release of bFGF remains lacking however. Data generated in this report suggest that the 27-kDa heat shock protein (HSP27) is involved in the non-lytic release of bFGF.

Endothelial cells express a basal level of the HSP27, which is further enhanced by transcriptional up-regulation in response to estrogens (14). Higher levels of HSP27 expression have been shown to affect microfilament assembly and morphology as well as cell growth (14, 15). For example, we have demonstrated that expression of human HSP27 in bovine arterial endothelial cells (BAECs) via transfection results in a 2–3-fold enhancement in the rate of cell growth (14). These characteristics make HSP27 a likely control point in angiogenic processes where estrogens play key regulatory roles, e.g. breast tumor angiogenesis (2, 3). HSP27 has several demonstrable functions that are attributed to its ability to act as a molecular chaperon, a property it shares with the highly homologous lens crystallins (16, 17) and other HSPs (18, 19). Putative ligands for the chaperoning activity of HSP27 may include steroid receptors (18, 20–22) and nascent or partially denatured proteins (23, 24).

To begin to investigate a role for HSP27 in breast tumor angiogenesis, stably transfected BAECs expressing human HSP27 or control BAECs (14) were co-cultured with the breast adenocarcinoma MCF-7 cells and the growth of each cell type was measured. We consistently found that the culture of HSP27 BAECs with the tumor cells resulted in increased MCF-7 growth, and analysis of HSP27 BAEC-conditioned media confirmed that bFGF was the paracrine factor released from the HSP27 BAECs. Reducing the expression of angiogenesis (8).
the endogenous HSP27 homolog (HSP25) by infection with HSP27 antisense adeno-virus resulted in decreased bFGF secretion. Immunoprecipitation using either anti-HSP27 or anti-bFGF antibodies co-precipitated the two proteins, suggesting that a direct interaction exists between HSP27 and bFGF. These data suggest that HSP27 may act as a chaperon of bFGF, facilitating bFGF release from endothelial cells.

MATERIALS AND METHODS

Culture of Bovine Arterial Endothelial Cells—Low passage bovine pulmonary arterial endothelial cells were a generous gift of Dr. W. Laug, Children's Hospital, Los Angeles, CA. All cell culture reagents were obtained from Bio Whittaker, Inc. except where otherwise noted. Cells were cultured under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM HEPES and supplemented with 10% fetal calf serum (Intergen) and 1 mM each of sodium pyruvate, penicillin, streptomycin, and nonessential amino acids. Cells were plated at a density of 0.7–2 × 104 cells/cm2 and passaged when confluent (approximately 1 × 106/cm2).

Plasmids and Stable BAEC Transfectants—Clones of stably transfected BAECs expressing human HSP27 were generated as described (14). Briefly, pHs2711, a plasmid containing a genomic clone of the human HSP27 gene (25) or the vector plasmid (Bluescript) were transfected into BAECs using cationic lipid (LipofectAMINE, Life Technologies, Inc.). The neomycin resistance-carrying plasmid, pCDM8neo, was co-transfected for the purpose of selecting transfected cells from non-transfected cells. Cells were cultured in the presence of 700 μg/ml Geneticin (G418-sulfate, Life Technologies, Inc.).

Culture of MCF-7 Cells—The breast adenocarcinoma cell line MCF-7 was obtained from the American Type Culture Collection at passage 149 (ATCC HTB-22). This cell line is estrogen receptor positive and exhibits a mitogenic response to β-estradiol. Cells were maintained in minimal essential medium containing 10% fetal calf serum, 1 mM sodium pyruvate, and 10 μg/ml bovine pancreas insulin. To generate estrogen-unresponsive cells (i.e. cells that do not exhibit a mitogenic response to β-estradiol), MCF-7 cells were subcultured into Phenol Red-free minimal essential medium containing sodium pyruvate, insulin, and 10% steroid-depleted fetal calf serum prepared by dextran/charcoal stripping (25). These cultures represent non-clonal populations of cells selected for the ability to grow in steroid-deficient media that express reduced estrogen receptor levels.

Tritiated Thymidine Incorporation into MCF-7 Cultured in BAEC-conditioned Media—Triplicate wells in 12-well plates were seeded with 2.5 × 104 HSP27 BAECs (14) or vector control BAECs in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Intergen) and 1 mM each of sodium pyruvate, penicillin, streptomycin, and 10% steroid-depleted fetal calf serum prepared by dextran/charcoal stripping (25). These cultures represent non-clonal populations of cells selected for the ability to grow in steroid-deficient media that express reduced estrogen receptor levels.

RESULTS

MCF-7 Growth Is Induced by bFGF in the Conditioned Media of β-Estradiol-treated HSP27-expressing BAECs—To demonstrate a role for HSP27 in breast tumor angiogenesis, breast adenocarcinoma cells (MCF-7 cells) were cultured in Transwell inserts above growing BAECs. We consistently found that the growth of the MCF-7 cells could be enhanced if these cells were either cultured with BAECs expressing human HSP27 (HSP27 BAECs) or cultured in the media conditioned by growing HSP27 BAECs, if the HSP27 BAECs were first treated with β-estradiol (Fig. 1). This mitogenic response of the MCF-7 cells was absolutely dependent on treatment of the HSP27 BAECs with β-estradiol. In the absence of β-estradiol, HSP27 BAEC culture media had no effect on MCF-7 growth. In addition, we obtained cultures of estrogen-unresponsive MCF-7 cells (see “Discussion”) in the conditioned media obtained from β-estradiol-treated HSP27 BAECs generated thymidine incorporation 1.54 ± 0.2 times that of control MCF-7 cultures (compare filled bars in Fig. 1, mean ± S.D., n = 5). Importantly, the addition of fresh β-estradiol to the conditioned media obtained from HSP27
BAECs not treated with β-estradiol failed to elicit a mitogenic response. In addition, MCF-7 culture in mock conditioned media containing β-estradiol (i.e., media cultured in the absence of cells) also failed to enhance growth (Fig. 1). This demonstrates that the estrogen-dependence of the enhanced MCF-7 growth is a consequence of β-estradiol affecting the HSP27 BAECs and not the MCF-7 cells. The enhanced MCF-7 growth was absolutely dependent on HSP27 expression in the BAECs, since co-culture with vector-transfected control cells or culture in their conditioned media did not result in enhanced thymidine incorporation into the MCF-7 cells. Thus, both enhanced HSP27 levels and incorporation into the MCF-7 cells. The enhanced MCF-7 growth was absolute.

To determine if the enhanced MCF-7 growth is correlative with increased bFGF secretion from the HSP27 BAECS, HSP27-expressing and control BAECs were placed in serum-free assay media (defined in Fig. 1), with or without β-estradiol, and cultured for 18 h. The conditioned media were cleared of cellular material and then precipitated with trichloroacetic acid, solubilized for SDS-PAGE, and subjected to immunoblot analysis using the monoclonal anti-bFGF antibody. Also included on the gels were Triton X-100 lysates of the cultures, the Triton-insoluble material (solubilized by SDS extraction), and, for the purpose of generating a standard curve, titrating amounts of rbFGF. In a representative experiment (Fig. 2, panel A), HSP27 BAECs and vector control BAECs, in the absence of β-estradiol, secreted 4.8 and 5.1 ng of bFGF/mg of protein in the Triton extract, respectively. Upon treatment of the transfectants with 100 nm β-estradiol, the amount of bFGF released by the HSP27 BAECs increased to 10.2 ng of bFGF/mg of Triton lysate protein, with increases in all the bFGF forms evident. The amount of bFGF in the conditioned media of the control BAECs treated with β-estradiol in Fig. 2 was 5.2 ng of bFGF/mg of Triton lysate protein. Estrogen treatment of the HSP27 BAECs resulted in an average 2.4 ± 0.2-fold enhancement (n = 4) of bFGF released into the media, increasing the media concentration from 240–320 pg/ml to 512–730 pg/ml. The higher concentration range, which supported a mitogenic response in our culture system, is above the ED50 of bFGF for other cell types in culture (e.g., 3T3 fibroblasts (11)). In contrast, the control BAECs did not release additional bFGF upon β-estradiol treatment (+estradiol/–estradiol = 1.1 ± 0.07, n = 4).

Although the transfectants released equivalent amounts of bFGF, HSP27 BAECs preferentially secreted HMW bFGF, with little or undetectable amounts of 18-kDa bFGF released. As demonstrated in Fig. 2, the HSP27 BAECs secrete a disproportionate amount of HMW bFGF. In the absence of β-estradiol treatment, the average percentage of the secreted bFGF repre-
Antisense Adenovirus Reduces the Amount of bFGF Secreted into the Media—Increased levels of HSP27 in endothelial cells are thus correlative with increased estrogen-induced bFGF release. To determine if reduced levels of HSP27 would abrogate this response, BAEC cells were infected with either non-replicating control or HSP27-antisense adenosivirus vectors. The expression of the bovine HSP27 homolog (HSP25) was dramatically decreased in the antisense-infected cells (Fig. 3). Infection of 10^7 virus particles/cm² of culture area for 2 days reduces HSP25 levels by a third to one-half as measured by densiometry of immunoblots using a rabbit polyclonal antibody raised against murine HSP25 (Fig. 3). Infection with control virus had no effect on HSP25 levels. The viability of all the infected BAEC cultures was 96 ± 1.3% (n = 4), and neither virus affected overall secretion of proteins into the conditioned media. The amount of radioactivity of [35S]methionine-labeled proteins secreted into the media were determined to be 13,000 and 15,200 cpm/mg of cellular protein for antisense- and control-infected BAECs, respectively. As shown in Fig. 3, infection of the antisense adenosivirus dramatically reduced the level of bFGF in the culture media, whereas the control virus had no effect. No quantitative differences were noted in the amount of bFGF present in either the Triton-soluble or Triton-insoluble cellular fractions (Fig. 3). It thus appears that, not only does HSP27 expression correlate with increased estrogen-induced bFGF release, but HSP27 may be necessary for bFGF release.

bFGF Co-precipitates with HSP27 by Immunoaffinity Chromatography—To begin to elucidate the role of HSP27 in bFGF release, immunoprecipitations of cell extracts using immobilized anti-HSP27 and anti-bFGF monoclonal antibodies were performed. Eluted material was then subjected to immunoblot analyses using the same antibodies. As demonstrated in Fig. 4, anti-HSP27 antibody precipitated HSP27 (panel C, lane 7) and anti-bFGF immunoreactive material (panel B, lanes 3 and 4). The latter was overwhelmingly HMW bFGF, whereas the anti-bFGF precipitated all forms (panel B, lane 5), with the main form representing the 18-kDa species. Anti-bFGF chromatography precipitated HSP27 (panel C, lane 8) along with cellular bFGF. Since the antibodies do not recognize a common determinant, as demonstrated by the failure of the anti-bFGF antibody to stain HSP27 in cell extracts (panel A, lane 1) and failure of the anti-HSP27 antibody to stain bFGF (panel A, lane 2), the co-precipitation of both proteins is the result of an interaction between the HSP27 and bFGF.

**DISCUSSION**

The small molecular weight heat shock protein (HSP27) is an estrogen-responsive protein, which has profound effects on cell

---

**FIG. 2.** Anti-bFGF immunoblot analysis. Panel A, the conditioned media (CM), Triton X-100 lysates (Tx), and the Triton-insoluble material (Tx-Ins) of HSP27 BAECs (lanes 1 and 2) or vector control BAECs (lanes 3 and 4) cultured with (lanes 1 and 3) or without 100 nM β-estradiol (lanes 2 and 4) were analyzed by immunoblot analysis using an anti-bFGF antibody. Panel B, serially diluted (2-fold) samples of bFGF, beginning with 15 ng of rbFGF, were subjected to immunoblot analysis for the purpose of generating a standard curve. Densiometric analysis was performed using the Eagle Eye II computerized digital camera and Eagle Sight 3.0 software (Stratagene). IDU, integrated density units.

**FIG. 3.** HSP27-antisense infection of BAECs. Triton X-100 lysates (Tx), the Triton-insoluble material (Tx-Ins), and trichloroacetic acid-precipitated culture media (Cult Med) of BAEC cultures infected with HSP27 antisense (A) or control (C) adenoviruses were subjected to immunoblot analyses using anti-HSP25 and anti-bFGF antibodies. The position of HSP25 (HS) and the HMW and 18-kDa forms of bFGF are indicated.
growth (14) and microfilament cytoskeletal dynamics (28). Expression of HSP27 in estrogen responsive breast tumors (i.e., tumors requiring β-estradiol for growth and therefore susceptible to anti-estrogen therapy) is correlative with the growth of these tumors (29, 30). The exact mechanisms by which enhanced HSP27 levels impart a phenotype of accelerated growth have not been firmly established.

The data presented in this report demonstrate that enhanced HSP27 expression facilitates the release of bFGF from endothelial cells (Fig. 2). This secreted bFGF, which was predominantly HMW bFGF, was mitogenic to breast tumor cells (Fig. 1). The 18-kDa and HMW bFGF have different capacities to modulate cellular function and may work through different binding mechanisms and intracellular signals (31). For example, HMW bFGF can support growth in low serum media at levels at which the 18-kDa bFGF form does not (31). This is consistent with the results of the assays detailed in this report in which MCF-7 growth is observed in the serum-free media obtained from the HSP27 BAECs (Fig. 1). The difference in cellular response to the two forms of bFGF may be due to unique signals induced from the binding of HMW bFGF to an intracellular or nuclear membrane receptor or from direct regulation of transcriptional activity in the nucleus (32, 33).

The MCF-7 cell line obtained for this study expresses an estrogen receptor and exhibits a mitogenic response to β-estradiol. Co-culture of these cells with the HSP27 BAECs accelerated the growth of the estrogen-responsive cells induced by β-estradiol alone (data not shown). Subculture of the MCF-7 cells in steroid-deficient media selected for cells that can grow in the absence of estrogens and generated cultures that exhibited decreased expression of the estrogen receptor. These cells no longer exhibited a mitogenic response to β-estradiol alone (Fig. 1) and thus roughly approximated estrogen-unresponsive breast tumors. Use of these cells in this study abrogated the estrogen-induced autocrine effects of β-estradiol and allowed the direct test of the effects of β-estradiol on the paracrine activity of the BAECs (Fig. 1).

The paracrine communication responsible for the results depicted in Fig. 1 may have relevance to the pathogenesis of breast adenocarcinomas. Endothelial cells isolated from a wide range of tissue and vessel types, including the BAECs employed in this study, possess demonstrable estrogen receptors and are capable of exhibiting genomic responses to β-estradiol (34–36). For example, culture of the BAECs employed in this study in the presence of β-estradiol for several days induced enhanced HSP27 expression (14). Thus, the potential exists for the capillary endothelial cells involved in the vascularization of breast tumors to respond in a similar fashion and increase HSP27 expression, which then would facilitate bFGF release.

The growth of estrogen-unresponsive tumors, therefore, would still be affected by β-estradiol present in the circulation even though the tumors are refractory to the steroid itself.

Secretion of bFGF has been postulated to occur via pathways that are independent of the ER-Golgi vesicular pathway (12, 13). Florkiewicz et al. (12) have demonstrated that the 18-kDa form of bFGF is translocated through the plasma membrane in an energy-dependent manner. This translocation could be arrested if the transmembrane domain of an integral membrane protein was affixed to the carboxyl terminus of the bFGF. A putative role for HSP27 in this model is suggested by the fact that a significant portion of cellular HSP27 is associated with the plasma membrane (2). The data presented in this report, however, demonstrate that HSP27 associates predominantly with HMW bFGF, whereas the model described is specific for the 18-kDa form of bFGF. Mignatti et al. (13) demonstrated that agents which stimulate exocytosis (e.g., treatment with the ionophore A23187) facilitate bFGF release and that conditions which inhibit exocytosis (e.g., culture at 18°C or with methylamine) inhibited the release of bFGF which stimulated cellular migration in an autocrine fashion. Since inhibitors of ER-Golgi protein trafficking failed to inhibit the autocrine induction of migration, it was concluded that bFGF is released via exocytosis that is independent of the ER-Golgi pathway. A role for HSP27 in this putative mechanism of bFGF release is suggested by the fact that overexpression of HSP27 in fibroblasts has been demonstrated to increase the rate of pinocytotic activity (15). This phenomenon was attributed to the role of HSP27 as a modulator of F-actin dynamics at the plasma membrane (15). If similar mechanisms are involved in exocytosis, increased HSP27 expression may facilitate the release of bFGF via this pathway.

The HSP27-dependent release of bFGF release requires β-estradiol treatment of the HSP27 BAECs. The nature of this estrogen-dependence is as yet unknown but is not due to greater bFGF (Fig. 2) or HSP27 expression (data not shown). The latter is consistent with the report demonstrating that the induction of HSP27 production by β-estradiol requires 72 h (14). β-Estradiol exerts a myriad of receptor- and non-receptor-mediated events that result in both genomic and non-genomic changes in the cell. For example, the BAECs employed in this study have a demonstrable estrogen receptor, the activation of which could lead to altered transcriptional activity and the up-regulation of specific genes (37). These gene products may influence HSP27-mediated bFGF release. Another of the many mechanisms by which estrogen may induce HSP27-dependent release is the possibility that activated estrogen receptor may directly associate with the HSP27-bFGF complex, acting as part of the putative chaperon machinery. Interactions of peptide hormones with the estrogen receptor have been proposed as one explanation for the peptide hormone induction of estrogen receptor-dependent transcription (38). Whatever the mechanism, the association of HSP27 with bFGF is likely to be an important step in the release of bFGF and/or the estrogen dependence of the release.

Acknowledgment.—Plasmid containing the genomic clone of human HSP27 was a gift of L. Weber, University of Nevada, Reno, Nevada.

REFERENCES
The 27-kDa Heat Shock Protein Facilitates Basic Fibroblast Growth Factor Release from Endothelial Cells
Randolph S. Piotrowicz, Jody L. Martin, Wolfgang H. Dillman and Eugene G. Levin

doi: 10.1074/jbc.272.11.7042

Access the most updated version of this article at http://www.jbc.org/content/272/11/7042

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 14 of which can be accessed free at http://www.jbc.org/content/272/11/7042.full.html#ref-list-1