The Barrett’s Antigen Anterior Gradient-2 Silences the p53 Transcriptional Response to DNA Damage*

Elizabeth Pohler‡, Ashley L. Craig‡, James Cotton‡, Laura Lawrie§, John F. Dillon‡, Pete Ross‡, Neil Kernohan‡, and Ted R. Hupp¶¶

The esophageal epithelium is subject to damage from bile acid reflux that promotes normal tissue injury resulting in the development of Barrett’s epithelium. There is a selection pressure for mutating p53 in this preneoplastic epithelium, thus identifying a physiologically relevant model for discovering novel regulators of the p53 pathway. Proteomic technologies were used to identify such p53 regulatory factors by identifying proteins that were overexpressed in Barrett’s epithelium. A very abundant polypeptide selectively expressed in Barrett’s epithelium was identified as anterior gradient-2. Immunochemical methods confirmed that anterior gradient-2 neutralizes the colony enhancing activity of the gene, suggesting a key role for this domain in enhancing cell survival. Deletion of the C-terminal 10 amino acids of anterior gradient-2 neutralizes the colony enhancing activity of the gene, suggesting a key role for this domain in enhancing cell survival. Constitutive overexpression of anterior gradient-2 does not alter cell-cycle parameters in unstimulated cells, suggesting that this gene is not directly modifying the cell cycle. However, cells overexpressing anterior gradient-2 attenuate p53 phosphorylation at both Ser15 and Ser392 and silence p53 transcriptional function in ultraviolet (UV)-damaged cells. Deletion of the C-terminal 10 amino acids of anterior gradient-2 permits phosphorylation at Ser15 in UV-damaged cells, suggesting that the C-terminal motif promoting colony survival also contributes to suppression of the Ser15 kinase pathway. These data identify anterior gradient-2 as a novel survival factor whose study may shed light on cellular pathways that attenuate the tumor suppressor p53. Molecular & Cellular Proteomics 3:534–547, 2004.

Cancer development is a multistep process involving sequential mutation in oncogenes and tumor suppressor genes that give selective advantage to the evolving cancer cells. The major clinical models that are giving novel molecular mechanistic insight into the multistep evolution of human neoplasia include colorectal and esophageal cancer. Colorectal cancer progression is perhaps the most well-characterized model whereby cancer development evolves through histopathological stages termed dysplasia, adenoma, and carcinoma, which can eventually metastasize. Classic molecular studies in colorectal cancer have indicated that RAS and APC mutations occur earlier in this progression sequence, while p53 mutations occur relatively later (1). Additional modifying factors include genome instability, (2), DNA methylation (3), and associated epigenetic changes in the expression of regulatory genes that can have profound effects on cancer incidence (4).

Esophageal adenocarcinoma cancer progression also proceeds through a set of morphological intermediates termed metaplasia and dysplasia, which are collectively called Barrett’s esophagus or Barrett’s epithelium (5, 6). Barrett’s epithelium is thought to develop as an adaptive response following exposure to gastric and duodenal contents refluxed into the esophagus. Barrett’s epithelium is a premalignant lesion, and although the progression from Barrett’s cell types to adenocarcinoma is not inevitable, the risk is estimated at an increase of 30- to 125-fold as compared with the normal population (7–9). The incidence of Barrett’s epithelium has dramatically increased over the last decade; however, more alarming is the parallel increase in adenocarcinoma of the esophagus over a similar period of time (10). Barrett’s epithelium being hyperproliferative is believed to be a fertile field for malignant transformation, and such early premalignant lesions produce biological and genetic heterogeneity as seen in previous studies by p53 mutations, aneuploidy, and abnormal methylation resulting in stepwise changes in differentiation, proliferation, and apoptosis (11). However, the environmental, genetic, and metabolic factors that regulate stress responses in normal and Barrett’s epithelium, as well as the associated transformation onward into adenocarcinoma, are as yet undefined.

In contrast to colorectal cancer progression, p53 mutations in the esophageal adenocarcinoma progression model occur earlier in the progression sequence within dysplastic or even preneoplastic Barrett’s metaplastic cells (12–14). The earlier mutation of the p53 gene in this tissue suggests a unique
requirement for inactivation of the p53 pathway and presumably involves, in part, the type of unique environmental damage imposed upon cells of the esophagus. Cells of the human esophageal epithelium are under relatively unique environmental pressures being exposed on a daily basis to thermal stress from hot food intake, unmetabolized chemicals or food products, and refluxed acid, pepsin, and bile from the stomach (15, 16). Types of naturally occurring environmental agents that can both activate p53 protein and that play a role in tissue transformation into Barrett’s epithelium include stresses such as lowered oxygen concentration, low extracellular pH, and thermal injury (17–19). Such cellular damage or trauma appears to be an initiating event in the evolution of ulceration, p53 mutation, metaplasia, dysplasia, and adenocarcinoma of the esophagus.

Cells of the normal esophageal epithelium and metaplastic cells have therefore presumably evolved specific mechanisms to tolerate or repair injury induced by exposure to these and other damaging agents that are relatively unique to this tissue (20). It is of interest therefore to develop an understanding of the cellular defense systems that operate in parallel to p53 in normal squamous and Barrett’s epithelium of the human esophagus. We have therefore previously identified a novel class of heat shock proteins in normal squamous esophageal epithelium; these include SEP70, SEP53, and glutamine-glutamyl transferase (21). The function and regulation of these genes are not yet clear, but their identification is the first step in understanding the unique stress responses in the esophageal microenvironment. In this study, we complement the original identification of the stress proteins that are expressed in normal squamous epithelium by using proteomics to identify gene products that are up-regulated in Barrett’s epithelium. This approach has identified a major protein overexpressed in Barrett’s epithelium as anterior gradient-2, an estrogen receptor-responsive gene known to be overexpressed in breast cancers but without a known function. We show here that anterior gradient-2 functions as a survival factor and that it acts as an inhibitor of p53, thus identifying a novel pathway from which to gain insights into the mechanisms of p53 silencing during cancer progression.

MATERIALS AND METHODS

Lysis of Squamous and Barrett’s Epithelium—Esophageal normal squamous or long-segment Barrett’s epithelial biopsies were obtained from patients undergoing upper gastrointestinal endoscopy. Biopsy specimens were taken from 28 to 34 cm from the teeth, and the tissue was macroscopically normal, unless indicated to be of Barrett’s type. All patients gave informed consent, and the study has been approved by the Tayside Medical Ethical Committee. Samples were collected into phosphate-buffered saline (PBS)1 prechilled at 0°C and frozen in liquid nitrogen in order to examine the steady-state pattern of protein synthesis using Coomassie-stained SDS-polyacrylamide gels. Epithelial samples were harvested quickly in Dulbecco’s modified Eagle’s medium and then frozen. Samples were lysed by homogenizing in lysis buffer (all reagents were obtained from Sigma (Poole, United Kingdom) unless otherwise noted; 7 mM urea, 1% nonidet P-40; 25 mM HEPES, pH 7.8; 5 mM dithiothreitol (DTT); 0.4 M KC1; 5 mM EDTA; 10 mM NaF; 2 μM/ml Pefabloc (Boehringer-Mannheim, Mannheim, Germany); 20 μg/ml Leupeptin; 1 μg/ml Aprotinin; 2 μg/ml Pepstatin; 10 μg/ml trypsin inhibitor; and 1 mM benzamidine) and incubated at 0°C for 15 min (20). Soluble supernatant was obtained after centrifugation at 14,000 rpm in a minicentrifuge at 2°C for 10 min, and protein concentrations were determined by the method of Bradford (22). One- or two-dimensional gel electrophoresis was carried out as described previously (21).

Mass Spectrometric Analysis of Proteins in Normal Squamous and Metaplastic Epithelium—Populations of normal squamous epithelium were lysed using urea lysis buffer (8 M urea; 50 mM HEPES, pH 7.6; 5 mM DTT; 0.4 M KC1; 1 mM benzamidine, 50 mM NaF) or lysis buffer (as above) to extract the polypeptides. Two-dimensional gel electrophoresis when necessary was carried out by adding 100 μg of total protein into 100 μl of solubilization buffer (8 M urea, 1% (v/v) Triton X-100, 40 mM Tris base, and 10 mM DTT), applied to an Immobiline Dryplate (pH 4.0–7.0; Pharmacia, Uppsala, Sweden) equilibrated in denaturation buffer (8 M urea, 0.05% (v/v) Triton X-100, 0.1 M DTT, and 0.5% (v/v) Pharmalyte, pH 3–10), and focused for 7 KWh with a circulating water-cooling unit set at 15°C. The second-dimension electrophoresis was performed using standard SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Polypeptides whose expressions were dramatically distinct were processed for mass spectrometric sequencing by the UK Protein Sequencing Facility in Aberdeen as described previously. Voyager-DE STR MALDI-TOF mass spectrometer was operated in the reflectron-delayed extraction mode (Biosystems, Warrington, United Kingdom). Spectra were calibrated internally using trypsin autodigestion products. A nonredundant protein sequence database (NCBI utilized by the Mascot search engine) was used for searches, with parameters including: cysteine as S-carbamidomethyl derivative and maximum peptide mass error of 50 ppm (23).

Immunochemical Methods—Following electrophoresis, protein from polyacrylamide gels was transferred to nitrocellulose (Hybond C, Amersham, Little Chalfont, United Kingdom), and protein blots were blocked by incubating with PBS/0.1% Tween-20/5% milk (PTM), incubated with purified monoclonal antibodies (2 μg/ml in PTM) overnight at 4°C, and developed with anti-mouse IgG-secondary antibody conjugated to peroxidase using enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden). The monoclonal antibodies were obtained as indicated: the polyclonal and monoclonal antibodies to anterior gradient-2 were derived from recombinant protein. p53 antibodies and phospho-specific antibodies were described previously (24, 25). The monoclonal antibody (MB.H2) with cross-reactivity to the major heat shock protein in normal squamous epithelium (named SEPlO7, squamous epithelial stress protein-70 kDa) was generated as described (21).

Cell Assays—Colonies formation assays were developed by transfecting the p53-null cell line, H1299, with 1–2 μg of the constructs indicated in Figs. 3, 4, and 9, which include genes subconed by recombination into the Gateway pCMV destination vector as indicated by the manufacturer (Invitrogen, Poole, United Kingdom): wild-type p53, mutant HIS175p53, and the various anterior gradient-2 constructs. Twenty-four hours post-transfection, cells were trypsinized, washed, and identical numbers of cells were plated into media containing 0.5 mg/ml neomycin or Geneticin. Two to 3 weeks post-plating, cells were washed with PBS and fixed for 30 min in methanol at –20°C, then stained with Giemsa stain and colonies counted. To construct stable cell lines overproducing anterior gradient-2 is a negative regulator of p53.

1 The abbreviations used are: PBS, phosphate-buffered saline; DTT, dithiothreitol; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; UV, ultraviolet; RLU, relative light unit; ER, endoplasmic reticulum.
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cDNAs transcribed from each sample RNA were also amplified with attB2. H1299 Anterior Gradient-2-positive and -negative cells were grown to flow cytometry, actinomycin D treatment of cells was as follows: 10,000 cells were collected by FACScan and analyzed with the p21-luciferase, and activity from the p21 promoter. H1299 cells were transfected with anterior gradient-2-encoding plasmid or control plasmid in the Gateway destination backbone (Invitrogen) and processed for colony formation as described above. Discreet colonies were ring-cloned by trypsinization and transferred into 24-well plates containing media plus 0.5 mg/ml Genetix. Each colony was expanded into larger dishes until sufficient cells were obtained for analysis by Western blotting and reverse transcription-PCR (RT-PCR) in order to determine their anterior gradient-2 status. For flow cytometry, cells were trypsinized, washed with PBS, then permeabilized in 70% ethanol for a minimum of 2 h. Cells were then washed with PBS and resuspended at a concentration of 1 × 10^6 cells per ml in 50 µg/ml propidium iodide in PBS. In each assay, 10,000 cells were collected by FACScan and analyzed with the CellQuest program (Becton Dickinson, Mountain View, CA). Prior to flow cytometry, actinomyein D treatment of cells was as follows: H1299 Anterior Gradient-2-positive and -negative cells were grown to 70–80% confluence in six-well dishes then treated with 1 µg/ml actinomycin D for 18 h. Cells were harvested 18 h later and processed for FACS analysis. For bromodeoxyuridine (BrdUrd) treatment, anterior gradient-2-positive and -negative cells were grown to 70–80% confluence, in pairs, in six-well dishes, then one of each pair was treated with 1 µg/ml actinomycin D for 18 h. Each culture was then pulsed with 30 µM BrdUrd for 15 min before harvesting and preparing for FACS analysis.

Recombinant Gene Construction—Human anterior gradient-2 was subcloned into the Gateway entry vector pDONR201 (Invitrogen), and the sequence was confirmed by DNA sequence analysis. The anterior gradient-2 gene was transferred by in vitro recombination into expression systems for use in bacteria, insect cells, and mammalian systems. Truncations of anterior gradient-2 were constructed using designed oligonucleotides that contained 5’ sequences from the coding region and that allowed their use in the Gateway cloning system and 3’ sequences that permitted removal of lengths at the 3’ end of the anterior gradient-2 gene. Thermal cycling conditions comprised an initial step of 95 °C for 15 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min then, a final extension at 72 °C. This truncated PCR amplimer was cloned into the Gateway pDONR201 vector (Invitrogen) then transferred by an in vitro recombination event into a mammalian expression system vector. This construct was used in a colony formation assay as described above. RT-PCR of anterior gradient-2 was carried out to measure its expression. H1299 (anterior gradient-2-positive and -negative cells) created above were grown to 70% confluence then harvested using trypsin. Cells were washed in PBS and total RNA was recovered (RNasey Mini Kit; Qiagen, West Sussex, United Kingdom). One microgram of RNA from each was used for reverse transcription using oligo(dT) and Superscript II reverse transcriptase (Life Technologies, Inc., Grand Island, NY). The PCR mixture contained 10% of the reverse transcription reaction mix, 0.5 µM oligonucleotide primers, 0.8 mM dNTP mix, 2.5 U of HotStarTaq (Qiagen), and 1.5 mM MgCl2. The sequences of the oligonucleotide primers used were derived from the attB1 and attB2 sequences of the Gateway cloning system (Invitrogen). The cDNAs transcribed from each sample RNA were also amplified with β-actin oligonucleotides to verify that equal quantities of cDNA were used in each AG-2 amplification reaction.

p53 Reporter Assays and p53 Quantitation—To measure p53 activity from the p21-luc promoter, H1299 anterior gradient-2-positive and -negative cell lines were grown to 70–80% confluence, in duplicate, in six-well plates then transfected with either 1 µg each of p53, p21-luciferase, and β-gal reporter constructs or control vector, p21-luciferase, and β-gal reporter constructs (26). Following an 18-h incubation, media was removed from all cultures, and one of each plate was treated with 50 J/m² UV-C. Fresh medium was applied to each dish, and cells were incubated for a further 6 h before preparing for analysis of luciferase and β-gal activity using luciferase and β-gal assay kits (Promega, Southampton, United Kingdom). Protein concentrations of the cell lysates were determined by the method of Bradford, then 10 µg of total protein per lane separated by electrophoresis on an SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose by Western blotting then probed with antibodies raised against anterior gradient-2, p53, phospho-SER392-p53 (24), or phospho-SER15-p53 (New England Biolabs, Beverly, MA). Immunoreactive protein bands were quantified using a Syngene Bioimaging System (Cambridge, United Kingdom).

RESULTS

Identification of a Novel Polypeptide Overproduced in Barrett’s Epithelium as Anterior Gradient-2—Lysates from normal squamous epithelium and Barrett’s intestinal-type metaplastic epithelial biopsies from the same patient were separated using two-dimensional gel electrophoresis. The major proteins that were differentially expressed in either normal or Barrett’s epithelium were excised from the gel and finger-printed after trypsinization using a mass spectrometer (Fig. 1A). A representative region of the two-dimensional gel is depicted where down-regulation of two signaling proteins were observed in Barrett’s epithelium (Fig. 1B). These two polypeptides were identified as the p53-inducible gene products 14-3-3-ρ (27) and HSP27 (28) (Table I), and their down-regulation in Barrett’s epithelium suggests that p53 may be attenuated as a transcription factor. Other proteins of interest identified by this method include galectin-7, annexin-1, and calgranulin A (Table II). Interestingly, galectin-7 is another p53-inducible gene product (29), thus bringing the total to three known p53-inducible gene products that are expressed in normal but not Barrett’s epithelium.

A representative region of the two-dimensional gel is depicted where up-regulation of protein expression was observed in Barrett’s epithelium (Fig. 1, C and D). These two polypeptides were identified as carbonic anhydrase II (Fig. 1C, right versus left panel; and Table III) and anterior gradient-2 (Fig. 1D, right versus left panel; and Table III). Anterior gradient-2 has no known function and was originally cloned from an RNA species expressed in the anterior region of the Xenopus egg (30), and anterior gradient-2 was identified independently as a gene induced by the estrogen receptor in a differential RNA display (31). A recent RNA microarray analysis also identified anterior gradient-2 as a major gene overexpressed in breast cancers (32, 33), thus confirming the original differential display analysis. Immunochemical Assays Confirm that Anterior Gradient-2 Is Overexpressed in Barrett’s Epithelium—Polyclonal and monoclonal antibodies were generated to anterior gradient-2 protein to be used for determining whether or not anterior gradient-2 is, in fact, overexpressed in Barrett’s epithelium. The antibodies can detect recombinant anterior gradient-2 specifically in lysates from insect cells infected with recombinant insect viruses encoding human anterior gradient-2 (Fig. 2A, lanes 7 and 8), but not in cells infected with a virus control.
Furthermore, some esophageal adenocarcinoma cell lines show a strikingly variable expression of anterior gradient-2 (Fig. 2A, lanes 5 and 6) indicating that the antibodies can be used to selectively detect anterior gradient-2 in lysates from human cancer cell lines. H1299 cells do not express detectable anterior gradient-2 (Fig. 2A, lane 2).
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TABLE I
Molecular masses of tryptic fragments produced from key stress proteins selectively expressed in normal but not Barrett’s epithelium

| Observed M, | Sequence | Start-end |
|------------|----------|-----------|
| HSP27 6    | GPSWDPFR | 13–20     |
| 987.61     | RVPFSLLR | 5–12      |
| 1075.63    | QLSSQVEIR | 80–89   |
| 1104.52    | ODEHGYISR | 128–136 |
| 1163.68    | LFDAQFLPR | 28–37   |
| 1906.04    | LATQSNETPVFSER | 172–188 |
| 14-3-3αv  |          |           |
| 1070.54    | EMPPTPNR | 161–169*(oxM) |
| 189.72     | DSTLMOQLIR | 215–224 |
| 1205.67    | DSTLMQQLIR | 215–224*(oxM) |
| 1617.96    | NLLSVAYKNNVGGQRR | 42–56  |
| 2439.24    | VETELQGVCDTVGLLLSDHSLIK | 88–1109 |
| 2696.49    | EKETELQGVCDTVGLLLSDHSLIK | 86–1109 |
| 2833.55    | LGALSNSVFHVHEYENANPSAEEKSALAK | 170–195 |

TABLE II
Major structural proteins differentially expressed

| Normal epithelium | Barrett’s epithelium |
|-------------------|----------------------|
| Keratin 13        | Tropomyosin          |
| Keratin 4         | Annexin A2           |
| Keratin 6D        |                      |
| Galectin-7        |                      |
| Annexin-1         |                      |
| Calgranulin A     |                      |

and polarized staining at the apical surface of the epithelium (Fig. 2G). This interestingly suggests a function for the protein at the cell membrane, especially relevant given a recent report demonstrating that a proportion of anterior gradient-2 can localize to the plasma membrane as well as endosomes (32, 33).

Anterior Gradient-2 Antagonizes the DNA Damage Cell-Cycle Checkpoint Pathways—It is critical to define the mechanism of function of anterior gradient-2 in order to give its overexpression in Barrett’s epithelium biological significance. Is anterior gradient-2 functioning as a growth suppressor, a proliferative factor, or a pro-apoptotic protein? For example, anterior gradient-2 may activate p53, but this is being silenced in Barrett’s epithelium by a dominant proliferative-promoting factor. Nevertheless, the simplest model we have takes in account anterior gradient-2 is overexpressed in Barrett’s epithelium, which suggests it may be involved in cell-cycle progression, because this epithelium is highly proliferative. Consistent with this hypothesis, anterior gradient-2 co-expression with the estrogen receptor in breast cancers (31–33) or in ovarian cysts (Fig. 2G) indicates that it can be overproduced in proliferative diseases other than Barrett’s epithelium. Selective pressures are beginning to be placed in the tumor suppressor p53 pathway in Barrett’s epithelium (6), and we examined whether anterior gradient-2 acts like a proto-oncogene in several biological assays: i) colony formation and cell survival; ii) cell-cycle perturbation; and iii) p53 pathway perturbation. Whether anterior gradient-2 antagonizes p53 or synergizes with p53 would give information on the selective pressures that drive p53 mutation in Barrett’s epithelium. Alternatively, this would give information on the selective pressures that drive p53 mutation in Barrett’s epithelium (6), and we examined whether anterior gradient-2 acts like a proto-oncogene in several biological assays: i) colony formation and cell survival; ii) cell-cycle perturbation; and iii) p53 pathway perturbation. Whether anterior gradient-2 antagonizes p53 or synergizes with p53 would give information on the selective pressures that drive p53 mutation in Barrett’s epithelium.
pressures that maintain the wild-type p53 gene, which is present ranging from a 30–80% frequency in both Barrett’s epithelium and breast cancers.

A classic assay to examine the function of an unidentified cancer gene is a colony formation assay, which measures the ability of a gene product to influence cell survival under the transient stress of cell attachment and growth under low-density conditions in vitro. For example, the transfection of the tumor suppressor p53 gene into cells can suppress the number of colonies formed, relative to vector DNA-only control (Fig. 3, B and A, respectively; Fig. 3G), indicative of a growth suppressor. By contrast, the mutant p53HIS175 protein, which can function as a proto-oncogene (34), enhances colony formation (Fig. 3, C versus A, Fig. 3G). The transfection of the anterior gradient-2 gene enhances colony formation (Fig. 3, D and G) at nearly identical levels to that produced by the p53HIS175 allele (Fig. 3G). Various deletions of the C terminus of anterior gradient-2 (trunc A and B) abolishes its activity as an enhancer of colony formation (Fig. 3, E and F) and gives a colony formation assay similar to vector only (Fig. 3, A and G). These data indicate that anterior gradient-2 can in fact function like a mutant oncogenic p53 allele and suggests that its co-expression with the estrogen receptor in breast cancers may promote cell survival (31–33).

From the colony formation assay, stable cells lines overexpressing anterior gradient-2 (AG-2+ cells) and cell lines from vector only (AG-2− cells) were established and used as an isogenic cell panel to further study anterior gradient-2 effects on proliferation. The lung cancer cell line H1299 was used for making the constitutively overproducing anterior gradient-2 for two reasons. First, the cell line does not produce detectable protein (Fig. 2A), and the esophageal cell lines OE19 and OE33 produce some AG-2 (Fig. 2). Second, although it may seem more appropriate to study anterior gradient-2 in an esophageal cell line, there are no esophageal cell lines available with a well-known p53 status. This is relatively important,
as our aim was to analyze the effects of anterior gradient-2 on
the p53 pathway, and H1299 cells are a very common p53-
null cell line used to study p53 activity and degradation after
p53 gene transfection. Furthermore, the expression of anterior
gradient-2 in breast cancers indicates that it is equally impor-
tant to analyze the function of anterior gradient-2 as a survival
factor not only in esophageal cells but in other cancers. As
such, the H1299 tumor cell line is a good model to begin to
dissect anterior gradient-2 functional links to p53.

Anterior gradient-2 protein is specifically detected in AG-2+/H11001
cells (Fig. 4A, from left, lane 1) compared with the isogenic
anterior gradient-2-negative cells (Fig. 4A, from left, lane 5).
Furthermore, the steady-state levels of anterior gradient-2
protein are controlled by the proteosome, because treatment
of cells with the proteosome inhibitor ALLN for 2 h can sta-
bilize the protein (Fig. 4A, from left, lanes 2–4 versus lanes
6–8). Using RT-PCR methods, anterior gradient-2 gene ex-
pression is also elevated in the AG-2+/H11001 cell line (Fig. 4B, lane
2), relative to AG-2−/− cells (Fig. 4B, lane 4), thus indicating that
the stable AG-2+/− cells express more AG-2 protein than the
AG-2− cells. Using this isogenic panel of AG-2+/−/− cells, the
cell-cycle parameters were examined to determine whether
stable overproduction of anterior gradient-2 affects cell-cycle
progression. Surprisingly, the G1/S and G2/M distribution of
the cell cycle in control AG-2−/− cells was similar to the AG-2+/−
cells (Fig. 5, A and B), indicating that anterior gradient-2
overproduction does not interfere substantially with cell-cycle
progression. There were also no effects on DNA synthesis
using BrdUrd-labeled cells (data not shown). The ability of
anterior gradient-2 to enhance colony survival (Fig. 3) is there-
fore apparently not due to its effect on basal cell-cycle
changes, but to its activity under stressful conditions of cells
surviving as single colonies in a colony suppressor assay.
That is, if anterior gradient-2 is functioning independent of
cell-cycle control it may function under “stressed” conditions.

We used standard DNA-damaging agents as a model stress
( actinomycin D, UV light), as these DNA-damaging agents are
used as models to activate the tumor suppressor protein p53.
When cells were exposed to low levels of the DNA-damaging
agent actinomycin D, there was a substantial shift in the
population of AG-2+/− cells accumulating in G2/M, relative to
control AG-2−/− cells (Fig. 5, D and C). The incubation of AG-2+/−
cells and AG-2− cells with BrdUrd and separation by flow
cytometry indicates that anterior gradient-2 is not permitting
DNA replication in the presence of damage (data not shown).
Furthermore, the esophageal stress protein SEP53-overpro-

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**Fig. 3.** Anterior gradient-2 enhances cell survival in a colony formation assay. H1299 cells were transfected with the indicated gene (1 μg) encoding: A, vector only; B, wild-type p53; C, mutant p53 encoded by the HIS175 allele; D, anterior gradient-2; E, anterior gradient-2 (Truncation A); F, Anterior Gradient-2 (Truncation B); and G, anterior gradient-2 (Truncation C). For a relative position of each truncation, see Fig. 9. Two days after transfection, cells were trypsinized and serially plated into media containing 0.5 mg/ml geneticin, grown for 2 weeks, fixed with dyes, and the colony number was determined as an average of three independent experiments (summarized in H).
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A. AG-2 protein levels

![AG-2 protein levels graph]

B. AG-2 RNA levels

![AG-2 RNA levels graph]

Fig. 4. Development of stable cell-overproducing anterior gradient-2. Because anterior gradient-2 can enhance colony survival, cells constitutively overproducing the protein were isolated and a representative matched cell panel was analyzed for protein and RNA expression. H1299 do not express anterior gradient-2 (Fig. 2) and were used for generating constitutively expressing cells. A. Anterior gradient-2-overproducing cells (lanes 1–4) and control cells (lanes 5–8) were treated with different concentrations of the proteosome inhibitor ALLN for 2 h (0, 2, 5, and 10 μM of ALLN). Cells were lysed and blotted to probe for anterior gradient-2 protein levels using immunoblotting methods. B. RNA was isolated from anterior gradient-2-overproducing cells (lanes 1 and 2) or control cells (lanes 3 and 4), and the levels of Anterior Gradient-2 RNA (lanes 2 and 4) or β-actin RNA (lanes 1 and 3) were determined using RT-PCR.

Reducing cells do not alter cell-cycle parameters after actinomycin D treatment, relative to and SEP53-negative cells (data not shown), indicating the cell-cycle perturbation is relatively specific for anterior gradient-2. Thus, alterations in cell-cycle progression in damaged cells is consistent with anterior gradient-2 being a survival factor under conditions where Barrett’s epithelium or breast cancers is stressed. Such an accumulation of cells at G2/M in damaged cells may subsequently enhance the survival or propagation of injured cells that can either lead to cell death as cells attempt to exit G2/M or lead to survival of mutant clones with enhanced proliferative capacity in a damaged microenvironment. However, not all stresses induce this cell-cycle change; as UV irradiation did not alter cell-cycle distribution in this anterior gradient-2-overproducing, p53-null cell line (data not shown).

Anterior Gradient-2 Attenuates the p53 Response.—Two biological assays have thus far defined a survival function for anterior gradient-2; enhanced activity in colony formation assays and alterations in cell-cycle parameters but only in damaged cells. Because anterior gradient-2 is expressed in Barrett’s epithelium where selective pressures are beginning to be placed on p53, it was critical to define whether anterior gradient-2 antagonizes or stimulates p53 activity. Two outcomes were possible. Stimulation of p53 transcription activity by anterior gradient-2 could be an important mechanism to arrest and repair injured cells, and this could eventually provide the selective pressure for p53 mutation in Barrett’s epithelium. Alternatively, a p53 silencing mechanism induced by anterior gradient-2 would suggest a direct mechanism to attenuate p53-dependent checkpoint control in the proliferating Barrett’s epithelium or in breast cancers. This could provide a selection pressure for maintaining a wild-type p53 gene in Barrett’s epithelium and breast cancers. In hindsight, silencing of p53 activity by anterior gradient-2 may be predicted from the start, because we detected down-regulation of the p53-inducible gene products galectin-7, 14-3-3σ, and HSP27 in Barrett’s epithelium compared with normal squamous epithelium (Fig. 1B; Table III).

The transfection of the p53 gene into p53-null cells along with a p53-responsive luciferase reporter gene can lead to a p53-dependent increase in gene expression (Fig. 6A, p53 panel versus vector only). Furthermore, the stimulation of p53 activity can be accomplished by co-transfection of the p53 gene with increasing amounts of its partner protein p300 (Fig. 6A, p53/p300 panels versus p53 panel). By contrast, the co-transfection of increasing amounts of anterior gradient-2 into cells with the p53 gene results in the attenuation of p53 activity from reporter templates (Fig. 6A, p53/AG2 panels). A similar inhibition of p53 activity can be achieved with the p53 inhibitor MDM2 (Fig. 6A, p53/MDM2 panels). Although these data indicate that anterior gradient-2 is a p53 inhibitor, we have found no evidence that it binds to p53 like MDM2. This hypothesis that anterior gradient-2 is a p53 inhibitor was further tested by examining p53 activity in the AG-2– and AG-2+ isogenic cell panel.

In undamaged AG-2– cells, the basal activity of p53 after the transfection of 0.12–0.5 μg of p53-expression DNA into cells with the p53-responsive luciferase reporter (Fig. 6B, No UV panels, ~48–70 relative light units (RLUs)) was reproducibly 2- to 4-fold higher than in the undamaged AG-2+ cells (Fig. 6C, No UV panels, ~17–21 RLUs). These data suggest that the overproduction of anterior gradient-2 can attenuate p53 activity, as seen in the anterior gradient-2 transfection (Fig. 6A). Furthermore, the activity of p53 after the transfection of 1.0 μg of p53 expression DNA into AG-2– cells with the p53-responsive luciferase reporter (Fig. 6B, No UV panels, ~3 RLUs) was attenuated relative to undamaged AG-2+ cells (Fig. 6C, No UV panels, ~70 RLUs). However, in UV-damaged AG-2– cells, there was nearly complete suppression of p53 activity (Fig. 6C, +UV panels versus no UV panels). By contrast, AG-2– cells did not show any differences in p53 activity in UV-damaged cells (Fig. 6B, no UV versus +UV panels). These data suggest that anterior gradient-2 activity is maximally recruited in injured cells to antagonize p53.

To determine a mechanism to account for how anterior gradient-2 silences p53 activity, we investigated whether anterior gradient-2 attenuates p53-activating kinases (ATM and CK2) or whether anterior gradient-2 stimulates MDM2-de-
dependent ubiquitination and degradation of p53. Both these two pathways are key controllers of the p53 response. Total p53 protein levels were similar as defined by densitometry after p53 transfection with reporter templates in undamaged AG-2\(^{+}\) cells (Fig. 7A, lanes 7–9) and undamaged AG-2\(^{-}\) cell panel (Fig. 7A, lanes 1–3). Furthermore, under conditions where p53 activity is silenced in UV-damaged AG-2\(^{+}\) cells (Fig. 6C), the p53 protein is not degraded in the AG-2\(^{+}\) cells and AG-2\(^{-}\) isogenic cell panel (Fig. 7A, lanes 4–6 and 10–12). These data indicate that the specific activity of p53 is decreased by anterior gradient-2 rather than a reduction in p53 activity by degradation of the protein. Consistent with this, we did not see any alteration in p53 ubiquitination by MDM2 in AG-2\(^{-}\) cells (data not shown). Furthermore, the decrease in the specific activity of p53 is not due to drastic overproduction of anterior gradient-2, because its levels are similar in undamaged or damaged the AG-2\(^{+}\) cells (Fig. 7B, lanes 7–12).

One of the key mechanisms whereby p53 protein is stimulated as a transcription factor is via changes in its phosphorylation at key phosphorylation sites, like Ser\(^{15}\) (modified by ATM/ATR) and Ser\(^{392}\) (modified by CK2). Because the specific activity of p53 can be changed by its phosphorylation (24, 35), we examined whether CK2 site phosphorylation at Ser\(^{392}\) was decreased. Using the same lysates from Fig. 7, A and B, where total p53 protein levels are not affected by UV damage in the AG-2\(^{+}\) cells (Fig. 7A, lanes 7–12), immunoblots were performed for Ser\(^{392}\) phosphorylation using a phospho-specific antibody for this site. In undamaged or damaged AG-2\(^{+}\) cells, there was little change in Ser\(^{392}\) site phosphorylation (Fig. 7C, lane 2 versus lane 1). However, in AG-2\(^{-}\) cells, UV damage induces an attenuation of Ser\(^{392}\) site phosphorylation.
A similar silencing of Ser\(^{15}\) phosphorylation was also observed (data not shown, see below). Instead of transfecting p53 into the anterior gradient-2-positive and -negative cell lines (Fig. 7), we transfected anterior gradient-2 into a p53 wild-type cell line to investigate whether anterior gradient-2 silenced endogenous p53 and associated Ser\(^{15}\) phosphorylation (Fig. 8). This latter phosphorylation is stimulated by an ATM- or ATR-dependent kinase pathway (36, 37) and is an assay that can be used to assess DNA damage to a cell. When A375 cells were damaged by UV irradiation, there was increases in Ser\(^{15}\) phosphorylation (Fig. 8A, lane 4 versus lane 1). p53 activation after irradiation by phosphorylation without changes in p53 levels has been observed previously in A375 cells (35). When A375 cells were transfected with anterior gradient-2, there was an attenuation of Ser\(^{15}\) phosphorylation (Fig. 8A, lane 5 versus lane 4) compared with total p53 levels (Fig. 8B, lane 5 versus lane 4).

Finally, we evaluated whether anterior gradient-2 activity as an enhancer of cell survival (Fig. 3) correlated with attenuation of p53 phosphorylation. The smallest truncation mutants of anterior gradient-2 that loose the endoplasmic reticulum (ER) retention sequence (Trunc D; Fig. 9H) do not support colony survival (Fig. 9, D versus C). In fact, one truncations mutant (Trunc F; Fig. 9H) acts like a dominant-negative effector and begins to suppress colony formation (Fig. 9F). The inactive truncation mutant of anterior gradient-2 (Trunc D) did not suppress Ser\(^{15}\) phosphorylation as well as mature anterior gradient-2 (Fig. 8A, lane 6 versus lane 5), suggesting that the colony survival activity of the protein is linked to its activity as a p53 kinase inhibitor.

**DISCUSSION**

The selective pressures that drive mutations in tumor suppressor genes like p53 in Barrett’s metaplasia are not clear, nor have the factors than maintain the wild-type p53 gene in metaplasia been defined (11). However, some of the key risk factors thought to be involved in Barrett’s pathogenesis and cancer progression are acid-bile reflux, heat shock, and oxi-
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A. p53 protein levels after p53 gene transfection

| Amount of p53 Gene (µg) | Control Cell | AG-2 Cell | UV Radiation |
|-------------------------|--------------|------------|--------------|
| 0.25                    | +            | +          | −            |
| 0.5                     | +            | +          | −            |
| 1.0                     | +            | +          | −            |

B. AG-2 protein levels after p53 gene transfection

| Amount of p53 Gene (µg) | Control Cell | AG-2 Cell | UV Radiation |
|-------------------------|--------------|------------|--------------|
| 0.25                    | +            | +          | −            |
| 0.5                     | +            | +          | −            |
| 1.0                     | +            | +          | −            |

C. AG-2 attenuates Ser15-phosphorylation of p53 in Irradiated cells

| P53 Gene (µg) | Control Cell | AG-2 Cell | UV Radiation |
|---------------|--------------|------------|--------------|
| 0.25          | +            | −          | +            |
| 0.5           | +            | −          | +            |
| 1.0           | +            | −          | +            |

Fig. 7. Anterior gradient-2 antagonizes p53-phosphorylation at Ser15 in damaged cells. A, anterior gradient-2 effects on p53 protein levels. AG-2- (lanes 1–6) and AG-2+ cells (lanes 7–12) were co-transfected with the increasing amounts of the p53 gene (0.25–1.0 µg), the reporter p21-luc, and pCMV-βgal. Twenty-four hours after transfection, cells were left untreated (lanes 1–3; 7–9) or damaged with UV (50 J/m²); lanes 4–6; 10–12), and 6 h later cells were harvested for quantitation of p53 protein levels by immunoblotting with the DO-1 monoclonal antibody. B, anterior gradient-2 protein levels in undamaged and irradiated cells. AG-2- (lanes 1–6) and AG-2+ cells (lanes 7–12) were co-transfected with the increasing amounts of the p53 gene (0.25–1.0 µg), the reporter p21-luc, and pCMV-βgal. Twenty-four hours after transfection, cells were left untreated (lanes 1–3; 7–9) or damaged with UV (50 J/m²); lanes 4–6; 10–12), and 6 h later cells were harvested for quantitation of anterior gradient-2 protein levels by immunoblotting with anterior gradient-2 polyclonal antibody. C, levels of p53 protein phosphorylation levels. AG-2- (lanes 1–2) and AG-2+ cells (lanes 3–4) were co-transfected with the increasing amounts of the p53 gene (0.25–1.0 µg, top, middle, and bottom panel, respectively), the reporter p21-luc, and pCMV-βgal. Twenty-four hours after transfection, cells were left untreated (lanes 1 and 3) or damaged with UV (50 J/m²); lanes 2 and 4), and 6 h later cells were harvested for quantitation of p53 phosphorylation at Ser15 by immunoblotting with FPS392-phospho-specific monoclonal antibody, as indicated previously (24).

By demonstrating that cell-cycle control proteins like p53 may be important modifiers of disease progression and/or patient variability. This hypothesis is supported by the later observations that the cell-cycle checkpoint transcription factor p53 is in fact mutated in pre-neoplastic Barrett’s metaplastic cells of the esophagus (12–14).

In order to gain novel insight into these two problems—the link between the protective stress proteins and p53 pathway perturbation—we have taken proteomics approaches to iden-
tify novel Barrett’s antigens (Tables I–III) to test in stress protein and p53 pathway assays. Apart from the cytoskeletal proteins that are differentially expressed in normal and Barrett’s epithelium (Table II), one striking feature to emerge was the switch in the type of abundant calcium-binding proteins that are used by normal squamous or Barrett’s epithelium. Normal epithelium expressed calgranulin A, which is a member of the S100 family of 2EF-hand calcium-binding motifs. S100 proteins are cytoplasmic and/or nuclear and involved in cell-cycle progression and differentiation. The second Ca$^{2+}$-binding protein expressed in normal epithelium was annexin 1, which belongs to a family of Ca$^{2+}$-dependent phospholipid-binding proteins that are located on the cytoplasmic face of the plasma membrane. It exhibits phospholipase A$_2$ inhibitory activity and may therefore affect synthesis of prostaglandins and leukotrienes, therefore modulating anti-inflammatory activity. Both of these two polypeptides were not expressed in Barrett’s epithelium, which in turn overproduced annexin A2 (Table I), which belongs to a family of Ca$^{2+}$-dependent phospholipid-binding proteins known to elevate osteoclast formation and bone resorption. Furthermore, the stress protein SEP53 also has some degree of homology in its N terminus to the S100 family of 2EF-hand calcium-binding motifs and SEP53 is differentially overexpressed in Barrett’s epithelium (unpublished data), indicating that SEP53 is not confined to squamous epithelium.

Anterior gradient-2 was the most novel protein up-regulated in Barrett’s epithelium, with the other two key proteins including carbonic anhydrase II and GRP94 (HSP90) (Table III). Anterior gradient-2 was first cloned by virtue of expression in *Xenopus* eggs, where the protein was reported to be secreted. Consistent with this, anterior gradient-2 can localize to the plasma membrane in Barrett’s epithelium and induction cysts (Fig. 2). Furthermore, a report cloning genes that are overexpressed in breast cancer membranes (31) identified an anterior gradient-2 homologue (32, 33). These later data confirm the original cloning of anterior gradient-2 as a gene cloned in a differential display of hormone estrogen receptor-induced genes and indicates that anterior gradient-2 is associated with proliferative cells. Consistent with this, our data show that anterior gradient-2 can enhance colony formation in survival assays, attenuate p53 activity, and suppress p53 phosphorylation after DNA damage, suggesting it is an inhibitor of the p53 pathway. Anterior gradient-2 therefore has the potential to act as a clinically relevant factor that modulates cell-cycle and p53 activity, presumably effecting patient responses to acid-reflux disease and altering selection for mutation in p53. The ability of anterior gradient-2 to suppress p53 activity could explain, in part, why there are lower frequency of mutations detected for p53 in breast cancers, a disease where anterior gradient-2 is overexpressed and where the wild-type p53 gene is maintained in a majority of cancers. On the other hand, if anterior gradient-2 is silencing p53 in Barrett’s epithelium, this does not explain why there is selection for mutation in p53 and further work will have to resolve this. However, in two key studies on the subject, the p53 mutation frequency in Barrett’s metaplasia is not 100%, but ranges from 20–70%, and we can speculate that the
metaplasia without p53 mutation has silenced p53 through a more active anterior gradient-2 pathway. Conversely, where p53 is mutated in Barrett’s epithelium, the anterior gradient-2 pathway may be less active. For example, there is variability in the levels of anterior gradient-2 and/or its cleavage to the smaller form (Fig. 2), suggesting that the protein is variable in patients in vivo. Future studies on dissecting the anterior gradient-2 pathway may identify modifiers that are themselves differentially expressed in Barrett’s epithelium that control the specific activity of the pathway.

The function and regulation of anterior gradient-2 are not known. However, we show here that anterior gradient-2 has a striking degree of homology to cytoplasmic thioredoxin, contains a hydrophobic ER leader sequence with a signal peptidase cleavage site at Ala^20/Lys^21 boundary, and the protein has an ER retention sequence in the C terminus that likely effects in intracellular trafficking (Figs. 1E and 9H). However, although Caenorhabditis elegans anterior gradient-2 has homology to the classic disulfide-active site region of thioredoxin (WCxxCK), human anterior gradient-2 gene lacks this classic disulfide-active site required for electron transfer (Fig. 1E). The mechanism of anterior gradient-2 attenuation of p53 appears not to be by direct binding to p53, but may relate to the ER retention sequence in anterior gradient-2 whose deletion inhibits the ability of the protein to reduce Ser^15 phosphorylation of p53. Our identification that anterior gradient-2 has homology to cytoplasmic thioredoxin gives two possible models to account for its function. The key observation, however, is that anterior gradient-2 has a peculiar loss of its disulfide center despite having strong homology to thioredoxin. Ribonucleotide reductase can be controlled by thioredoxins, and this former protein is a key modifier of p53-dependent responses to anticancer drugs (41). If anterior gradient-2 acts like a dominant-negative thioredoxin because it does not transfer electrons, it could poison the anti-oxidant electron transfer system, create a pro-oxidizing environment, and enhance the fixation of cell damage associated with cancer progression. An alternate function for anterior gradient-2 also relies on the loss of its disulfide center. The ER-resident chaperone named ERP29 has a strong degree of homology to the protein disulfide isomerase family of reducing agents, except that ERP29 also has only one of the two sulfurs that are required for electron transfer (42). Furthermore, ERP29 has an ER retention sequence, like anterior gradient-2, that is linked to its transport to the membrane where it cooperates with heat shock proteins in the maturation and secretion of thyroglobulin (43). This is intriguingly similar to anterior gradient-2; it only has one of two sulfurs in the active site despite a large degree of homology to a redox protein, it has a classic ER retention sequence, it localizes to the plasma membrane, and it can be secreted (30). Thus, it may be possible for anterior gradient-2 localization at the plasma membrane to attenuate the ATM and CK2 kinase pathways, by an undefined signaling pathway, that in turn reduces the specific activity of p53 as a transcription factor.

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¶ To whom correspondence should be addressed: Cancer Research UK Laboratories in the Department of Molecular and Cellular Pathology, University of Dundee, Dundee DD1 9SY, United Kingdom. Tel.: 44-1382-496-430; E-mail: Ted.Hupp@cancer.org.uk.

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