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Mammoglobin and Lipophilin Related Molecules in Normal and Tumor Human Breast Tissue: Expression, Hormone Regulation and Functional Analysis

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Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammoglobin (MGB1), hSBEM (Human Small Breast Epithelial Mucin), Psoriasin, Estrogen receptor beta (hERB) and SRA (Steroid receptor RNA activator), are currently studied in our group. We found that:

MGB1 expression correlates with ER (an established breast cancer prognostic and predictive factor), but is not modified by hormone treatments, suggesting it might be a new independent breast cancer prognostic marker.

hSBEM, which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics can now be detected using specific antibodies.

SRA, previously believed to believed to belong to the family of non-coding RNA and able to activate steroid receptor activity, is translated into a protein which function remains to be determined.

Psoriasin, highly expressed in high risk DCIS, can contributes to breast tumor progression through modulation of Jab1 activity.

hERB isoforms have promoter-specific differential activities suggesting that they may have a role in differentially modulating estrogen action during breast tumor progression.
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INTRODUCTION

Breast cancer remains one of the most frequently diagnosed cancers today. One in eight women is expected to present with breast cancer within her lifetime in developed countries. An estimated 1,000,000 cases are detected each year worldwide and in Canada alone, an estimated 21,200 women will be diagnosed with breast cancer and 5,300 will be lost to this disease in 2003 (1). For women with recurrent disease, the median time of survival is about two years (2). Despite such striking statistics, breast cancer related mortality is slowly decreasing as continuing research has led to earlier detection, more treatment options for breast cancer patients and an improved chance of long-term survival. Improving the diagnosis and clinical management of breast cancer requires access to and characterization of biomarkers that are able to reflect the molecular phenotype of breast tissue. Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammaglobin (MGB1), hSBEM (Human Small Breast Epithelial Mucin), Psoriasin, Estrogen receptor beta (hERB) and SRA (Steroid receptor RNA activator) are currently studied in our group.

BODY

MGB1: Mammaglobin A was first identified in 1996, as a breast specific member of the uteroglobin gene family overexpressed in some breast tumors (3, 4). As shown in APPENDIX 1, we evaluated MGB1 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively (5). Both MGB1 mRNA and protein expression were significantly higher in estrogen receptor positive compared to estrogen negative tumors (Mann-Whitney rank sum test, p = 0.04; Chi-square test, p = 0.01; respectively). In contrast, MGB1 expression did not correlate with progesterone receptor levels or Nottingham grade. As estrogen and antiestrogen treatment of estrogen positive breast cancer cell lines does not modify MGB1 expression we suggest that MGB1 could be a new independent breast cancer prognostic marker.

hSBEM: We reported last year the identification of a novel putative breast-specific gene (hSBEM, Human Small Breast Epithelial Mucin), which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics (6, APPENDIX 2). We have now obtained mammary cells MCF-7 stably expressing V5-tagged hSBEM protein as well as antibodies recognizing the transfected as well as the endogenous protein. We have just initiated a new project, funded by the CIHR and abstract of which is included in APPENDIX 3, to further explore the potential of this protein as a breast cancer biomarker.

Psoriasin: We have previously identified psoriasin (S100A7) as a differentially expressed gene between DCIS and invasive breast carcinoma (7). In collaboration with Dr. Watson, we have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) and we suggest that intracellular psoriasin influences breast cancer progression through stimulation of Jab1 activity (8, APPENDIX 4).
hERB: Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression. Estrogen action is mediated mainly through two estrogen receptors (ERs): ER-alpha (9) and ER-beta (10, 11). Several variant forms of ER-beta have been identified in breast tissues (for reviews see Refs. 12-15). In collaboration with Dr. Murphy, we investigated the putative functional characteristics of human receptor beta isoforms (16, APPENDIX 5). We showed that only ER-beta was able to bind ligand whereas all ER-beta isoforms bind to DNA even though their binding abilities differ. ER-beta isoforms inhibition of ER-alpha and ER-beta transcriptional activity is promoter specific. Overall, our data suggest that ER-beta isoforms may have a differentially modulating estrogen action. As shown in APPENDIX 6 we also found that the expression of hERB isoforms differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy suggesting a possible role of ERB related protein in hormone resistance (17).

SRA: SRA is a steroid receptor co-activator acting as a functional RNA and is classified as belonging to the growing family of functional non-coding RNAs (18). None of the different SRA transcripts described to date encode a detectable SRA protein following in vitro and in vivo translation experiments. We have previously shown that SRA RNA was differentially expressed in normal and tumor breast tissues (19, 20). We have identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5' extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines (21, APPENDIX 7).

KEY RESEARCH ACCOMPLISHMENTS

- We hypothesized that MGB1 could be a new independent prognostic marker of breast cancer.
- We obtained breast cancer cells stably expressing tagged hSBEM protein.
- We obtained antibodies recognizing both transfected and endogenous hSBEM protein.
- We established a possible mechanism of action for psoriasin in breast tissues.
- We confirmed that hERB isoforms are likely to play a role in response to hormone therapy.
- We have identified new SRA isoforms encoding stable SRA proteins.
- We obtained breast cancer cells stably expressing tagged SRA proteins.
- We obtained antibodies recognizing transfected and endogenous SRA proteins

REPORTABLE OUTCOMES

* Five articles published (APPENDICES 1, 4, 5, 6, 7)
CONCLUSION

Two projects are currently funded within the laboratory: hSBEM and SRA. We are now characterizing the different transfected cell lines and investigating the possible use of SBEM to detect in vivo breast cancer cells and the putative function of SRA protein.

REFERENCES


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APPENDIX 1: Endocrine. 2003. 21: 00-00
Mammaglobin (SCGB2A2) is a breast-specific member of the secretoglobin (SCGB) gene family. SCGB2A2 has previously been found overexpressed in breast tumors but possible associations between its expression and established prognostic tumor characteristics such as the levels of estrogen and progesterone receptors have not yet been investigated. We evaluated SCGB2A2 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both SCGB2A2 mRNA and protein expression were significantly higher in estrogen-receptor-positive compared to estrogen-receptor-negative tumors (Mann-Whitney rank sum test, \( p = 0.04 \); chi-square test, \( p = 0.01 \); respectively). In contrast, SCGB2A2 expression did not correlate with progesterone receptor levels or Nottingham grade. As estrogen and antiestrogen treatment of estrogen-positive breast cancer cell lines does not modify SCGB2A2 expression we suggest that SCGB2A2 may be a new independent breast cancer prognostic marker.

Key Words: SCGB2A2; MGB1; estrogen receptor; progesterone receptor; Nottingham grade; breast cancer.

Introduction

Mammaglobin (MGB1, SCGB2A2) was first identified in 1996, using differential display analysis, as a breast-specific member of the secretoglobin (SCGB) gene family overexpressed in some breast tumors \( (1,2) \). Today, a search for breast-specific expressed sequence tags (ESTs) performed using the Differential Gene Expression Display (DGED) tool at the Cancer Gene Anatomy Project (CGAP) website (http://cgap.nci.nih.gov/Tissues/GXS) shows that SCGB2A2-related ESTs have been found in nine different breast cDNA libraries but only two non-breast libraries, further confirming the relative breast specificity of SCGB2A2 expression. Using a subtractive hybridization approach, we previously identified SCGB2A2 mRNA as overexpressed in the \textit{in situ} compared to the invasive element within an individual breast tumor \( (3,4) \). Further \textit{in situ} hybridization analysis, performed in breast tumors selected to include normal, \textit{in situ}, and invasive primary tumor elements revealed that SCGB2A2 expression, restricted to epithelial cells, could be detected in all elements and was significantly increased in tumor cells compared to normal cells \( (4) \). This higher SCGB2A2 expression in malignant versus nonmalignant breast epithelium has also been confirmed at the protein level by immunocytochemistry \( (5) \). In this latter study, Watson et al. concluded that SCGB2A2 expression was independent of tumor grade and histological type.

It has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of SCGB2A2 mRNA \( (6-9) \). Even though its biological function remains unknown, SCGB2A2 is now considered as a relatively specific marker of axillary lymph node breast metastases as well as of occult breast cancer \( (10-13) \). Interestingly, Zach et al. detected SCGB2A2 mRNA expression by nested reverse-transcription PCR (RT-PCR) more frequently in the blood of patients with estrogen-receptor-positive (ER+) breast tumor than in the blood of estrogen-receptor-negative (ER−) breast cancer patients, suggesting a possible relationship between SCGB2A2 and ER levels in primary breast tumors \( (6) \). In order to investigate further possible associations between SCGB2A2 expression and estrogen and progesterone receptors in primary breast tumors, we assessed SCGB2A2 expression at the mRNA and at the protein level in a cohort of breast tumors.

Results

Assessment of SCGB2A2 mRNA Expression in a Cohort of 52 Human Breast Tumor Samples

To establish whether SCGB2A2 mRNA expression paralleled established known prognostic parameters such as ER and PR levels, a cohort of 52 cases was selected from the
Fig. 1. RT-PCR analysis of SCGB2A2 and GAPDH mRNA expression in primary breast tumors. Total RNA was extracted from frozen tissue sections corresponding to ER positive (ER+) and ER negative (ER−) cases, reverse-transcribed and PCR amplified as described in the Materials and Methods section using SCGB2A2- or GAPDH-specific primers. PCR products were then separated on 2% agarose gels prestained with ethidium bromide. Black arrow: product corresponding to SCGB2A2, grey arrow: product corresponding to GAPDH. M: Molecular weight marker (Φ×174 RF DNA/HaeIII fragments, Gibco BRL, Grand Island, NY).

Fig. 2. Quantification of SCGB2A2 mRNA expression in different breast tumor subgroups. Total RNA was extracted from frozen tissue sections corresponding to 52 cases and analyzed as described in Fig. 1. SCGB2A2 mRNA expression was quantified relative to GAPDH mRNA as described in the Materials and Methods section. Tumors were grouped according to their ER status (ER+, ER−), their PR status (PR+, PR−) or their grade (Low Gr: Nottingham scores between 5 and 7; High Gr: Nottingham scores between 8 and 9). Difference between subgroups was tested using the Mann–Whitney rank sum test, two-sided.

NCIC-Manitoba Breast Tumor Bank. For each case, clinical characteristics of the tumor (i.e., ER and PR levels, Nottingham grade) were known (see Materials and Methods for a summary of tumor subgroup characteristics). Total RNA was extracted from frozen primary tumor sections, reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically SCGB2A2 cDNA, and chosen to span intrinsic regions. As shown Fig. 1, SCGB2A2 corresponding signal can be detected in the majority of cases, even though levels of expression varied from one sample to another. Amplification of the ubiquitously expressed GAPDH cDNA in the same cDNA samples was performed in parallel and, for each case, a normalized SCGB2A2 mRNA expression value was calculated (see Materials and Methods). SCGB2A2 expression was found to strongly correlate with ER levels (n = 52, Spearman coefficient r = 0.282, p = 0.042) but not with PR levels or grade (data not shown). Similarly (Fig. 2), using the established clinical cut-off of ER positivity (ER positive tumors have a binding higher than 3 fmol/mg of total protein), SCGB2A2 mRNA expression was significantly (Mann–Whitney rank sum test, two-sided, p = 0.040) higher in ER+ (n = 33, median value SCGB2A2 = 0.62) than in ER− (n = 19, median SCGB2A2 value = 0.33).

Assessment of SCGB2A2 Protein Expression in a Cohort of 32 Human Breast Tumor Samples

In order to determine whether SCGB2A2 protein expression correlated with SCGB2A2 mRNA expression and whether a similar association between ER status and SCGB2A2 expression could be observed at the protein level, paraffin
blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression (see Materials and Methods). Slides were scored blindly for SCGB2A2 protein expression by a pathologist as described in the Materials and Methods section. Some sections showed no (Fig. 3A, SCGB2A2 score = 0) or low (Fig. 3B, SCGB2A2 score = 1) SCGB2A2 expression, whereas others presented strong SCGB2A2 protein signal (Fig. 3C, SCGB2A2 score = 3; Fig. 3D, SCGB2A2 score = 2). Comparison of SCGB2A2 protein scores and previously obtained normalized SCGB2A2 mRNA levels revealed a strong correlation (n = 32, Spearman r coefficient \( r = 0.575, p = 0.0006 \)) between protein and mRNA levels. Tumors were classified as low (scores between 0 and 1) and high (1.5 and 3) SCGB2A2 protein expressers, and differences between tumor subgroups (ER+/ER−, PR+/PR−, low grade/high grade) were assessed using chi-square test. As observed for SCGB2A2 mRNA, SCGB2A2 protein positivity was associated (chi-square test, \( p = 0.017 \)) with ER status but not with PR status or grade (Fig. 4).

**Absence of Estrogen Regulation of SCGB2A2 Expression**

These data suggested that estrogen might regulate SCGB2A2 expression. In order to address the question of a possible regulation of SCGB2A2 expression in breast cancer cells, ZR-75 cells, known to express SCGB2A2 (14), were treated by estradiol-17β \( 10^{-8} M \) or the antiestrogen ICI-182,780 \( 10^{-6} M \) for 6, 24, and 48 h as described in the Materials and Methods section. Total RNA was extracted and analyzed by RT-PCR using primers recognizing GAPDH, SCGB2A2, or psoria-
sin cDNAs. Psoriasin was chosen as its expression has previously been shown to be regulated by estrogen treatment (15, our unpublished data). SCGB2A2 mRNA expression was not changed under any treatment condition (data not shown), whereas, as expected, psoriasin signal was found to be increased by estradiol and decreased by antiestrogen treatment as soon as 6 h of treatment, with a maximum effect after 24 and 48 h of treatment (estradiol treatment: 1.5-, 2.8-, and 4.5-fold control and antiestrogen treatment 0.90-, 0.80-, and 0.70-fold control, respectively).

**Discussion**

Assessment of SCGB2A2 expression at the mRNA and the protein levels in a cohort of breast tissue samples showed a statistically significant relationship between SCGB2A2 levels and ER status. However, within the same cohort, no association was found between SCGB2A2 expression and other known prognostic marker such as PR levels or Nottingham grade.

To the clinician, a factor is considered a prognostic factor when it is associated with the outcome of the disease, i.e., predicts how the disease would evolve if not treated, whereas a predictive factor is associated to the degree of response to therapy, i.e., predicts the likelihood of response to a particular treatment. A high level of ER in tumor tissue has a good prognostic value and also predicts a good likelihood of responding to hormonal adjuvant therapy such as tamoxifen (16,17). As PR expression is positively regulated by estrogens, higher PR levels in ER+ tumors support the hypothesis of an operational ER signaling pathway and is therefore also considered as a good prognostic and predictive parameter. Whereas the parallel between SCGB2A2 and ER expression suggested that SCGB2A2 could be a new ER target gene, the lack of association with a known regulated gene such as PR suggested that SCGB2A2 expression was independent of ER signaling pathway. This latter hypothesis was further supported by the absence of estrogen and antiestrogen regulation of SCGB2A2 expression in ZR-75 cells, even though ER signaling pathway appears functional, as shown by the induction of a known ER-regulated gene, psoriasin. It should be noted that a similar absence of regulation was also observed in another ER+ breast cancer cell line MCF-7 cells (our unpublished results; 18). However, even though the SCGB2A2 gene was not grossly rearranged in MCF-7 cells (18), these cells do not express endogenous SCGB2A2 (our unpublished results; 2). It might therefore be hypothesized that SCGB2A2 expression in MCF-7 cells is negatively regulated by other factors, resulting in an absence of estrogen regulation in these cells. Further experiments performed on other breast cancer cell lines and primary cells (19) are needed to confirm these preliminary results.

Interestingly, the general expression of SCGB2A2 as well its association with ER levels observed in vivo in breast tissue contrasts with in vitro observations made on mammary epithelial cancer cell lines. Indeed, looking at a panel of different breast cancer cell lines, Watson et al. reported the detection of SCGB2A2 transcripts only in few cell lines (MB361, MB415, MB468, BT474, MB175) with no expression in MCF7, MB134, MB231, or MCF10A cells (2). Similarly, we did not detect SCGB2A2 expression in breast cell lines such as BT20, T47D, or MCF10AT1 even though a strong signal was seen in ZR-75 (our unpublished observation). As cells such as MB468 and MB361 are ER− and cells such as ZR-75 or BT474 are ER+, SCGB2A2 expression does not appear related to ER status in cells grown in vitro. Overall, this suggests that most of cell lines, through
selection, medium conditions, and/or dedifferentiation lost their ability to express SCGB2A2 in vitro. Presently, no data are available regarding the possible biological function of SCGB2A2. It has however recently been reported that SCGB2A2 existed in a tetrameric complex with BU101 (lipophillin B), another member of the secretoglobin family, the expression of which correlated with SCGB2A2 expression in breast tissue (20). The role of this complex as well as the possible regulation of its components remains to be determined.

In conclusion, we found that SCGB2A2 expression correlated with ER levels in breast tumor tissue. As ER is considered as a good prognostic factor and as SCGB2A2 does not appear to be directly regulated by the ER signaling pathway, we hypothesize that SCGB2A2 expression may be a new independent prognostic marker in breast cancer. Further experiments performed on a larger cohort of patients and completed with follow up studies are needed to test this hypothesis.

Materials and Methods

Human Breast Tissues and Cell Lines

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As it has been previously described (21), tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in hematoxylin and eosin (H&E)-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks. Fifty two tumors were selected, spanning a wide range of estrogen and progesterone receptor levels, as determined by ligand binding assay. Within these tumors, 9 were ER-/PR- (ER < 3 fmol/mg total protein; PR < 10 fmol/mg), 10 were ER-/PR+ (ER < 3 fmol/mg; PR > 10 fmol/mg), 10 were ER+/PR- (ER > 3 fmol/mg; PR < 10 fmol/mg), and 23 were ER+/PR+ (ER > 3 fmol/mg, PR > 10 fmol/mg). These tumors also spanned a wide range of Nottingham grade for ER- (n = 19, grade ranging from 5 to 9, median 8) and ER+ (n = 33, grade ranging from 5 to 9, median 6) tumors. SCGB2A2 mRNA expression was assessed by RT-PCR on total RNA extracted from frozen tissue sections. Paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression.

ZR-75 cells, ER+ breast cancer cells known to express SCGB2A2, were grown and treated with estradiol-17β 10^-8 M in charcoal-stripped medium or with the antiestrogen ICI 182,780 (10^-6 M) in regular medium for 6, 24, or 48 h, as previously described (22). Total RNA was extracted from frozen tissue sections or cell lines using Tri-reagent (MRCl, Cincinnati, OH).

RT-PCR Analysis

One microgram of total RNA was reverse transcribed in a final volume of 20 µL and 1 µL of the reaction mixture subsequently amplified by PCR as previously described (23, 24). Primers used corresponded to SCGB2A2 (sense 5'-CGACAGCAGCAGCCTCAC-3', located in SCGB2A2 sequence between bases 41 and 59, and antisense 5'-TCCG TAGTTGTTCCTCAC-3', located between bases 401 and 383) (2); to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCCACTTCCACCTTTG-3' and antisense 5'-CTCTTGTGCCTTGCTGGG-3'); and to psoriasin (24) gene (sense 5'-AAGAAGAGATGA GCAACAC-3' and antisense 5'-CCAGCAAGAGACAGAACT-3'). To amplify cDNA corresponding to SCGB2A2, GAPDH, and psoriasis, 30 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) of PCR were used. Ten microliters of PCR products were loaded on prestained (15 µg/mL ethidium bromide) 2% agarose gels. Identity of fragments corresponding to SCGB2A2, GAPDH, and psoriasin had previously been confirmed by sequencing.

Three independent PCRs were performed using SCGB2A2, psoriasin, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/ChemiDoc System (Biorad), were quantified by densitometry using the Quantity One software (Version 4.2, Biorad). SCGB2A2 and psoriasis expression were expressed relative to GAPDH expression as previously described (25). Briefly, three independent PCRs were performed using each set of primers. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of SCGB2A2 were then expressed relative to the GAPDH signal corresponding to each individual tumor sample. Correlation between normalized SCGB2A2 expression and tumor characteristics was tested by calculation of the Spearman coefficient, r. Comparison between tumor subgroups was performed using the Mann–Whitney rank sum test, two-sided.

Immunohistochemical Analysis of SCGB2A2 Expression

Detection of SCGB2A2 protein was performed using an antibody previously characterized and kindly provided by Dr. Timothy Fleming (1, 2, 5, 18). Paraffin-embedded breast tissue sections were processed using the automated Discovery Staining Module, Ventana System (Tucson, Arizona) and the Research IHC DAB paraffin protocol according to the manufacturer’s instructions. All steps were performed automatically: briefly, following deparaffinization of tissue
sections, slides were incubated 60 min at 42°C in the presence of rabbit anti-SCGB2A2 antibody (1/1000 final concentration), washed, incubated with biotinylated secondary anti-rabbit antibody (14 minutes 42°C), washed, incubated 8 min with avidin–HRPO complex subsequently detected with DAB-H₂O₂ solution. Counterstaining was also performed automatically by the Ventana apparatus (hematoxylin/bluing reagent).

Levels of mammaglobin expression were assessed by bright-field microscopic examination at low-power magnification and using a previously described semiquantitative approach (25). Scores were obtained by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score. Cases with a score lower than or equal to 1 were considered negative or weakly positive, whereas tumors with scores higher than 1.0 were classified as positive for SCGB2A2 expression. Statistical comparisons between tumor subgroups have been performed using the chi-square test.

Acknowledgments

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APPENDIX 2: Cancer Res. 2002. 62: 2736-2740
Identification of a Novel Breast- and Salivary Gland-specific, Muclin-like Gene
Strongly Expressed in Normal and Tumor Human Mammary Epithelium

Richard J. Miksicek, Yvonne Myal, Peter H. Watson, Christina Walker, Leigh C. Murphy, and Etienne Leygue

Abstract

Expression profiling using the public expressed sequence tag (EST) and serial analysis of gene expression (SAGE) databases resulted in the identification of a putative breast-specific mRNA that we term small breast epithelial mucin (SBEM). Hybridization analysis performed on 43 normal human tissues revealed that the SBEM gene was only expressed in mammary and salivary glands. Further reverse-transcription PCR analyses confirmed SBEM expression in most of established human breast epithelial cell lines analyzed (7 of 8) but not in cell lines of non-breast origin (8 of 9). SBEM mRNA expression was detected in >90% of invasive ductal carcinomas and correlated with the expression of a previously characterized breast-specific gene, mammaglobin-1 (n = 54; Spearman r = 0.33, P = 0.011). Interestingly, a higher SBEM/mammaglobin-1 ratio was observed in primary tumors with axillary lymph node metastasis than in node-negative tumors (n = 46; Mann-Whitney, P = 0.04). In a subset of 20 primary breast tumors and their matched axillary lymph nodes, a high concordance (Fisher’s exact test, P < 0.001) was seen between PCR detection of SBEM mRNA and lymph node tissue and their histopathological status, indicating that SBEM mRNA expression is conserved in nodal metastasis. The SBEM gene is predicted to code for a putative low molecular weight, secretedialoglycoprotein, potentially useful for the diagnosis of metastatic breast cancer.

Introduction

Early detection remains a central goal in breast cancer treatment to enable intervention at a localized and potentially curable stage and to maximize the opportunity for breast conservation. The 5-year survival rate for women with breast cancer increases dramatically when it can be diagnosed at an early stage, from >95% in patients with a localized tumor to ~75% with regional disease and <25% in women with disseminated cancer (1). Nevertheless, only 60% of all breast cancers are diagnosed at a local stage, and any improvement in early detection would have a significant impact on reducing overall breast cancer mortality.

Improving the diagnosis and clinical management of breast cancer requires access to a wider range of biomarkers able to reflect the molecular phenotype of breast tissue. A special need exists to identify novel genes whose expression is restricted to the mammary epithelium, because these genes have the greatest potential to enhance detection of micrometastatic disease and the potential to report on proliferative changes in the breast, analogous to the ability of elevated serum prostate-specific antigen levels to indicate the presence of hyperplasia or cancer of the prostate gland (2).

The identification of new tissue-specific markers has benefited especially from expansion of public and private databases for ESTs (3, 4) and by large-scale efforts to profile patterns of gene expression using techniques such as serial analysis of gene expression (5). Using sequence analysis software and web-based tools developed for molecular profiling, we have identified a novel putative breast-specific gene, belonging to a recently regrouped cluster (UniGene identifier Hs.348419), which represents an attractive candidate for a breast tumor marker with obvious potential for cancer diagnostics.

Materials and Methods

Database and Sequence Analysis. The eDNA xProfiler tool was used to search for novel breast-specific ESTs. Protein sequence analysis used the SignalP algorithm to search for signal peptide or transmembrane domain, the PredictProtein tool to predict sites of potential glycosylation sites (7).

RNA Hybridization Analysis for Tissue Specificity. A 3²P-labeled SBEM probe, generated using the cloned SBEM PCR product (395 bp) and the RadPrime DNA labeling system (Life Technologies, Inc., Burlington, Ontario, Canada), was hybridized to a commercially available RNA Master Blot (Clontech, Palo Alto, CA), containing poly(A)+ RNA (100–500 μg) isolated from a variety of adult and fetal human tissues, according to the manufacturer’s instructions.

Cell Culture and RNA Preparation. Cell lines were obtained from the American Type Culture Collection or other sources and were cultured as follows: DMEM with 10% fetal bovine serum (MCf7, MCF10AT1, MCF10AT2, SK-UT-1B, and Hep2); DMEM with 10% calf serum (MDA MB-231, Hec 1A, and HeLa); DMEM/Ham’s F12 (1:1) with 10% fetal bovine serum (ZR-75-1 and RL-95-2); RPMI 1640 with 10% fetal bovine serum (T-47D and LNCAp); or MUSI-1 medium (8) with 5% fetal bovine serum (M13SV-1). All media were supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and hepes (20 mM). All cultures were also transfected with the following gene constructs: Green Fluorescent Protein (GFP), Herpes Simplex Virus (HSV), and HIV-1 LTR. Media and sera were obtained from Life Technologies, Inc. (Life Technologies, Inc., Grand Island, NY). RNA was extracted from cultured cells using guanidinium isothiocyanate, followed by centrifugation through a 5-M sodium chloride cushion as described (9). RNA from cultured primary BMDCs obtained by reduction mammoplasty was a kind gift from P. Erwin (Biotherapeutics, Inc., Ann Arbor, MI).

Breast Tumors and Axillary Lymph Nodes. Fifty-four invasive ductal carcinomas were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Cases spanned many ER (0–298 fmol/mg protein) and PR (0–1198 fmol/mg protein) levels, as determined by ligand binding assay. Tumors also spanned many grades (Nottingham grade scores from 5 to 9). For

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4 The abbreviations used are: EST, expressed sequence tag; SBEM, small breast epithelial mucin; HMMEC, human mammary epithelial cell; ER, estrogen receptor; PR, progesterone receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; MUC1, mucin 1.

5 Interested address: http://www.ncbi.nlm.nih.gov/UniGene; UniGene is a system for automatically partitioning GenBank sequences, including ESTs, into a nonredundant set of gene-oriented clusters.

6 Interested address: http://cgap.nci.nih.gov/CGAP/Tissues/Profiles.

7 Interested address: http://www.obst edu/db/services/SignAl.

8 Interested address: http://www.obst.edu/services/NetOgyle.
46 tumors, pathological axillary lymph node status (presence or absence of metastasis) was known.

In a subset of cases (n = 20), frozen primary human breast tumor samples and their matched frozen lymph node tissues containing (n = 14) or not (n = 6) histologically detectable metastatic cancer cells were available from the Manitoba Breast Tumor Bank. For the primary tumor samples, the ER levels, determined by ligand binding assays, ranged from 2.3 fmol/mg protein to 298 fmol/mg protein, whereas PR levels ranged from 10.1 fmol/mg protein to 112 fmol/mg protein.

**RNA Analysis by RT-PCR.** Total RNA was extracted from 20-μm frozen tissue sections (five sections/tumor) and reverse transcribed as described previously (10). The primers used for SEBM amplification consisted of SEBM-D (5'-CTTGGATCCTTTGATCTGGT-3'; sense) and SEBM-E (5'-AAGGTGTCAGCGGATGATAT-3'; antisense). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, aliquots of each reverse transcription mixture (2 μl for Fig. 2 and 0.8 μl for Fig. 3) were amplified in a final volume of 20 μl, in the presence of 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 1.5 mm MgCl₂, 200 μM of each deoxynucleotide triphosphate, 5 ng/μl of each SEBM primer, and 0.5 unit of Taq DNA polymerase. Each PCR consisted of 35 cycles (15 s at 94°C, 30 s at 58°C, and 60 s at 74°C).

Primers used for mammaglobin-D were: Mam-1 (5'-CCGACACGACGACACCTCAC-3'; sense strand) and Mam-2 (5'-TCCGTAGTGGTTCTCCAC-3'; antisense strand). Primers for the ubiquitously expressed GAPDH gene were GAP-1 (5'-ACCACCCTCCTCCTCTTCTGG-3'; sense strand) and GAP-2 (5'-CTTGGTGTGCTTGTCTGGG-3'; antisense strand). To amplify cDNA corresponding to mammaglobin-D and GAPDH, 30 cycles of PCR were performed (30 s at 94°C, 30 s at 58°C, and 60 s at 72°C). All buffers were the same as for SEBM PCR, except that 2 mm MgCl₂ was used when amplifying mammaglobin-D cDNA. PCR products were then separated on a 1.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml).

**Quantification and Statistical Analysis.** Three independent PCRs were performed for tumor specimens using SEBM, mammaglobin-D, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/ChemDoc System (Bio-Rad), were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). SEBM and mammaglobin-D mRNA expression was normalized to GAPDH expression as described previously (11). Correlation between SEBM expression and tumor characteristics or mammaglobin-D expression was tested by calculation of the Spearman coefficient r. Differences between tumor subgroups were tested using the Mann-Whitney two-tailed test or Fisher's exact test.

**In Situ Analysis of SEBM mRNA Expression.** In situ hybridization was performed on adjacent paraffin-embedded breast tumor tissue sections corresponding to a case shown to express high levels of SEBM mRNA by RT-PCR, using SEBM 32P-labeled sense and antisense probes, as described previously (12).

**Results**

Identification of a Putative Novel Breast-specific Gene. The cDNA xProfiler tool (see "Materials and Methods") was used to identify tissue-restricted cDNAs with preferential representation in libraries prepared from normal breast tissue and breast tumors. This search identified a new cluster of ESTs now grouped under the UniGene identifier number HS.348419. Of the 30 ESTs found in this cluster, 15 are restricted to breast cDNA libraries, 9 were isolated from random activation of gene expression or pooled tissues, 5 were isolated from fetal sources (fetal heart and fetal skin), and 1 came from a head and neck tumor cDNA library. Alignment of these ESTs led to the construction of a 500-bp consensus cDNA sequence containing a 90-aminoc acid open reading frame in which the initiating methionine is framed by a nearly perfect consensus motif for translation initiation (5'-CCACCATGGA-3'; Ref. 13). Further database analysis showed that this sequence, interrupted by three introns, is present on chromosome 12q13.2. Primers were designed to span the open reading frame, and we cloned a 396-base fragment from both MCF-7 cells and breast tissue, which we called SEBM (GenBank accession number AF414087). The presence of a hydrophobic signal peptide (residues 1-19; Ref. 6) within the protein sequence (GenBank accession number AAA02119) suggests that SEBM is a secreted protein subject to proteolytic processing. The NetOGlyc glycosylation algorithm (7) further predicts this protein to be N-glycosylated on most of its 16 threonine residues. The SEBM protein contains three tandem copies of a neutral octapeptide core repeat (ThrThrAlaAlaAxxThrThrAla, where Xxx corresponds to Ala, Pro, or Ser). The NH₂ and COOH termini of the processed polypeptide are otherwise charged and fairly polar. These features suggest strong similarity to many sialomucins, although this protein lacks a transmembrane domain and is substantially shorter than most other known epithelial mucins (14, 15).

Expression of SEBM mRNA Is Restricted to the Mammary and Salivary Glands. Database searches suggested that SEBM expression was mainly restricted to breast tissue. To confirm this prediction, we performed hybridization analysis with an RNA MasterBlot containing highly purified polyadenylated RNA from 43 adult and 7 fetal human tissues arrayed on a nylon membrane. A SEBM cDNA probe hybridized exclusively to mRNA from the mammary and salivary glands (Fig. 1). Of note, no expression was observed in colon, lung, uterus, ovary, liver, pancreas, kidney, or prostate, all of which represent common primary tumor sites. Additionally, no hybridization to any of the fetal RNAs was observed.
...SEBM mRNA Is Expressed in Breast Cancer Cell Lines but not in Cell Lines of Non-Breast Origin. The profile of SEBM mRNA expression was further assessed using RT-PCR, followed by PCR amplification, in a panel of human breast and non-breast cell lines. A SEBM PCR product of the expected size (396 bp) was readily detected in MCF7 and ZR-75-1 breast tumor cells (data not shown). Lower but reproducible expression was also observed in primary HMECs and in several established breast epithelial cell lines including T-47D, M135V-1 (8), MCF10A1, and MCF10AT3c (16). MDA MB-231 breast tumor cells were negative for SEBM expression, as were six tumor cell lines of non-breast origin (uterus: RL95-2, SK-UT-1B, Hec 1A; cervix: HeLa; prostate: LNCaP; and liver: HepG2). As controls, we also examined the expression of a housekeeping gene (GAPDH) and mammmaglobin-1, an established mammary-specific gene that is being independently investigated as a promising marker for breast tumor diagnosis and nodal metastasis (12, 17). Of the cell lines tested, only HMEC and ZR-75-1 cells expressed mammaglobin-1, consistent with published reports.

Analysis of SEBM mRNA in Human Breast Tumors. Northern blot analyses performed on a small series of 10 cases revealed that SEBM mRNA was 600 bp long and differentially expressed from one sample to another (data not shown). To determine whether SEBM mRNA was widely expressed in human breast tumor tissue, 54 human breast tumors, spanning many ER and PR levels as well as tumor grade and nodal status, were selected from the Manitoba Breast Tumor Bank. Total RNA was extracted from frozen tissue sections and reverse transcribed. PCR amplification of GAPDH (control), mammaglobin-1, and SEBM cDNA was then performed. A PCR product, 396-bp long was detected in all but three tumors (data not shown) when using SEBM-specific primers. After cloning and sequencing, this product was shown to correspond to SEBM cDNA. Quantification of the SEBM signal relative to the GAPDH signal was performed as described in "Materials and Methods." No correlation was found between SEBM expression and tumor characteristics such as ER (n = 54; Spearman r = -0.01, P = 0.89) and PR (n = 54; Spearman r = -0.03, P = 0.77) levels or tumor grade (n = 44; Spearman r = -0.06, P = 0.68). Interestingly, however, the SEBM signal correlated positively with mammaglobin-1 expression (n = 54; Spearman r = 0.340, P = 0.011). Subgroup comparison of SEBM and mammaglobin-1 expression confirmed our previous observation that mammaglobin-1 expression is higher in ER-positive and low-grade tumors (Table 1). Interestingly, although not statistically significant (P = 0.09), higher SEBM expression was found in lymph node-positive compared with node-negative tumors. Also of interest is the fact that the SEBM:mammaglobin-1 ratio is significantly (n = 46; Mann-Whitney, P = 0.04) higher in these lymph node-positive tumors.

...SEBM mRNA Expression in Primary Breast Tumors and Their Corresponding Axillary Nodes. Next we investigated the possibility that SEBM mRNA could be a tissue marker of axillary lymph node metastasis. Twenty independent cases were selected, including 14 tumors that were axillary lymph node positive and 6 that were node negative. Total RNA was extracted from frozen primary tumor sections and frozen node sections of corresponding axillary lymph nodes. The histological status of all tissues was confirmed in paraffin sections cut from adjacent mirror image paraffin tissue blocks that had been processed in parallel to the frozen blocks. These RNAs were reverse-transcribed and analyzed by RT-PCR using SEBM-specific primers. PCR was performed three times, giving the same result. A representative experiment is shown in Fig. 2. A signal corresponding to SEBM was detected in all lymph nodes containing metastatic cells by histopathological assessment (14 of 14 cases). In contrast, no signal was detectable in lymph nodes from cases without histologically detectable tumor cells (0 of 6 cases). RT-PCR detection of SEBM mRNA in axillary lymph nodes is therefore strongly associated (Fisher's exact test, P < 0.001) with the histopathological detection of lymph node metastases. The higher sensitivity afforded by RT-PCR detection therefore indicates that SEBM, perhaps together with mammaglobin-1, represents an excellent marker for the detection or confirmation of occult breast tumor metastasis, where histopathology may not be definitive.

...SEBM mRNA Is Expressed in Mammary Epithelial Tumor Cells in Vivo. To further establish whether SEBM was expressed by mammary epithelial cells in vivo, paraffin breast tumor tissue sections corresponding to a case shown to strongly express SEBM mRNA by RT-PCR were studied by in situ hybridization. No signal was detectable when using a sense probe (Fig. 3A). In contrast, a signal was observed in epithelial tumor cells when using an antisense probe (Fig. 3B). SEBM mRNA was not detected in stromal or inflammatory cells in any of the sections studied.

Discussion

This article reports the identification, cloning, and preliminary characterization of a cDNA encoding a novel mucin-like protein that displays an unusually narrow pattern of expression. Hybridization analysis revealed that SEBM mRNA was only detectable in two normal tissues, breast and salivary gland. Interestingly, the tissue-
specific expression that we observed experimentally directly reflects the distribution of ESTs within the Hs348419 cluster. Indeed, as mentioned above, only two adult tissues (breast and head/neck tumors) have been shown to express SBEM-related ESTs. The fact that SBEM is also expressed in salivary tissue does not undermine the possible use of SBEM as a marker of breast cancer, because tumors of the salivary gland are less common and can readily be distinguished clinically.

Among the primary breast tumors examined in this study (representing mostly invasive ductal carcinoma), SBEM mRNA was observed by RT-PCR analysis in the majority (>90%) of cases. Despite a significant overall correlation between the expression of SBEM and mammaglobin-I mRNA, a significantly higher SBEM:mammaglobin-I ratio was observed in primary tumors associated with positive axillary lymph nodes as compared with node-negative tumors. This was mostly attributable to a trend toward higher SBEM expression in node-positive tumors. Although further analysis of a larger number of tumors will be required to confirm these observations, this may suggest differences in the biology of these tumors and also a possible role of SBEM and mammaglobin-I in the mechanisms involved in tumor metastasis. Our findings indicate, however, that SBEM expression is a common feature of breast cancer and can furthermore serve as a useful marker for breast nodal metastasis, both for detection of micrometastatic cells within lymph nodes as well as in the differential diagnosis of the primary origin of an unknown metastasis. This potential is enhanced by the conserved SBEM expression in high grade and ER/PR-negative tumors that are most likely to metastasize.

The potential diagnostic relevance of SBEM is also increased by its predicted biochemical structure. The SBEM cDNA sequence codes for a 90-amino acid polypeptide that contains a distinctive tandem repeat, rich in alanine and threonine residues, that represents a probable target for O-glycosylation. Consistent with such posttranslational modification is the presence of a well-defined signal peptide, leading us to predict that SBEM is likely to be processed at the apical surface of luminal epithelial cells and to be secreted into the alveolar or ductal lumen. Further study is needed to ascertain whether higher SBEM expression occurs in association with tumors.

Secreted (or transmembrane) proteins that contain internally repeated, densely glycosylated neutral core motifs such as this are characteristic of mucins, which are typically expressed by the surface epithelium of secretory mucous and by exocrine glands (14, 15). The role of mucins is primarily one of hydrating and lubricating epithelial linings, although several mucins have been implicated in modulating both cell adhesion and growth factor signaling (18, 19). Furthermore, mucins have a well-established link to cancer, best illustrated by the product of the MUC1 gene. MUC1 is overexpressed in a variety of epithelial tumors including breast cancer and gives rise to several well-characterized tumor antigens including CA15.3 and CA27.29 (20, 21). Combined with the loss of cell polarity and changes in glycosylation patterns observed in transformed epithelial cells, overexpression of MUC1 results in the appearance of mucin-derived tumor antigens in the sera of cancer patients that are not observed in normal controls (14, 22). We hypothesize that a similar situation may hold for SBEM expression in the human mammary epithelium and in human breast tumors. However, MUC1 displays relatively broad expression among epithelial tissues including the colon, breast, pancreas, ovary, prostate, tracheobronchial tree, stomach, and uterus. For this reason, MUC1-derived tumor antigens have relatively poor specificity for individual tumor types, and their clinical utility is limited to monitoring the efficacy of cancer therapy and warning of tumor relapse or malignant spread (21–23).

Parallels between SBEM and known epithelial mucins such as MUC1, together with its more narrowly restricted pattern of expression, suggest that this novel gene represents an attractive candidate for a breast biomarker with potential for cancer diagnostics, as well as being a possible future target for the development of a breast tumor vaccine. Moreover, the absence of SBEM expression in normal lymph node tissue suggests that this gene could also be used to detect breast micrometastasis in axillary lymph nodes.

References


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Appendix 3: Summary, CIHR 2003-2005
SUMMARY OF RESEARCH

In Canada alone, an estimated 21,000 women will be diagnosed in 2002 with breast cancer, and 5,400 women will be lost to this disease. There is an urgent need to identify novel genes whose expression is restricted to the mammary epithelium. Indeed these genes have the greatest potential (alone or combined with existing markers) to report on proliferative changes in the breast which may herald increased risk of breast cancer development, to enhance the detection of micrometastatic disease, and to be used for targeting specifically breast cancer cells.

We have identified a novel putative breast-specific gene, called hSBEM (human Small Breast Epithelial Mucin) which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics. We have indeed shown that hSBEM mRNA was expressed by breast tumor epithelial cells in 94% of primary breast tumors, independently of tumor characteristics such as steroid receptor levels or grade. We also demonstrated that hSBEM expression was conserved in breast tumor cells that metastasized in axillary lymph nodes. Interestingly, the protein encoded by this new gene shares structural similarities with MUC1, a heavily glycosylated protein over-expressed in epithelial tumors including breast and lung cancer. Change in MUC1 glycosylation patterns observed in transformed epithelial cells results in the appearance of mucin-derived tumor antigens in the sera of cancer patients. However, as MUC1 displays a broad expression among many normal epithelial tissues (including colon, breast, pancreas, ovary, prostate, and uterus), its clinical utility as a specific marker for breast cancer is limited. No data are available to date regarding the detection, the level of expression or the glycosylation states of the hSBEM protein during breast tumorigenesis.

HYPOTHESIS: hSBEM glycosylated protein, exists in both trans-membrane and C-terminally clipped soluble forms. As shown for MUC1, glycosylation patterns are changed during tumorigenesis. hSBEM protein is over-expressed during breast tumorigenesis and its increased concentration in vivo on the membrane of breast cancer cells and in their immediate vicinity can be used to pin-point in vivo the position of these cells.

GOAL: To establish whether the expression and/or the glycosylation pattern of hSBEM protein are modified during breast tumorigenesis and to develop an assay allowing the non invasive in vivo detection of breast cancer cells.

SPECIFIC AIMS

1 Investigation of hSBEM protein expression and glycosylation in breast cancer cell lines
2 Investigation of hSBEM protein expression and glycosylation in normal and tumor breast tissues
3 In vivo detection of hSBEM protein

AIMS 1-2 hSBEM protein expression will be analyzed by Western blot and 2D gels following differential sugar removal in human breast cell lines and breast normal and tumor tissue. Data will determine whether hSBEM gene expression is altered and whether changes in glycosylation profile occur during breast tumorigenesis.

AIM 3 Iodinated or iron nanoparticles-coupled anti-SBEM antibodies will be injected in SBEM expressing tumor bearing mice. Preferential sites of antibody accumulation will then be located by counting and radiography or MRI. Data will determine whether anti-hSBEM antibodies can be used to detect non invasively breast cancer cells in vivo in primary tumors and metastases.

Research Module, Page 9
Psoriasis Interacts with Jab1 and Influences Breast Cancer Progression

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ABSTRACT

Psoriasis (S100A7) is expressed at low levels in normal breast epithelial cells but is highly expressed in preinvasive ductal carcinoma in situ. Persistent psoriasis expression occurs in some invasive carcinomas and is associated with poor prognostic factors. Whereas there is evidence that secreted psoriasis can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions, an intracellular biological function is unknown. We have found that psoriasis physically interacts with Jab1 (c-jun activation-domain binding protein 1) in the yeast two-hybrid assay and confirmed this by coimmunoprecipitation assay in breast cancer cells. Psoriasis-transfected breast cancer cells showed increased nuclear Jab1 and demonstrated several features consistent with an alteration in Jab1 activity including an increase in activator protein-1 (AP-1) activity, increased expression of AP-1 and HIF-1-dependent genes, and reduced expression of the cell-cycle inhibitor p27Kip1. Psoriasis overexpression was also associated with alteration of cellular functions that are associated with increased malignancy, including increased growth, decreased adhesion, and increased invasiveness in vitro, as well as increased tumorigenicity in vivo in nude mice. We conclude that intracellular psoriasis influences breast cancer progression and that this may occur through stimulation of Jab1 activity.

INTRODUCTION

We have identified psoriasis (S100A7) previously as a differentially expressed gene between DCIS and invasive carcinoma (1). The expression of psoriasis is low in normal breast and benign pathologies (1), but psoriasis is among the most highly expressed genes in high grade DCIS (2, 3). Whereas expression is often reduced in invasive carcinoma, persistent high expression is associated with markers of poor prognosis (4). This profile of gene expression raises the possibility that psoriasis may be functionally involved in invasion and early tumor progression (5). Psoriasis is a small calcium-binding protein belonging to the S100 gene family (6, 7), among which several other members have been associated with breast tumor progression (8, 9). Most interest has been focused on S100A4 (10), which was also initially identified as a differentially expressed gene between non-metastatic and metastatic rodent mammary tumor cell lines (11). In later studies by several groups, S100A4 has been shown to directly influence the invasive and metastatic phenotype in breast cancer cell lines (12–14) and tumors (15), and expression is also associated with poor prognostic factors and patient survival in human breast tumors (16).

Psoriasis was originally described as highly expressed within psoriatic skin lesions (17) and found to be a secreted protein (18), but has since been observed to be present in the cytoplasm and nucleus of both abnormally differentiated keratinocytes (19) and breast carcinoma cells (2, 4). Whereas there is evidence that secreted psoriasis can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions (18), a function for intracellular psoriasis also appears likely but has yet to be established.

We sought to identify proteins that might interact with psoriasis in breast epithelia by using the yeast two-hybrid system (20). Jab1 (21) was found to specifically interact with psoriasis in the yeast system, and this interaction was confirmed by biochemical assay in breast cancer cells. Jab1 is a component of a multimeric protein complex (22, 23), the CSN/COP9 signalosome, which is involved in signal transduction and protein degradation via the Ub-26S proteasome (24, 25). The effect of overexpression of psoriasis on Jab1 distribution and function in a breast cancer cell line was studied. Psoriasis overexpression resulted in redistribution of Jab1 to the nucleus and multiple functional changes that can be attributed to activation of Jab1, as well as enhanced tumorigenesis and metastasis in an in vivo assay. These data support our hypothesis that psoriasis enhances early tumor progression and the process of invasion in breast cancer cells in part by interacting with Jab1 and positively enhancing its activity.

MATERIALS AND METHODS

Yeast Two-Hybrid System. For yeast two-hybrid studies, the coding region of human psoriasis protein was fused in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech). The resulting bait plasmid (pGBT9-psor) was used to screen a normal human mammary epithelium cDNA library (Clontech) by the yeast two-hybrid method as we have described previously (26). Clones were isolated that could grow on "Trp"-"Leu"-"His" medium, did not autoactivate the β-galactosidase reporter gene, and demonstrated specificity for their interaction with psoriasis. This was done by testing the interaction of psoriasis with specific "prey" constructs not identified in the screen. Jab1 was analyzed in a similar fashion. The NH2-terminal "bait" psoriasis plasmid used to define the region of psoriasis involved in Jab1 binding encoded amino acids 1–52 (pGBT9-N-term-psor), and the COOH-terminal bait psoriasis plasmid encoded amino acids 43–101 (pGBT9-C-term-psor).

Cell Culture, Transfections, and Antibodies. The human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 were cultured in DMEM supplemented with 10% FBS under standard conditions (4). The former cell line is negative for psoriasis, whereas the latter expresses psoriasis mRNA and protein (confirmed by RT-PCR and Western blot; data not shown). The full psoriasis protein coding sequence was cloned into pcDNA3.1 (Invitrogen) and transfected into MDA-MB-231 cells using Superfect (Qiagen) followed by G418 selection. Resistant colonies were isolated and expanded. Psoriasis protein expression was determined by Western blot using a rabbit antisera against psoriasis antibody generated by our laboratory and directed against the epitope KAS- GAAPSBSIGQ corresponding to amino acids 88–101. The specificity of the antibody was established by comparison with a similar antibody generated previously against the same epitope (4), and by immunohistochemistry and Western blot, using transfected breast cancer cell lines and tumors as described previously (4). Three MDA-MB-231 clones were found to express psoriasis.
PSORIASIS ALTERS JAB1 FUNCTION IN BREAST CANCER CELLS

* (designated as clone 231-LP1 exhibiting low psoriasis expression, and clones 231-HP1 and 231-HP2 both exhibiting similar high levels of psoriasis expression). Wild-type MDA-MB-231 and clone 231-neo (generated by transfection with the empty vector) do not express psoriasis. Jab1 and p27Kip1 antibodies were obtained from Santa Cruz Biotechnology, Inc. Hypoxic stimulation of cells was performed in a Forma Scientific Model 1025 Anaerobic System containing an atmosphere of 0.7% O2, 5% CO2, and 5% H2 at 37°C for 24 h as we have described previously (27).

Immunoprecipitation and Western Blot. Human breast cancer cell lines expressing psoriasis (231-HP2 and MDA-MB-468) were lysed on ice in 25 mM HEPES (pH 7.7), 0.4 M NaCl, 1.5 mM MgCl2, 2 mM EDTA, 1% Triton X-100, 0.1 mM DTT, and protease inhibitor mixture (Roche). Complexes were immunoprecipitated by Jab1 antibody/protein G-Sepharose (Pierce) at 4°C for 2 h. Binding and washes were performed in the same buffer, except the NaCl concentration was diluted 4-fold (28). Coinmunoprecipitated psoriasis protein was detected by immunoblotting using the psoriasis-specific antibody. Total protein lysates were extracted from the cell-line pellets in SDS-Isolation Buffer [50 mM Tris (pH 6.8), 20 mM EDTA, 5% SDS, 5 mM β-mercaptoethanol, and a mixture of protease inhibitors (Roche)]. Protein concentrations were determined using the Micro-BCA protein assay kit (Pierce). Protein lysates were run on a 15.5% SDS-PAGE mini gel using Tricine SDS-PAGE to separate the proteins, and then transferred to 0.2 μm nitrocellulose (Bio-Rad). After blocking in 10% skimmed milk powder in Tris-buffered saline-0.05% Tween, blots were incubated with primary antibodies (~15 μg/ml in Tris-buffered saline-0.05% Tween) followed by incubation with appropriate secondary antibodies and visualization by incubation with Supersignal (Pierce) as per the manufacturer’s instructions and exposure on X-ray films.

Reporter Gene Assay and Transcription of AP-1-dependent Genes. MDA-MB-231 parental cells and clones stably transfected with psoriasis were transfected with an AP-1-driven luciferase reporter gene (Stratagene) and a β-galactosidase expression vector, in triplicate experiments using Effectene (Qiagen). Luciferase was measured in cell lysates (Promega) 18 h after transfection and standardized to β-galactosidase activity (Promega). Total RNA from the MDA-MB-231 clones was isolated using TRizol (Sigma) and reverse transcribed in duplicate from triplicate samples as described (4). Specific primers for VEGF, MMP13, and GAPDH were used for PCR as follows: VEGF-UPPER (sense) CGG AGA CGT GT AAA TCT GCC TGG G and VEGF-LOWER (antisense) AAC AAA AAT AAA ATG GCG AAT CC; MMP13-UPPER (sense) ATG CGG GGT TCC GTA T and MMP13-LOWER (antisense) CGC AGC AAC AAG AAA CAA; and GAPDH-UPPER (sense) ACC CAC TCC TCC ACC TTT G and GAPDH-LOWER (antisense) CTC TTG GTC TCT TGC TTT GTT G. Reactions were stopped during the log-linear stage with data amplification and samples electrophoresed through an agarose gel that was poststained with ethidium bromide for band visualization. Images were captured using anLAB camera and MCID software (Imaging Research, St. Catharine’s, Ontario, Canada).

Immunohistochemistry. Cultured cells were grown on microscope slides for 24 h, and then fixed and processed as described previously (4). Immunohistochemical staining for psoriasis was performed essentially as described previously, using an automated tissue immunostainer (Ventana Medical Systems, Phoenix, AZ), and 3,3’-diaminobenzidine immunohistochemistry kit and bulk reagents supplied by manufacturer. Briefly, the staining protocol was set to “Extended Cell Conditioning” procedure, followed by 12 h incubation with primary antibody (concentration 1:3000) and 32-min incubation with secondary antibody. Positive staining was assessed by light microscopy.

Adhesion, Growth, and Invasion Assays. MDA-MB-231 clones were trypsinized from flasks that were 60–70% confluent. Cells (10,000) were plated in triplicate on three different days in 96-well plates having fibronectin, collagen I, or uncoated plastic surfaces (Becton Dickinson). After 1 h at 37°C, nonadherent cells were gently washed away with PBS. Adherent cells were stained with crystal violet, and their relative abundance determined by spectrophotometric absorbance. For growth assay, 1000 cells/well were plated in plastic 96-well plates in triplicate on three different days and allowed to grow for 18, 24, 48, and 72 h. Cells were stained with crystal violet and their relative abundance determined by spectrophotometric absorbance. Invasion assays were performed in triplicate on a Matrigel-coated modified Boyden-invasion chamber (24-well plate inserts with 8-μm pores; Becton Dickinson). FBS DMEM (10%) was added to the upper chamber, and allowed 12 h to deplete the Matrigel and invade through the porous membrane. Cells that invaded and were adhering to the bottom of the membrane were stained with crystal violet. Invaded cells were visualized by light microscopy and enumerated by counting the number of cells per high power field in five random fields.

In Vivo Mouse Studies. Breast cancer cells (four experimental groups comprising MDA-MB-231 parental cells, 231-neo control, 231-LP1, and 231-HP1) were grown in culture and then suspended in 0.2 ml of PBS at a concentration of 3 × 106 cells before injection into mammary fat pads of female nude mice according to a protocol approved by the University of Manitoba Animal Care Committee. Each experimental group included 5 animals, and two injections were sited bilaterally in each animal to achieve a total of 10 possible tumor sites per group. Tumor diameters were measured by calipers at weekly intervals, and the tumor volume was calculated from the formula: volume = 4/3 π (0.5 × smaller diameter)2 × 0.5 × larger diameter. The experiment was continued for up to 8 weeks at which time all of the animals were euthanized, and all of the injection sites, tumors, and multiple organ tissues (abdominal lymph nodes, lungs, liver, and spleen) were examined grossly for the presence of tumor. Representative tissue blocks from all of the primary injection sites and all of the organ sites suspicious for metastatic tumor were subsequently processed by 10% formalin fixation, paraffin embedding, and preparation of H&E-stained sections for light microscopic examination.

RESULTS

Identification and Confirmation of Psoriasis Interacting Proteins. We used full-length psoriasis fused to the GAL4 DNA-binding domain as bait in a yeast two-hybrid assay (20) and screened 1.74 × 107 colonies from a normal human breast cDNA library. Among 4 true positive clones (26), 1 (Fig. 1a) contained almost the full protein sequence (amino acids 42–335) for Jab1. As shown in Fig. 1a, controls including unrelated bait (Rad18) and prey (Mad2) constructs, and empty bait and prey vectors did not show any activation of reporter genes. We noted that a Jab1-binding motif common to several Jab1 interacting proteins described recently is also contained within psoriasis (Fig. 1b), so we tested whether this region was necessary for the psoriasis-Jab1 interaction. As shown (Fig. 1e), only the COOH-terminal portion of psoriasis that contains this motif interacted with Jab1. To additionally confirm the psoriasis-Jab1 interaction in breast cancer cells, psoriasis was stably transfected into MDA-MB-231 cells, and coimmunoprecipitation experiments performed using Jab1 and psoriasis antibodies. Psoriasis-Jab1 protein complexes were detected in both psoriasis-transfected MDA-MB-231 cells (231-HP2) and the breast cell line MDA-MB-468 (which exhibits endogenous psoriasis expression) when Jab1 antibody was used for immuno precipitation (Fig. 1c). However, no psoriasis-containing complex was detected in control lanes in the absence of Jab1 antibody or protein G beads. Psoriasis-specific antibody immunoprecipitated psoriasis from cell lysates but was unable to coimmunoprecipitate Jab1 (data not shown), presumably because of the proximity and partial overlap of the epitope recognized by the antibody (amino acids 88–101 of psoriasis) and the proposed Jab1-binding domain (amino acids 57–89 of psoriasis).

Psoriasis and Jab1 Cellular Localization. We localized psoriasis and Jab1 in the MDA-MB-231 clones by immunohistochemistry. Jab1, like psoriasis, has been found previously to be both nuclear and cytoplasmic in cell types other than breast. In MDA-MB-231 cells and all 4 of the transfected cell lines (231-neo, 231-LP1, 231-HP1, and 231-HP2) Jab1 is expressed at comparable levels in the cytoplasmic compartment (Fig. 2, right panel). However, in all three of the psoriasis-expressing clones, 231-LP1, 231-HP1, and 231-HP2 (Fig. 2, left panels), there is a relative increase in Jab1 within the nucleus. However, the total amount of Jab1 protein as detected by Western blot is similar in all of the cell clones and does not change in the presence of psoriasis (Fig. 3a). Psoriasis can also be detected by immunoprecipitation of medium conditioned by 231-HP2 and MDA-MB-468.
PSORIASIN ALTERS JAB1 FUNCTION IN BREAST CANCER CELLS

Fig. 1. Psoriasis specifically interacts with Jab1 in yeast two-hybrid assay and breast cancer cells, a, the psoriasis-Jab1 interaction was confirmed in yeast, by testing psoriasis fused to the GAL4-BD (binding domain) with selected proteins fused to the GAL4-AD (activating domain). Yeast plates (+ and - Histidine) are shown on the left, and the plating schema for each sector is shown on the right. Activation of reporter genes and colony growth is only present when the full-length psoriasis or the COOH-terminal half of psoriasis is combined with Jab1. b, the COOH-terminal half of psoriasis contains sequences similar to a Jab1-binding domain identified previously in p27Kip1, LFA-1, and c-jun. c, psoriasis can be communoprecipitated with Jab1 complexes from breast cancer cell lysates. Western blot using antipsoriasis antibody to detect psoriasis, which coinmunoprecipitated with Jab1, from a panel of breast cell lines (see “Materials and Methods”).

cells (data not shown), suggesting that psoriasis is also secreted by breast cancer cells in culture.

Psoriasis Overexpression Influences Several Jab1-related Functions. Jab1 influences a number of cellular proteins. Among these, Jab1 affects the level of the negative cell cycle-regulating protein p27Kip1 by promoting the export of p27Kip1 from the nucleus to the cytoplasm and the subsequent degradation by the Ub-28S proteasome (29). Therefore, we first examined p27Kip1 expression in our MDA-MB-231 clones and found that psoriasis-overexpressing clones showed a consistent reduction in levels of p27Kip1 relative to wild-type and control cells (Fig. 3a).

To determine whether psoriasis influences other Jab1 functions in breast cancer cells we examined AP-1-dependent transcription in the MDA-MB-231 clones using an AP-1-driven luciferase reporter (Fig. 3b). AP-1 activity was increased in all 3 of the psoriasis-transfected clones in close proportion to the level of psoriasis expression (Fig. 3a). In the high psoriasis-expressing clones (231-H1P1 and 231-H2P2) there was a 6.5-fold increase in luciferase activity (P < 0.0001). These psoriasis-expressing cells showed a difference in total Jab1 levels assessed by Western blot, compared with non-psoriasis-expressing controls (Fig. 3a). However, the effect on AP-1 activity is consistent with the redistribution and relative increase in nuclear Jab1 protein detected by immunohistochemistry (Fig. 2) and the findings of others (21). Expression of endogenous AP-1-dependent genes was next examined by RT-PCR (Fig. 3c). Psoriasis expression is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF (30) and MMP13 (31), and this increase is proportional to the levels of psoriasis in the MDA-MB-231 control and transfected cells.

Jab1 also interacts with HIF-1 (32) and enhances its activity. Expression of HIF-1 and the HIF-1-regulated gene CAIX (27) was examined by Western blot. Under hypoxic conditions (0.7% O2), psoriasis-expressing clones showed a marked and higher induction of HIF-1 compared with control cells (Fig. 3d, top panel) and a parallel increase in CAIX protein (Fig. 3d, middle panel). However, it was noted that CAIX expression was also increased in psoriasis expressing 231-H1P1 and 231-H2P2 cells under normoxic conditions. The latter observation is consistent with the recent finding that CAIX can also be regulated by AP-1 (33) and indicates that a component of the CAIX induction seen under hypoxic conditions might be attributable to AP-1, given the involvement of AP-1 as well as HIF-1 in the cellular hypoxic response (34, 35).

Psoriasis Overexpression Influences Breast Tumor Progression in Vitro. We next looked for a relationship between psoriasis expression and biological events relevant to tumor progression in breast cancer cells. The effect of psoriasis on growth of MDA-MB-231 cells was examined and found to be associated with a modest but significant increase in growth rate (Fig. 4a) of up to 1.3 fold (P = 0.0009). The influence of psoriasis on cellular adhesion, an important parameter of invasion, was measured in an in vitro assay. We observed a consistent reduction in cell-substrate adhesion (Fig. 4b) in psoriasis-expressing clones plated on plastic (0.42-fold reduction; P < 0.0001), collagen I (0.20-fold reduction; P < 0.0001), and fibronectin (0.18-fold reduction; P < 0.0001). The influence of psoriasis on invasion was then assessed in a modified Boyden chamber assay. There was a 1.4-fold increase in invasiveness in the high psoriasis-expressing clones (P < 0.0001) after 12 h (Fig. 4c), at which time there was no significant difference in growth (data not shown).

Psoriasis Overexpression Influences Breast Tumor Progression in Vivo. To determine whether psoriasis expression can also influence invasion and metastasis in vivo, psoriasis-overexpressing cells (231-LP1 and 231-H1P1) and control cells (parental 231 and 231-neo) were injected into the mammary fat pad of nude mice, and the generation of tumors and metastasis was assessed (Fig. 5). Control cell lines (231 and 231-neo) generated tumors in 2 of 10 and 3 of 10 sites, respectively, after 8 weeks. These tumors were first noted between 2 and 3 weeks after injection, and increased slowly in size (Fig. 5a). Both psoriasis-expressing cell lines (LP1 and HP1) generated grossly detectable tumors in 7 of 10 and 6 of 10 sites. These tumors were also first noted between 2 and 4 weeks after injection but
increased rapidly in size (Fig. 5, b and c). By week 8 there was no difference in incidence or mean tumor size between parental 231 cells and 231-neo controls, or between the two psoriasis-expressing clones (Fig. 5e). However, both psoriasis-expressing clones were significantly different from both parental and neo-transfected control cells ($P = 0.017$ and $P = 0.024$, Mann Whitney; Fig. 5j). Overall mean tumor sizes (mm$^3$) for each experimental group were: MDA-231 = $21^{11}$, 231-neo = $54^5$, LP1 = $336^{22}$, and HP1 = $370^{70}$. When control groups and psoriasis transfected groups were combined, the mean tumor sizes (mm$^3$) were also significantly different: MDA-213 + 231-neo = $40^{30}$ and LP1 + HP1 = $352^{335}$ combined ($P = 0.0016$, Mann Whitney test). Microscopic examination of primary injection sites identified one additional microscopic tumor in the LP1 cell line group. The primary tumors derived from both control and psoriasis-expressing cells showed similar histological appearances. Expression of psoriasis was confirmed in representative tumors derived from psoriasis-transfected cell clones by immunohistochemistry (data not shown) and by Western blot (Fig. 5f). Psoriasis expression was only detected in tumors from psoriasis-transfected cells (although only a very weak signal was detected in the LP1 cell line), p27 expression was reduced in both psoriasis-transfected cell clone tumors. Grossly evident metastasis was identified and confirmed by microscopy in abdominal lymph nodes distant from the primary injection sites in 2 of 10 mice injected with psoriasis-overexpressing cells (both in the HP1 cell line group) compared with 0 of 10 mice in the control experimental groups.

**DISCUSSION**

The transition from normal epithelium through DCIS to invasive breast cancer is likely to involve many complex processes that are influenced by dynamic changes in gene expression (36). Perhaps the most critical of these processes is the acquisition of the invasive phenotype (37) that occurs with the transition from DCIS to invasive disease, because this event transforms an otherwise local disease into one that is capable of distant spread to threaten the host. It is likely that some of those genes that show alterations in expression between premalignant and invasive components of breast tissues may be relevant to the process of invasion and offer markers of risk of early tumor progression (36). In this study we demonstrate that the psoriasis gene,
which is highly expressed in DCIS and associated with poor prognosis factors when expressed in invasive disease, can enhance growth, adhesion, and invasiveness of a breast cell line in \textit{in vitro} assays and tumorigenicity in nude mice \textit{in vivo}. Furthermore, we describe a potential mechanism for these effects through a direct interaction between psoriasis and the multifunctional intracellular protein Jab1 (21).

Jab1 was originally identified in mammalian cells as a factor influencing \textit{c}-jun transcription of AP-1-regulated genes (21). It soon became clear that Jab1 was also a component (CSN5) of a multimeric protein complex (22, 23). The CSN/COP9 signalosome had been studied previously in other systems and shown to be involved in protein degradation via the Ub-26S proteasome (24, 25). Jab1 has since been shown to be involved in a diversity of interactions with components of cell signaling pathways in \textit{in vitro}, yeast, and human cell line model systems. These interactions appear to result in either translocation of Jab1 from cytoplasm to nucleus (integrin LFA-1 [38], erbB-2 [39] signaling), enhanced activity of transcription factors (including \textit{c}-jun/AP-1 [21], HIF-1 [32], steroid receptors and cofactors [40, 41]) or the promotion of degradation of the interacting protein (including Smad4 [42], p53 [43], HIF-1 [32], MIF1 [28], and p27\textsuperscript{kip1} [29, 43]), often but not always associated with translocation from nucleus to cytoplasm. However, the physiological relevance of some of these interactions, and specifically in the context of breast epithelial cells, is mostly unknown.

In ovarian tumors, increased nuclear Jab1 is associated with progression and poor outcome (44), and altered Jab1 has also been implicated in renal cancer (45). A direct role for Jab1 in breast cancer has not been identified previously; however, several proteins including p53 and erbB-2, which are known to interact with or to influence Jab1, are altered at an early stage within high-risk DCIS (46–49) and may exert some of their effects through Jab1. The interaction between psoriasis and Jab1 also has the potential to directly facilitate several aspects of early tumor progression. We have shown here that expression of psoriasis is associated with translocation of Jab1 to the nucleus, alterations in expression of several Jab1 "downstream" genes, and increased proliferation, altered response to hypoxia, and promotion of invasion. Increased proliferation may be specifically attributable to increased AP-1 activity and down-regulation of the cell cycle inhibitor p27\textsuperscript{kip1} in this model. Alteration of Jab1 might also lead to increased activation of estrogen receptor and progesterone receptor, and up-regulation of cyclin D1 and alteration of transforming growth factor \beta signaling in other cell models (39, 50, 51), but these aspects of Jab1 function remain to be examined in the context of breast cancer. Increased capacity to survive hypoxic stress may occur through augmented HIF-1 activity and hypoxic response. Increased invasiveness may result from activation of AP-1 and HIF-1-dependent genes (52, 53), such as matrix metalloproteinases and VEGF, which are already implicated as critical factors in breast tumor progression (37, 54).

The estrogen receptor-negative MDA-MB-231 breast cell line was selected to reflect the context of psoriasis expression that we and others have observed previously in breast tumors \textit{in vivo} (2, 4). The modest although significant increase in proliferation and invasiveness seen in our \textit{in vitro} assays may reflect the fact that this cell line is already a highly proliferative and invasive cell in \textit{in vitro} assay. More
Fig. 5. Effect of psoriasis expression on tumor growth in nude mice. Groups of 5 mice for each cell line received an injection of 5 x 10^3 cells into the mammary fat pads. The top panels (a–c) show representative mice at 8 weeks from each treatment group that received (a) MDA-MB-231 cells, (b) 231-LP1 cells, or (c) 231-HP2 cells. The expression of psoriasis and p27 protein determined by Western blot on extracts from representative tumors. e, relative growth curves for mice in each group. Lanes and data points indicate the mean tumor volumes at each time point. f, distribution of tumor volumes at 8 weeks. Statistical significance was determined by Mann-Whitney test.

striking increases in growth and invasiveness were observed in vivo in the nude mice experiments, where metastasis was also associated with psoriasis-expressing tumors. This difference is consistent with the anticipated effects of enhanced metalloproteinase and VEGF expression on extracellular matrix and angiogenesis, spheres of influence that are not adequately replicated in vitro assays, and has been observed by others studying the effects of overexpression of VEGF in breast cell lines (55). Nevertheless, additional detailed studies will be necessary to confirm the direct relationship and functional role of these specific factors in the enhanced growth and invasiveness seen in this model in vivo.

Alteration of Jab1 activity in tumors could be attributable in part to alterations in either the cytoplasmic-nuclear distribution (Refs. 38, 45, 56; as appears to be the case for the effect of psoriasis), the ratio of free Jab1:COP9-associated Jab1 (56), competition between different interacting proteins (42), or direct elevation of Jab1 expression and activation. The relevance of these potential mechanisms of action to breast cancer remains to be resolved, both for psoriasis and several other Jab1-interacting proteins. Nevertheless, it has been demonstrated that the many important activities of Jab1 can be influenced by competition between different interacting proteins (42). For example, p53 can compete with and down-regulate Jab1 activation of c-jun (57), and inhibition of Jab1 causes reciprocal up-regulation of p53 (42) and down-regulation of c-jun in HeLa cells (57). It is also interesting to note that the chemokine MIF can exert the opposite effect on Jab1 to psoriasis (28) with respect to modulation of AP-1 activity and p27 expression. This raises the question of whether these different chemokine molecules might compete to modulate Jab1 activity.

Whereas our data support the involvement of Jab1 in mediating many of the biological actions of psoriasis, additional experiments will be needed to confirm that a direct interaction occurs between the putative Jab1 binding motif (29) on psoriasis and the Jab1 protein, and that direct alterations of Jab1 indeed exert effects on specific target genes and pathways. It is also possible that some of the functions of psoriasis are mediated through other pathways (58). For example, it has been shown that other secreted S100 proteins (S100B and S100A12) can bind to and stimulate the receptor for advanced glycation end products, leading to activation of intracellular signaling pathways including up-regulation of ras, mitogen-activating protein kinase and nuclear factor κB in immune cells (59, 60). Expression of receptor for advanced glycation end products is also associated with invasion in gastric carcinoma (61) and is functionally involved in metastasis (62). Unlike some other S100s with chemokine activities such as S100A9 and S100A12, which are expressed by both epithelial and stromal inflammatory cells (63), expression of S100A7 (psoriasis) is restricted to epithelium, at least in skin and breast. However,
psoriasis is also secreted and could potentially interact with cell surface receptors on immune or epithelial cells.

In summary, we have shown that psoriatic cells can contribute to breast tumor progression and that its action may be mediated, at least in part, through Jab1. Although other important cellular proteins also influence and may compete for Jab1, psoriasis is one of the most abundant proteins in high-risk DCIS (2) and is likely to exert an important effect on Jab1 activity in breast tumor cells at an early stage of tumor progression. Thus, therapies aimed at modulating the effect of psoriasis may have important potential in the treatment of early breast cancer.

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Putative functional characteristics of human estrogen receptor-beta isoforms

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Abstract

Estrogen receptors (ERα and ERβ) are clearly multifaceted in terms of structure and function. Several relatively abundant ERβ isoforms have been identified, which can be differentially expressed in various tissues. In order to provide insight into the possible role of the ERβ family in breast tissue a study of the putative functions of the human (h) ERβ1, hERβ2 and hERβ5 isoforms was undertaken. Only hERβ1 was found to bind ligand, which induced conformational changes as determined by protease digestion assays. All ERβ isoforms could bind to and bend DNA although the relative efficiency with which they bound DNA differed with hERα>hERβ1>hERβ2>>hERβ5. All ERβ isoforms inhibited ERα transcriptional activity on an estrogen-response element (ERE)-reporter gene. The relative activities were hERβ1>hERβ2 >hERβ5; however, only hERβ1 had transcriptional activity of its own. Both LY117018-hERα and LY117018-hERβ1 complexes alone could activate transcription on a TGF-β3-CAT gene. Although hERβ2 and hERβ5 had no activity alone, they inhibited ERα but not hERβ1 transcriptional activity of transforming growth factor (TGF)-β3-CAT. In marked contrast to activity on an ERE-CAT reporter gene, hERβ1 did not modulate ERα transcriptional activity on a TGF-β3-CAT reporter gene. These data support promoter-specific differential activities of hERβ isoforms with respect to models of ERα regulated gene expression, and suggest that they may have a role in differentially modulating estrogen action.

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Introduction

The estrogen receptor family of steroid hormone receptors is clearly multifaceted (Hall et al. 2001) and more complex than originally thought. There are two genes which encode estrogen receptors (ER), ERα and ERβ. Both are ligand regulated transcription factors which classically modulate target gene transcription by binding as homo- and/or heterodimers to estrogen responsive sequences in target gene promoters (Cowell et al. 1997). These receptors likely have distinct roles in estrogen action, independent of each other when they are expressed separately (Couse & Korach 1999) but can also have direct interactions due to heterodimerization when the receptors are expressed together in the same target cell (Enmark et al. 1997). In addition, both ERs may encode variant isoforms generated by alternative splicing mechanisms (Lu et al. 1998, Moore et al. 1998). In particular there are data to support variant isoforms of ERβ at the protein level (Fuqua et al. 1999, Fujimura et al. 2001). Furthermore, we have shown in human breast tissues that variant forms of ERβ are more abundant than the wild-type at least at the RNA level (Leygue et al. 1999).

Human (h) ERβ2 (also called hERβcx (Ogawa et al. 1998b)) and ERβ5 variant mRNAs are missing the wild-type exon 8 sequences and contain extra sequences which are distinct from each other, followed by sequences that are then identical with each other (see Fig. 1). They are predicted to encode C-terminally truncated ERβ-like proteins identical to wild-type until amino acid residue 468 (by reference to the long form of hERβ1) (Ogawa et al. 1998a). After amino acid 468 hERβ2 is predicted to encode 28 novel amino acids, with the full-length protein having a predicted molecular mass of 55.5 kDa. In contrast, after amino acid 468 hERβ5 is predicted to encode only 5 novel
amino acids with the full-length protein having a predicted molecular mass of 53 kDa.

Although total ERβ expression appears to decrease between normal breast and ER-positive breast tumors (Leygue et al. 1998b, Roger et al. 2001), the relative expression of the variant ERβ isoforms to the wild-type ERβ can also change during breast tumorigenesis, at least at the RNA level (Leygue et al. 1999). This suggests that the expression and/or the activity of the ERβ family of receptors changes during breast tumorigenesis and may have a role in this process as well as having a role in the altered estrogen action that occurs during breast tumorigenesis. In order to provide insight into the possible role of the ERβ family in breast tissue we have undertaken a study of the putative functions of the hERβ1, hERβ2 and hERβ5 isoforms.

Materials and methods

Materials

17β-Estradiol (E2), 4-hydroxytamoxifen (4-OH-TAM) and CAPS (3-cyclohexylamino-1-propanesulfonic acid) were from Sigma Chemical Co. (St Louis, MO, USA). ICI 182,780 was a gift from AstraZeneca Pharmaceuticals, Macclesfield, UK. LY117018 was a gift from Eli Lilly Co. (Indianapolis, IN, USA). [3H]17β-Estradiol, [14C]chloramphenicol, and [35S]-methionine were from New England Nuclear (Boston, MA, USA). [α-32P]dCTP, [γ-32P]ATP, and [35S]-ATP were from ICN Pharmaceuticals (Irvine, CA, USA). All cell culture reagents were obtained from GIBCO/BRL (Burlington, Ontario, Canada).

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI, USA). Reactions were performed according to the manufacturer's instructions.

Scatchard analysis

Human ERα (pcDNA3-1/wild-type human ERα from HEGO (Green et al. 1986)), human ERβ1 (pcDNA3-1 hERβ1, long form of 530 amino acids (Leygue et al. 1998a, Ogawa et al. 1998a)), human ERβ2 (pcDNA3-1 hERβ2, long form (Ogawa et al. 1998b)) and human ERβ5 (pcDNA3-1 hERβ5, long form) proteins were synthesized by in vitro transcription–translation as described above. Ligand binding studies were conducted as previously described (Lu et al. 2000). In vitro-generated receptor was diluted 10-fold in buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction that contained varying concentrations of [3H]E2 (0.01–100 nM), followed by overnight incubation at 4°C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabelled E2. Unbound steroid was removed by addition of 500 µl 0.5% charcoal–0.05% dextran in the above dilution buffer for 30 min at 4°C followed by centrifugation at 10 000 × g for 10 min at 4°C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [3H]E2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, Kd.

Limited proteolytic digestion analysis

Conformational studies were performed as described previously (Beekman et al. 1993). In vitro-synthesized ERs were incubated with agonists (E2, diethylstilbestrol) and antagonists (4-OH-TAM, LY117018) overnight at 4°C. The liganded receptors were then diluted 1:10 (v/v) in TE buffer, then 20 µl of this ER solution were treated with increased concentrations of trypsin (0.2 to 5 µg) for 20 min at room temperature and stopped by the addition of loading buffer. The samples were boiled and were analyzed directly by SDS-polyacrylamide gel electrophoresis (10% w/v). The gel was dried and the digested bands were visualized by autoradiography.

Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized human ERs were used for EMSA. Typically 1 µl programmed lysates containing equal amounts of each receptor as determined
by polycrylamide gel electrophoresis of \(^{35}\)S-methionine-labeled protein generated in parallel in \textit{vitro} transcription-translation assays, was assayed in EMSA. One microliter lysate was incubated in a final volume of 20 \(\mu\)l, and the reaction solution was 5 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 \(\mu\)g poly[dI-C]. The binding reaction was initiated by adding 1 \(\mu\)l (approx. 10 fmol) 5'-\([\text{\textsuperscript{32}}\text{P}]\)-end-labeled, double stranded estrogen-response element (ERE) oligonucleotide (35 mer, 5'-AACTTTGATGAGTCAGTGTGAACCTTGGAC-3' containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20 \(^\circ\)C for 30 min. DNA-bound complexes were electrophoretically separated on a 4-5% lowly cross-linked acrylamide gel (1:29 bisacrylamide) at 150 V for 90 min at 20 \(^\circ\)C in 0.5 \(\times\) TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 \(\mu\)g ER antibody (usually 1 \(\mu\)l H222 for ER\(\alpha\), or 1 \(\mu\)l PAI-310 for ER\(\beta1\) and ER\(\beta2\)) were run to determine the presence of super-shifted antibody-bound ER–ERE complexes (data not shown). Reticulocyte lysates containing \textit{in vitro}-translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4 \(^\circ\)C overnight to allow receptors to bind ligand, followed by EMSA.

### DNA bending assay

The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL, USA) (Nardulli & Shapiro 1992) was digested with EcoRI and EcoRV to produce a 430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of
[γ-32P]ATP, and purified on a G50 Sephadex column. Gel mobility shift assays were carried out essentially as described above. Aliquots of the binding reactions were run on 8% non-denaturing acrylamide gels, dried and exposed to X-ray film. The degree of DNA bending was determined using the method of Thompson and Landy (1988).

**Cells, cell culture and transient transfection**

For transient transfection analysis, tagged ER expression vectors were generated. Human ERTα, ERβ1, ERβ2 and ERβ5 were tagged at their N-terminus with a polyhistidine and an Xpress epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cos-1 and Cos-7 cells were obtained from the ATCC (Manassas, VA, USA). The cells were routinely cultured in DMEM containing 5% v/v fetal calf serum (FBS), 1% w/v glucose, glutamine and penicillin-streptomycin (5%CM). To obtain estrogen-depleted cells, the culture medium of stock cells was changed to phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin-streptomycin (5%CS) and replaced every 2 days. Six days later the medium was replaced by 10%CS until required for experiments. For transient transfection experiments, the cells were transfected using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, Ontario, Canada). Briefly, the day before transfection, the estrogen-depleted cells were seeded in 6-well plates at 2·5 × 10⁵ cells per well in 2 ml 5%CS and left overnight. The plates were 70–80% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer's protocol, then fresh medium (5%CS) was added to the transfection mixture and 0·6 ml per well of the above mixture with either ERE-II-TCO-CAT (a gift from P Webb (Webb et al. 1995)) or transforming growth factor (TGF)-β3-CAT-reporter plasmid DNA (Yang et al. 1996) was added. ER expression plasmid (50–450 ng) or empty vector and 100 ng β-gal pCH110 plasmid DNA (Pharmacia Canada, Mississauga, Ontario, Canada) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA-Effectene complexes. Vehicle (ethanol), estradiol-17β or LY117018 was then added 20 to 30 min later. The cells were left for 48 h and then harvested. Cell extracts were prepared by freeze/thawing and were used to determine chloramphenicol acetyl transferase (CAT) and β-galactosidase activity as previously described (Dotzlaw et al. 1992).

**Western blot analysis**

For Western blot analysis, 2·5 × 10⁵ Cos-1 cells were set up in 6-well plates, then transiently transfected with plasmids and treated with estrogen or antiestrogen under the same conditions as for the CAT assay described above. Cells were harvested 48 h after transfection, washed once with Isoton II and then the washed cell pellets were resuspended in 200 μl Isoton II. Aliquots of cell suspension (150 μl) were extracted and used for Western blots and the remainder was used for determination of β-galactosidase activity. For Western blotting, the cells were pelleted and then extracted using 40 μl hot (95°C) extraction buffer J with shaking for 20 min at 95°C as previously described by Joel et al. (1998). The entire extract was subjected to 10% SDS-polyacrylamide gel electrophoresis as previously described (Adeyinka et al. 2002). The separated proteins were transferred to nitrocellulose membranes and processed as previously described (Adeyinka et al. 2002). Detection of the tagged estrogen receptor proteins was by incubation of blots with anti-Xpress antibody (1/5000 in TBST, Cat#R310-25, Invitrogen Canada Inc.) overnight at 4°C, followed by washing and incubation with secondary antibody (horseradish peroxidase conjugated goat anti-mouse antibody, 1/5000 in TBST, Jackson Immuno Research Labs Inc., West Grove, PA, USA) at room temperature for 2 h. Visualization was carried out using the SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

**Statistical analysis**

Differences in variance were tested using ANOVA, where appropriate. Differences between individual mean values were then determined using Student’s t-tests. All tests were performed using GraphPad Prism statistical analysis software (GraphPad Software Inc., San Diego, CA, USA).
Results

Identification and organization of hERβ1, hERβ2 and hERβ5 cDNA sequences at the hERβ locus on chromosome 14

The estrogen receptor beta gene has been localized to human chromosome 14q22–24 and the genomic structure of 8 exons comprising hERβ1 has previously been published (Enmark et al. 1997). However, the previously described variant hERβ2 (also called hERβcx) and hERβ5 mRNA contain only sequences corresponding to exons 1 to 7 of hERβ1 and then they diverge (see Fig. 1A). They do not contain exon 8 sequences of hERβ1, but contain sequences termed exon 9, which are located downstream of exon 8 on chromosome 14 (Fig. 1B), identified using database sequences of chromosome 14 (accession numbers CNSO1 RHJ and AF215937) and the Human Genome Working Draft. It should be noted that neither of these genomic sequences contain an extra A 5’ of the start site of translation for hERβ, that would place another upstream ATG in frame with the known coding region and introduce 18 amino acids to the N-terminal of the known coding region, as recently described (Wilkinson et al. 2002). Interestingly, hERβ5 mRNA also contains sequences between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA. These hERβ5 mRNA specific sequences can be found immediately following exon 7 sequences in intron 7 of the human ERβ gene (Fig. 1B), suggesting that the normal splice donor site is not recognized and a cryptic splice donor site is present in intron 7. Furthermore the exon 9 sequences present in hERβ5 cDNA start 28 nucleotides downstream of those present in hERβ2, suggesting a cryptic splice acceptor site is present within exon 9. There are also multiple non-coding exons 5’ to exon 1 as previously identified (Enmark et al. 1997), since several hERβ cDNAs contain sequences in their 5’ UTR which are found further upstream of the previously described exon 1 on chromosome 14 (Fig. 1B; 1H–1C seen in AB006589, 1B seen in NM_001437, AX234658, AF05428, AF060555, AB006589 references). The sequences of a recently characterized promoter region of hERβ (Li et al. 2000) are found immediately upstream and overlapping with exon 1B. However, the presence of hERβ mRNAs whose 5’UTR contain exonic sequences found upstream of this documented promoter suggest that there are alternative promoters for the hERβ gene. This is similar to the hERα gene and suggests that regulation of expression of these genes is complex (Kos et al. 2001).

The predicted open reading frames for hERβ1, hERβ2 and hERβ5 are shown in Fig. 1A. hERβ2 contains amino acids 1–468 which are identical to hERβ1; the sequence then diverges containing another 28 novel amino acids encoded in the open reading frame. hERβ5 was isolated as a partial cDNA but is likely also to be identical to hERβ1 from amino acids 1–468 and then diverges containing another 5 novel amino acids. Both these variant hERβ proteins would be truncated at the C-terminus, disrupted in helix 11 and missing helix 12 and therefore unlikely to bind ligand or have AF2-mediated transcriptional activity. Lack of ligand binding has been confirmed using in vitro generation of these proteins as outlined below.

Ligand binding activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Human ERβ1 has previously been shown (Enmark et al. 1997) to bind E2 with high affinity and specificity, and our data confirm these findings. Figure 2A shows specific saturable binding of [3H]E2 to in vitro translated hERβ1 with a calculated \( K_d = 0.11 \) nM. However, the open reading frames of hERβ2 and hERβ5 cDNA predict for C-terminally truncated proteins compared with hERβ1 and are predicted not to bind ligand. As shown in Fig. 2B and C no saturable binding of [3H]E2 to in vitro-translated hERβ2 or β5 was observed.

Human estrogen receptor isoform conformational status and ligand induced changes

To determine the possible conformational status of variant hERβ isoforms, a previously used limited trypsin digestion assay (Beckman et al. 1993, McDonnell et al. 1993) was employed to compare the proteolytic digestion patterns of variant 35S-methionine labeled hERα, hERβ1, hERβ2 and hERβ5 in the presence and absence of estrogens and antiestrogens (4-OH-TAM and LY117018). The results are shown in Fig. 3. In the absence of any ligand, all ER isoforms were sensitive to
proteolysis. In the presence of estradiol both hERα and hERβ1 become more resistant to digestion, and a 32-5 kDa resistant band (shown by asterisks in Fig. 3) was observed. In contrast addition of the antiestrogens 4-OH-TAM and LY117018 did not significantly affect the sensitivity of the receptors to trypsin compared with the receptors in the absence of ligand. The sensitivity of the variant isoforms hERβ2 and hERβ5 was not affected by ligand, consistent with their inability to bind ligand and suggesting that the variant isoforms are unlikely to be in an activated conformation.

DNA binding and bending activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Similar amounts of each recombinantly produced ER isoform protein, determined as described in the Materials and methods section, were used in the electrophoretic mobility shift assays. As previously demonstrated hERβ1 and hERβ2 can bind to an ERE in a gel mobility shift assay (Fig. 4A), although the efficiency of hERβ2 DNA binding was less than hERβ1 (Moore et al. 1998). In contrast, Ogawa et al. (1998b) showed no DNA binding activity for hERβ2. hERβ5 also has the ability to bind an ERE in gel mobility shift assays (Fig. 4A), but was less efficient than hERβ2. The specificity of the binding was determined by competition with excess unlabeled ERE whereas no competition was seen with an excess of unlabeled nonspecific 33 mer oligonucleotide.

DNA bending assays demonstrated that hERα, hERβ1 and hERβ2 were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end (E) of the DNA fragment (Fig. 4B and C; Nardulli & Shapiro 1993, Lu et al. 2000). The calculated bending angle for hERα was 64±8 (mean ± s.e.m., n=3), for hERβ1 it was 53±6 and for hERβ2 it was 54±6. A lower overall signal of the retarded complexes was seen with hERβ1 and hERβ2 compared with hERα which likely reflects the lower efficiency of the hERβ isoforms of binding to an ERE compared with the hERα. Furthermore, the DNA binding ability of hERβ5 was too low to obtain accurate data for DNA bending calculations. No effect of ligand was observed (data not shown).
Figure 3 Sensitivity of human estrogen receptor isoforms to protease digestion. Radiolabeled ER was made in vitro as described in the Materials and methods section, and digested with increasing levels of trypsin, with and without ligand (E₂, 4-OH-TAM or LY117018 (LY)). The products were visualized by autoradiography after SDS-PAGE. Resistant bands are shown by arrows. The asterisks show the agonist induced resistant 32.5 kDa bands.

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Transcriptional activity of hERβ1, hERβ2 and hERβ5

The ability of tagged ERs to activate transcription was initially investigated using Cos-1 cells and an ERE containing reporter gene, ERE_{452-delta-TCO-CAT}, which has two vitellogenin A2 (−333/−288) EREs upstream of a CAT reporter (Webb et al. 1995). Epitope tagged receptors were used so that relative expression of all the ERs could be measured using antibodies to the epitope tag, and preliminary experiments demonstrated that the tagged ERα and ERβ1 were similar to their untagged counterparts in activating transcription with and without ligand (data not shown). Preliminary studies showed that transfection of...
50 ng ERα expression vector gave maximal estradiol-induced transactivation of this reporter gene. hERα and hERβ1 activated transcription in a ligand inducible manner (Fig. 5), but hERβ1 was overall less active than hERα (P<0.0001, n=5), and increased expression of hERβ1 did not alter this relationship. These data are consistent with previous findings. As shown in Fig. 5, low doses of estradiol (0-1 nM) which significantly activated hERα did not activate hERβ1 (P<0.0001, n=5), and the apparent ligand-independent activity (zero ligand added) of hERα was significantly higher than that of hERβ1 (P=0.024, n=5). This ligand-independent activity was inhibited by 0.1 and 100 nM of the antiestrogen LY117018 (a raloxifene analog) as well as by hERβ1 (50-450 ng) and hERβ2 (450 ng) (data not shown). These results are not due to over-expression of hERβ1 protein relative to hERα, since under conditions where similar levels of hERα protein (50 ng hERα expression plasmid; see Fig. 6, lane 4) and hERβ1 are expressed (150 ng hERβ1 expression plasmid; see Fig. 6, lane 4) the ligand-independent activity of the receptors is still significantly different (see Fig. 5, compare histogram bar 1 with 7) and the estrogen-inducible (0.1 and 100 nM) activity of hERβ1 (see Fig. 5, compare histogram bars 2 and 3 with histogram bars 8 and 9) is not further increased. The expression of the variant isoforms hERβ2 and hERβ5 alone demonstrated little if any transcriptional activity under these conditions (Fig. 5).

ERα and hERβ isoforms can heterodimerize (Cowley et al. 1997), which may underlie the functional interactions between ER isoforms. All hERβ isoforms tested inhibited the transcriptional activity of hERα on an ERE containing promoter (Fig. 7A and B) but the various hERβ isoforms had different efficiencies with hERβ1 > hERβ2 > hERβ5. Ligand activation of hERβ1 did not affect its ability to decrease the activity of hERα, since under conditions when it was not activated (0 or 0.1 nM estradiol, see Fig. 5) hERβ1 activity was similar to that under conditions when it was activated (100 nM estradiol). Variant isoforms of hERβ had little if any effect on hERβ1 activity on ERE-containing promoters (data not shown).

The transcriptional activity of ER isoforms was next examined on the non-ERE-containing promoter, TGFβ-3-CAT, where the DNA binding domain of ERα is not required for activity (Yang et al. 1996). This promoter was shown to be preferentially activated by the raloxifene-bound hERα compared with estradiol in cultured cells (Yang et al. 1996), and we have previously shown differential abilities of murine ERβ isoforms to affect this promoter compared with ERE-containing promoters (Lu et al. 2000). Therefore, the activity of hERβ isoforms on TGFβ-3-CAT was examined (Fig. 8). Optimal activity for hERα was obtained with transfection of 50 ng expression plasmid (data not shown). A significant increase in transcription was obtained with 0.1 nM LY117018 that was not further increased with 100 nM LY117018 treatment (P=0.0061, n=3). LY117018 significantly increased the transcriptional activity of hERβ1 on the TGFβ-3-CAT reporter gene at the lower levels of hERβ1 expression (50 ng, P=0.0008; 150 ng, P=0.02, n=3) but at high levels of hERβ1 expression (450 ng), a significant increase in ligand-independent activity was seen, and no further increase was seen due to ligand. Overall, hERβ1 was significantly less active than hERα in inducing TGFβ-3-CAT (P<0.0001, n=3).

Figure 4 (A) Determination of the ability of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) to bind to DNA. Autoradiograph of an electrophoretic mobility gel shift analysis of in vitro-transcribed/translated hERα, hERβ1, hERβ2 and hERβ5 proteins binding to a 35 mer double stranded ERE oligonucleotide containing the vitilogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate ER isoform in the shifted complex was determined by the ability of a specific antibody (H222 for ERα, PA1 for the ERβ proteins) to super-shift the complex (data not shown). Specificity of the complexes was determined by the ability of a 200-fold excess of the unlabeled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200-fold excess of unlabeled nonspecific 33 mer oligonucleotide (nonspecific competitor). (B and C) Comparison of the ability of (B) hERα (hERα) and hERβ1 (hERβ1) and (C) hERβ1 and hERβ2 (hERβ2) to bend DNA. In vitro-transcribed/translated ER isoforms were preincubated with 10 nM estradiol-17β followed by incubation with radiolabeled ERE Bend fragments as described in the Materials and methods and were subjected to electrophoretic gel mobility shift analysis. DNA bending was demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison to the mobility of complexes when the ERE is at the end of the DNA fragment (E).
Although there was a trend towards inhibition of TGFβ-3-CAT with increasing expression of hERβ2 or hERβ3 (data not shown), this was not statistically significant.

When the ability of hERβ isoforms to affect hERα activity was investigated at the TGFβ-3 promoter, differences between the wild-type and variant isoforms were observed. The wild-type hERβ1 did not significantly affect hERα transcriptional activity at any level of expression tested (Fig. 9A and B). However, under the same conditions hERβ2 significantly inhibited hERα transcriptional activity on TGFβ-3-CAT (P=0.0002, n=3), and as expected the effect was not influenced by LY117018, since hERβ2 does not bind ligand. However, hERβ2 inhibits both the ligand activated and the non-ligand activated (data not shown) hERα (P=0.017, n=3) at the TGFβ-3-CAT promoter. hERβ5 also inhibited hERα transcriptional activity on TGFβ-3-CAT but only at the highest expression of hERβ5 (Fig. 9B, P=0.038, n=3). Similar to their action at an ERE-containing promoter, the truncated ERβ variants ERβ2 and ERβ5 do not modulate wild-type hERβ1 transcriptional activity on TGFβ-3-CAT (data not shown).

**Discussion**

There is a growing body of evidence that ERα and ERβ can be expressed together in some cell types and independently expressed in others (Dotzlaw et al. 1997, Jarvinen et al. 2000, Saji et al. 2000). If expressed together they form heterodimers, which under experimental conditions are preferred over homodimerization (Cowley et al. 1997). Further, transient coexpression of ERα and ERβ in cell lines results in ERβ1-induced reduction of ERα activity.
at low ligand concentrations, as measured using ERE-regulated reporters (Hall & McDonnell 1999). A conclusion from these data is that ERβ can directly modulate ERα activity. This has significance since many reports exist of differential expression of the two receptors under conditions of altered estrogen sensitivity. For example, ERβ expression is significantly downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ's ability to modulate ERα is significantly altered during breast tumorigenesis (Leygue et al. 1998b, Roger et al. 2001). In addition, current data show that in normal and neoplastic breast tissues, the level of expression of the C-terminally truncated ERβ variants, ERβ2 and ERβ5, is markedly higher than the ligand binding ERβ1. These data suggest that the variant ERβ isoforms may also have a role in modulating estrogen and possibly antiestrogen action in human breast cells. The experiments described in this manuscript were undertaken to gain insight into the possible role of the truncated ERβ variants.

Our data show that only hERβ1 is able to bind ligand. Steroid hormone receptors are known to undergo conformational changes during the process of activation especially due to ligand binding, and differences are seen between agonist and antagonist binding (Beckman et al. 1993, McDonnell et al. 1995). Recent structural analyses of the ligand binding domain (LBD) of several nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonists and antagonists bind to the same site but induce different conformational changes that are now known to affect transcriptional function, providing structural evidence for antagonism (Brzozowski et al. 1997). The variant hERβ isoforms, while not binding ligand, may exist in an activated state in the absence and presence of ligand; however, our data suggest that hERβ2 and hERβ5 are unlikely to be in an activated conformation, and this is consistent with their inability to activate transcription of either a 'classical' or a 'non-classical' estrogen receptor regulated reporter gene.

All ERβ isoforms examined (ERβ1, ERβ2, and ERβ5) inhibit the transcriptional activity of ERα on ERE-containing promoters, while only ERβ1 has any activity alone. This confirms and extends previous data and demonstrates that the relative inhibitory activity of the ERβ isoforms is
Figure 7 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from an ERE (vitellogenin A2) regulated CAT reporter gene in the presence and absence of ligand following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency) ± S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.
ERβ1 > ERβ2 > ERβ5. This correlates with the relative efficiencies with which ERβ homodimers bind to DNA and may suggest a competition of the beta isoform homodimers with ERα homodimers for DNA binding. However, since heterodimers are preferred under these conditions, it is likely that these predominate under our experimental conditions and the intrinsically lower transcriptional activity of the heterodimers are predominant. Cowley et al. (1997) demonstrated that when hERα and hERβ1 are expressed at both a 1:1 and 1:2 ratio the ERα/ERβ1 heterodimer was predominant. This heterodimer had a DNA binding affinity similar to that of the ERα homodimer, and was capable of recruiting steroid receptor coactivator-1 (SRC-1). However, the heterodimer has less transcriptional activity than the ERα/ERα homodimer, suggesting that it may be less efficient in recruiting coactivators than the ERα homodimer. In contrast, the C-terminally truncated hERβ2 has markedly reduced ability to bind to DNA and likely the ERα/ERβ2 heterodimer also binds less well than ERα/ERα homodimers to an ERE (Moore et al. 1998, Ogawa et al. 1998b). But in contrast to hERβ1, hERβ2 does not recruit coactivators (Ogawa et al. 1998b). Our data show that hERβ5 is less efficient than hERβ2 in binding to DNA, and is also unlikely to recruit coactivators. However, at an ERE the wild-type hERβ1 is more potent than either of the two variants in inhibiting the ability of ERα to activate transcription. So it appears that the inability to
recruit coactivators is not correlated with the ability of ERβ isoforms to inhibit ERα activity. Since DNA activity is also a reflection of efficiency of dimerization, it is speculated that the truncated ERβ isoforms have reduced ability to dimerize with ERα and form stable heterodimers than the wild-type ERβ1. Together with our Western blot data it seems that significant inhibition of ERα transcription occurs at levels of ERβ1 expression that are less than or equivalent to ERα (50 ng ERβ1 plasmid makes less protein than 50 ng ERα plasmid, but still significantly affects ERα transcription activity). Therefore our data would be consistent with the mechanism of inhibition being related to a high efficiency of dimerization and reduced efficiency in recruiting coactivators, but not the inability to recruit coactivators.

Interestingly, marked differences in the ability of the ERβ isoforms to affect ERα activity are seen at an estrogen receptor responsive site where the mechanism of transcriptional regulation is quite distinct from that operating at a classical ERE, e.g. the so-called raloxifene responsive element in the TGF-β3 promoter (Yang et al. 1996). This is in marked contrast to the results seen at an ERE regulated reporter gene. The ER responsive site in the TGF-β3 promoter is poorly activated by the estradiol–ERα complex, but is strongly activated by the raloxifene–ERα complex. In addition, the DNA binding domain of the ER is not required for this activation. It is assumed that protein–protein interactions between ERα and other transcription factors bound to this promoter are involved in regulation. However, the identity of these ‘other’ transcription factors is unknown. Using an analog of raloxifene, LY17018 (Lu et al. 2000), we have confirmed that this promoter is poorly activated by the estradiol–ERα complex (and this was not altered in our hands by treatment of the transfected Cos-1 cells with epidermal growth factor (Lu & Giguere 2001); data not shown) but was significantly activated by the LY17018–ERα complex. Similarly, the LY17018–hERβ1 complex was found to activate transcription from the TGF-β3 promoter, but in contrast to the murine ERβ1 (Lu et al. 2000), is less active than the LY17018–hERα complex. Human ERβ2 and hERβ5 alone could not activate this promoter. This is in contrast to the murine ERβ2 variant (Lu et al. 2000) which is structurally quite different to the hERβ2. Furthermore, no murine equivalent to either hERβ2 or hERβ5 isoforms, that are frequently expressed in human tissues, has as yet been identified. However, coexpression of increasing amounts of hERβ2 and hERβ5 with ERα resulted in inhibition of LY17018–ERα transcriptional activity but not LY17018–ERβ1 activity from the TGF-β3 promoter. In contrast to an ERE-containing promoter is the observation that the wild-type hERβ1 did not significantly inhibit the transcriptional activity of the LY17018–ERα complex at the TGF-β3 promoter. At this promoter the differences in the hERβ isoform activity on LY17018–ERα complexes were correlated to the ability to recruit coregulatory factors. Significant effects of hERβ2 on hERα were seen under conditions of equimolar expression, as determined by Western blot analysis of the similarly tagged proteins, but hERβ5 was less active than hERβ2 and this is consistent with a reduced efficiency of dimerization. There appears to be a differential expression of hERβ isoforms at least at the RNA level in different human tissues, as well as altered relative expression during breast tumorigenesis (Leygue et al. 1999, Omoto et al. 2002), and altered levels of hERβ6x (hERβ2) as well as hERβ1 during prostate cancer progression (Fujimura et al. 2001). Therefore, it is possible that the differential activities of hERβ isoforms on some genes may have both physiological and pathophysiological importance.

In conclusion we have characterized some potential functions of several commonly expressed hERβ isoforms. Generally, the ligand binding wild-type hERβ1 has transcriptional activity alone on both ‘classical’ and ‘non-classical’ estrogen responsive promoters, although it is less efficient than ERα. Furthermore, the hERβ family of receptors generally negatively modulate ERα.

Figure 9 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from a TGF-β3-CAT reporter gene in the presence and absence of the raloxifene analog LY17018 following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±SEM of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.

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transcriptional activity when coexpressed at 'classical' as well as 'non-classical' ER responsive promoters. However, promoter specific differential activity of the various hERβ isoforms was found, in particular between the wild-type hERβ1 and its C-terminally truncated variants hERβ2 and hERβ5. The possibility that there is differential expression of the hERβ isoforms suggests that they may have a role in differentially modulating estrogen action.

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Relationship of coregulator and oestrogen receptor isoform expression to de novo tamoxifen resistance in human breast cancer

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This study addresses the hypothesis that altered expression of oestrogen receptor-beta and/or altered relative expression of coactivators and corepressors of oestrogen receptors are associated with and may be mechanisms of de novo tamoxifen resistance in oestrogen receptor positive breast cancer. All cases were oestrogen receptor +, node negative, primary breast tumours from patients who later had no disease progression (tamoxifen sensitive) or whose disease progressed while on tamoxifen (tamoxifen resistant). Using an antibody to oestrogen receptor-beta that detects multiple forms of this protein (total) but not an antibody that detects only full-length oestrogen receptor-beta 1, it was found that high total oestrogen receptor beta protein expression was more frequently observed in tamoxifen sensitive tumours than resistant tumours (Fisher's exact test, P=0.046). However, no significant differences in the relative expression of oestrogen receptor alpha (ER alpha), oestrogen receptor beta 5 and full-length oestrogen receptor beta 1 RNA in the tamoxifen sensitive and resistant groups were found. Also, when the relative expression of two known coactivators, steroid receptor RNA activator and amplified in breast cancer 1 RNA to the known corepressor, repressor of oestrogen receptor activity RNA, was examined, no significant differences between the tamoxifen sensitive and resistant groups were found. Altogether, there is little evidence for altered coregulators expression in breast tumours that are de novo tamoxifen resistant. However, our data provide preliminary evidence that the expression of oestrogen receptor beta protein isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.

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Keywords: SRA; AIB1; ROA; coregulators; OR isoforms; human breast cancer; tamoxifen

The ability of anti-oestrogens such as tamoxifen to compete with oestrogens for binding to OR and to antagonise their mitogenic action provides the basic rationale for endocrine therapy and prevention (for a review see (Osborne, 1998b) in breast cancer. Adjuvant tamoxifen post-operative therapy reduces the number of recurrences and prolongs survival in women whose primary tumours are oestrogen receptor (OR) positive (Group, 1998). However, even though OR level is considered a marker for predicting the likelihood of responding to adjuvant hormonal therapies, some patients, whose primary tumours are OR positive do not respond to tamoxifen treatment. Such apparent de novo tamoxifen resistance does not depend upon the level of OR within the primary tumour. As well as many of those patients whose disease initially responds to tamoxifen, progress while still under treatment having acquired resistance and this occurs despite continued expression of OR. Thus suggesting other components of the oestrogen signalling pathway may be altered. Recent observations using laboratory models (Hall and McDonnell, 1999; Lanz et al, 1999; McKenna et al, 1999; Montano et al, 1999) have demonstrated that altered levels of OR isoforms and/or alteration of expression of coactivators and corepressors can deregulate oestrogen and antioestrogen activity in target cells, suggesting the hypothesis that altered levels of OR isoforms and/or coregulators in vivo could be a mechanism of tamoxifen resistance. Previously we have demonstrated that the relative expression of OR alpha/alpha.5 as well as the relative expression of some OR coactivators to corepressors is significantly altered during breast tumorigenesis in vivo (Leygue et al, 1998; Murphy et al, 2000). Furthermore, since these alterations parallel the marked changes in oestrogen action that accompany breast tumourigenesis, they may have a role in this process. To explore the hypothesis that such changes could underlie de novo tamoxifen resistance in vivo, the expression of OR isoforms, two known coactivators (steroid receptor RNA activator (SRA), (Lanz et al, 1999) and amplified in breast cancer-1 (AIB1) (Anzick et al, 1997)) and one corepressor (repressor of oestrogen receptor activity (ROA) (Montano et al, 1999)) of OR activity have been investigated in primary breast tumours from node negative patients whose tumours were OR

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positive and that subsequently responded or had disease progression while on adjuvant tamoxifen therapy.

MATERIALS AND METHODS

Human breast tumours

All breast tumour cases used for this study were selected from the NCIC-Manitoba Breast Tumour Bank (Winnipeg, Manitoba, Canada). As previously described (Hiller et al., 1996), tissues are accrued to the Bank from cases at multiple centres within Manitoba, rapidly collected and processed to create matched formalin-fixed-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by coloured inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks from cases for this study. For each case, interpretations included an estimate of the cellular composition (including the percentage of invasive epithelial tumour cells and stroma), tumour type and tumour grade (Gardener scoring). Steroid receptor status was determined for all cases by ligand binding assay performed on an adjacent portion of tumour tissue. Tumours with oestrogen receptor levels above 3 fmol mg⁻¹ of total protein were considered OR positive.

To identify cases that responded divergently to tamoxifen, review of approximately 1000 consecutive cases was undertaken to identify cases that were OR positive, node negative and that had been treated with adjuvant tamoxifen following surgery +/- local radiation.

Immunohistochemistry

Immunohistochemistry was performed on serial 5 µm sections from a representative, formalin fixed paraffin embedded archival tissue block from each tumour. Immunohistochemical staining for ORβ was performed using two different primary antibodies. IgYERB503 (a gift from Dr Jan-Ake Gustafson) detects total ORβ isoforms (Horvath et al., 2001; Saji et al., 2000) and GC17 (a gift from Dr Shuk-Mei Ho) detects only the full-length ORβ (Leav et al., 2001). The GC17 polyclonal antibody was raised in rabbits against a peptide sequence in the F domain of the human OR-β receptor (amino acids 449 to 465) and its specificity validated previously (Leav et al., 2001). The epitope to which the IgYERB503 antibody is directed is not known, but this polyclonal chicken antibody was raised to an ORβ recombinant protein which was disrupted in the ligand binding domain by insertion of 18 additional amino acids, but was subsequently shown to also recognize the full-length non-inserted ORβ protein (Saji et al., 2000). Antibodies were applied using an automated tissue immunostainer (Discovery module, Ventana Medical Systems, Phoenix, AZ, USA), DAB immunohistochemistry kit and bulk reagents that were supplied by the manufacturer. Briefly, the Discovery staining protocol was set to 'Standard Cell Conditioning' procedure, followed by 12 h incubation with primary antibody and 32 min incubation with secondary antibody. Concentrations of primary antibodies initially applied to the Ventana instrument were 1:200 for IgYERB503 and 1:50 for GC17, which translates into final concentrations of 1:600 and 1:150 after a 1:3 dilution with buffer dispensed onto the slide with the primary antibody. Levels of nuclear ORβ expression were scored semi-quantitatively, under the light microscope. Scores were obtained by estimating average signal intensity (on a scale of 0–300) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall IHC-score. Cases with a score lower than or equal to 100 were considered negative or weakly positive, whereas tumours with scores higher than 100 were classified as positive for ORβ expression (Al-Haddad et al., 1999).

RNA Extraction and RT–PCR conditions

Total RNA was extracted from 20 µm frozen tissue sections (20 sections per tumour) using Trizol™ reagent (Life Technologies, NY, USA) according to the manufacturer’s instructions and quantified spectrophotometrically. One µg of total RNA was reverse transcribed in a final volume of 25 µl as previously described (Leygue et al., 1996).

Primers and PCR conditions

Coregulators The primers used were: SRAcoreU primer (5'-AGGAACGCGGGTGGAGAAGA-3'; sense; positions 35–33, Genbank accession number AF092038) and SRAcoreR primer (5'-AGTCTGGGAAACCAGGAT-3'; antisense; position 696–678, Genbank accession number AF092038); AIBI-U primer (5'-ATACATTGCTGATGTTGACT-3'; sense; positions 110–130, Genbank accession number AF012108) and AIBI-L primer (5'-TCTGTCCTTTATAGGC-3'; antisense; positions 458–438, Genbank accession number AF012108); ROA-U primer (5'-CGAAAAATCTCTCCCTCGACCA-3'; sense; positions 385–405, Genbank accession number AF156962) and ROA-L primer (5'-CCTCTTGGCTTTTCTTACCA-3'; antisense; positions 781–761, Genbank accession number AF156962).

Radioactive PCR amplifications for SRA were performed and PCR products analysed as previously described (Leygue et al., 1999b) with minor modifications. Briefly, 1 µl of reverse transcript-
tion mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of 32P-dCTP (3000 Ci mmol⁻¹), 4 ng μl⁻¹ of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For SRA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and exposed 2 h to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA, USA).

PCR amplifications for AIB1 and ROA were performed and PCR products analysed as previously described (Leygue et al, 1996) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl, in the presence of 4 ng μl⁻¹ of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For AIB1, each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 55°C and 30 s at 72°C). For ROA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 57°C and 30 s at 72°C). PCR products were then separated on agarose gels stained with ethidium bromide as previously described (Leygue et al, 1996).

Primers for OR isoforms

ORx-U primer (5'-TGTGCAATGACTATGCTCA-3'; sense; located in ORx 792–811) and ORx-L primer (5'-GCCTTTCTCCCTGTTTTTAA-3'; antisense; located in ORx 940–922). Nucleotide positions given correspond to published sequences of the human ORx cDNA (Green et al, 1996). PCR amplifications were performed and PCR products analysed as previously described with minor modifications (Dotzlaw et al, 1997). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi of 32P-dCTP (3000 Ci mmol⁻¹), 2 ng μl⁻¹ of ORx-U/ORx-L and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C).

A previously validated triple primer assay was used to determine the relative expression of ORβ1 and its variant isoforms ORβ2 and ORβ5 (Leygue et al, 1999a). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi of 32P-dCTP (3000 Ci mmol⁻¹), 4 ng μl⁻¹ of each primer (ORβ1U, ORβ1L and ORβ2L) and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). All ORβ PCRs consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Three independent PCRs were performed.

Quantification of SRA and OR RNA expression

Exposed screens were scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA, USA) and the intensity of the signal corresponding to SRA or the appropriate OR isoform fragments was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumour measured in each set of PCR experiments (always the same tumour sample) and all signals were expressed relative to this signal. Levels of SRA were expressed relative to ROA (SRA/ROA), AIB1 (SRA/AIB1) or ORx (SRA/ORx) in each individual tumour sample. Levels of ORβ isoforms were expressed relative to other ORβ isoforms shown under statistical analysis and as previously described (Leygue et al, 1999a).

Quantification of the relative expression of the deleted SRA variant RNA

It has previously been shown that the coamplification of a full-length and a deleted variant SRA (SRA-Del) cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (Leygue et al, 1999b). For each sample, the signal corresponding to the SRA-Del was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA) and expressed as a percentage of the corresponding core SRA signal. For each case, three independent assays were performed and the mean determined.

Quantification of ROA and AIB1 RNA expression

Following analysis of PCR products on pre-stained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA, USA). At least, three independent PCRs were performed. A value of 1 was arbitrarily assigned to the ROA or AIB1 signal of one particular tumour and is the same tumour as described above and all signals were expressed relative to this signal. Levels of AIB1 were expressed relative to ROA (AIB1/ROA) and ORx (AIB1/ORx), and levels of ROA were expressed relative to ORx (ROA/ORx).

Statistical analysis

Differences between tamoxifen sensitive and tamoxifen resistant cases were tested using the Mann-Whitney rank sum test, two-tailed. Potential differences in expression between the two groups with respect to each ORβ isoform RNA relative to other ORβ isoform RNA expression (e.g. log ORβ1/control ORβ; log ORβ2/control ORβ; log ORβ3/control ORβ, as previously described (Leygue et al, 1999a)), and the relative expressions of coregulators (i.e. logAIB1/ROA; logSRA/ROA; logSRA/AIB1; logAIB1/ORx; logSRA/ORx; logROA/ORx) were determined.

Tumours were classified as low (scores between 0 and 100) and high (150–300) ORβ expressions, and differences between tamoxifen sensitive and tamoxifen resistant cases were tested using Fisher's exact test. Correlation between ORβ protein expression (HIC-score) and tumour characteristics was tested by calculation of the Spearman correlation coefficient r.

RESULTS

Expression of ORβ protein in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment

ORβ protein was determined immunohistochemically on adjacent sections from each tumour, using two different antibodies. GC-17 is an antibody recognizing an epitope in the C-terminus of full-length ORβ1 (Leav et al, 2001). Normal breast tissue was used as a positive control and is shown in Figure 1A. Examples of staining in human breast tumour sections are shown in Figure 1B–D. Some tumour sections showed no (Figure 1B, full-length ORβ score=0) or low (Figure 1C, full-length ORβ score=100), while others showed strong full-length ORβ signals (Figure 1D, full-length ORβ score=300). Tumours were classified as low (scores between 0 and 100) and high (150–300) full-length ORβ protein expressions, and differences between tamoxifen sensitive and resistant tumours determined by Fisher's exact test. No significant differences were found.

IgYERbeta503 is an antibody that recognizes ligand binding and non-ligand binding ORβ protein isoforms (Horvath et al, 2001; Saji et al, 2000) and which we refer to as total ORβ protein. Normal breast tissue was used as a positive control and is shown in Figure 2A. Examples of staining with this antibody in human breast tumour sections are shown in Figure 2B–D. Some sections showed no (Figure 2B, total ORβ score=0) or low (Figure 2C, total ORβ score=100) total ORβ expression whereas others had strong total ORβ protein signal (Figure 2D, total ORβ score=300).

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Tumours were classified as low and high total ORβ protein expressing, and there was a statistically significant difference in high total ORβ protein between the Tamoxifen sensitive and resistant groups (Fisher’s exact test, P=0.046). High total ORβ protein expressing were more frequently observed in tamoxifen sensitive tumours than resistant tumours.

Correlation between ORβ protein expression and tumour characteristics was tested by calculation of the Spearman coefficient. A positive correlation between ORβ1 (GG17) protein and progesterone receptor (PR) levels (Spearman r=0.44, P=0.022) was found when each was examined as continuous variables. When tumours were divided into PR+ (>10 fmol mg⁻¹ protein) or PR− (<10 fmol mg⁻¹ protein) groups there was a significantly higher level of ORβ1 (GG17) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0268; median for PR+ tumours=55, range 5–150 and median for PR− tumours=10, range 0–75). As well, there was also a significantly higher level of total ORβ (tgS903) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0085; median for PR+ tumours=125, range 25–270 and median for PR− tumours=50 range 0–100).

Relative expression of ORβ isoform RNA in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment. To determine if the differences described above in ORβ protein expression were correlated with differences in ORβ variant isoform RNA expression, we compared the relative expression of ORβ variant RNA to full-length ORβ RNA in the tamoxifen sensitive and resistant groups. Unfortunately, frozen tissue samples corresponding to many of the paraffin blocks from patients in the cohort used for immunohistochemistry were not available. Therefore additional cases selected were selected from the tumourbank as described in Materials and Methods. Using previously validated assays (Leygue et al., 1998; 1999a) the relative expression of ORβ2, ORβ5 and full-length ORβ1 RNA in the tamoxifen sensitive and resistant groups was not significantly different.

Relative expression of coregulators in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment. To address the hypothesis that altered relative expression of steroid receptor coactivators and corepressors could underlie altered tamoxifen sensitivity in human breast tumours, and since we previously showed that the relative expression of two coactivators (SRA and AIB1) to a corepressor (ROA) is altered in ORβ breast tumours compared their adjacent normal breast tissue, we chose these coregulators to study. They were measured by RT–PCR in the above tumour cohorts. SRA, AIB1, and ROA mRNAs were detectable in most samples, even though their level of expression differed from one sample to another. Consistent with our previous results (Leygue et al., 1999b), an additional fragment, migrating at an apparent size of 459 bp was also observed in most tumours when using SRA specific primers. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038). There were no significant differences between the tamoxifen sensitive and the de novo tamoxifen resistant breast cancers in the relative expression of any of the coactivators to corepressor RNA, or in the relative expression of SRA/AIB1 RNA, or in expression of any of these coregulator RNAs relative to ORβ or total ORβ RNA expression. As well, there was no significant difference in the relative expression of variant SRA/full-length SRA between the groups either.

Tumour characteristics. No statistically significant differences were found between the tamoxifen sensitive and tamoxifen resistant cohorts in any of the tumour characteristics described in the Materials and Methods section except for PR. PR levels were statistically significantly different (P=0.044) between the two groups.
using a Mann–Whitney rank sum test (two sided). PR levels were higher (median PR was 32 fmol mg⁻¹ protein; range 8–216 fmol mg⁻¹ protein) in the tamoxifen 'sensitive' group compared to the tamoxifen 'de novo resistant' group (median PR was 14 fmol mg⁻¹ protein; range 4–288 fmol mg⁻¹ protein). This was a consistent finding in both selected cohorts (that used for immunohistochemistry and that used for the RNA study), and provides strong support for differences in oestrogen signalling pathways in these two groups since PR is a marker of OR signal transduction (Horwitz et al, 1975; Osborne, 1998a).

**DISCUSSION**

We and others have shown that the relative expression of ORs and ORβ is significantly altered during breast tumourigenesis (Leygue et al, 1998; Roger et al, 2001), and a similar mechanism has been proposed to underlie tamoxifen resistance in breast cancers (Paech et al, 1997). The current study shows no significant differences in expression of full-length ORβ (ORβ/f1) between tamoxifen sensitive and resistant tumours. Interestingly, in this small cohort of tumours when total ORβ expression was examined, there were significantly more high total ORβ expressors in the tamoxifen 'sensitive' compared to the 'resistant' group. This suggests the possibility that increased and altered ORβ isoform protein expression have a role in de novo tamoxifen resistance, or at least together with other parameters may provide better markers of endocrine sensitivity. The increased expression of ORβ proteins in the tamoxifen sensitive group is also consistent with recently published data where patients with ORβ positive tumours (determined using an antibody to an N-terminal epitope of the ORβ protein, and defined as nuclear staining in >10% of cancer cells) had a significantly better overall survival than patients with ORβ negative tumours while receiving adjuvant tamoxifen therapy (Mann et al, 2001). Both these latter data and those presented currently in this manuscript are in contrast to data showing increased ORβ RNA expression in tamoxifen resistant tumours versus tamoxifen sensitive tumours previously published (Speirs et al, 1999). Together these studies suggest that the ORβ status and the nature of ORβ isoforms together with ORα status in human breast cancers may be important biomarkers of endocrine sensitivity, and warrants further study, in larger, prospectively gathered cohorts. The association of increased ORβ isoform expression with tamoxifen sensitivity, suggests a possible mechanistic role, and one possible mechanism may be suggested by several publications which have shown that ORβ isoforms have a modulatory effect on ORα, both in normal tissues (Welhau et al, 2000) as well as in cell culture models (Ogawa et al, 1998; Hall and McDonnell, 1999).

The potential difference between tamoxifen sensitive and resistant groups with respect to ORβ-like proteins, was not correlated with differences in the relative expression of full-length ORβ and two known variants ORβ2 and ORβ5 at the RNA level between the tamoxifen 'sensitive' versus the tamoxifen 'resistant' groups, however. This may be due to differential regulation of protein versus RNA level or the likelihood that there are other potential ORβ isoforms (known and unknown) expressed in breast tissues in addition to ORβ1, ORβ2 and ORβ5 (Lu et al, 1998; Fuqua et al, 1999), whose cognate proteins would be detected by the antibody but not measured in the triple primer RT–PCR assay.

Another mechanism for differential tamoxifen sensitivity in OR+ breast tumours could be altered coregulator expression. Although the relative expression of OR coregulators SRA, AIB1 and ROA is altered between normal breast and OR+ breast tumours, there were no significant differences in the ratios of any of the coactivators/corepressors or any of the ratios of these coregulators to ORα RNA levels between primary breast tumours from patients who were later found to be disease free (sensitive) or have disease progression (resistant) while on adjuvant tamoxifen treatment. These data suggest that altered relative expression of these coregulators is unlikely to be a marker of tamoxifen sensitivity in OR+, node negative, primary breast tumours, and unlikely to have a functional role in de novo tamoxifen resistance. Although SRA is functional as an RNA molecule, ROA and AIB1 are functional as proteins. Furthermore, other factors can affect protein activity for example phosphorylation in the case of AIB1 (Mora and Brown, 2000) or sequestration by other proteins such as prothymosin-alpha in the case of ROA (Martin et al, 2000). Our studies do not exclude differences at the protein and/or activity levels of ROA and AIB1 being involved in de novo tamoxifen resistance, nor do they exclude altered expression of these factors having a role in acquired tamoxifen resistance (Lavinsky et al, 1998). Altogether, there is little evidence for altered coregulators expression in breast tumours that are de novo tamoxifen resistant. However, our data provide preliminary evidence that the expression of ORβ proteins isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy. As well our data support distinct differences in the OR signalling pathways between these two groups of patients since the expression of a known oestrogen responsive gene PR is significantly different between the two groups, the precise mechanisms underlying these differences remain to be elucidated.

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Identification of new human coding steroid receptor RNA activator isoforms

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Abstract

SRA is a steroid receptor co-activator which acts as a functional RNA and is classified as belonging to the growing family of functional non-coding RNAs. None of the different SRA transcripts described to date encode a detectable SRA protein following in vitro and in vivo translation experiments. We have identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5' extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines.

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Keywords: SRA; Steroid receptor co-activator; Normal and tumor human breast tissue; Polymorphism; Tumorigenesis; PCR

Endogenous steroid hormones such as estrogen, progesterone, and androgen regulate the growth and the development of several organs and tissues including brain, bones, and reproductive organs such as uterus, testis, and breast. Steroid hormone actions are mainly mediated through specific receptors that belong to the steroid/thyroid/retinoic acid receptor super-family and act as ligand-dependent transcription factors [1–3]. The transcription of target genes by hormone-liganded receptors depends upon interactions between these receptors and several members of a complex co-activator population (see [2] and references herein). Among co-activators, co-activators are proteins defined as enhancing hormone induced transactivation without altering basal transcriptional activity and as reversing squelching between different receptors when overexpressed [3]. To an already long list of nuclear receptor co-activators, Lanz et al. [4] recently added SRA, a steroid receptor specific activator that differs from other co-activators in two main features. First, SRA co-activates steroid receptors as an RNA and not as a protein. These authors were unsuccessful in trying to generate in vitro or in vivo stable SRA protein but demonstrated that SRA-RNA existed in a ribonucleoprotein complex activating steroid receptor induced transcription in the absence of a translated SRA protein [4]. Second, as opposed to most positive co-regulators that interact with and co-activate both class I and class II nuclear receptors, SRA appears to be specific for steroid receptors. SRA expression is modified during breast tumorigenesis and breast tumor progression and we have suggested that this co-activator could be involved in the molecular mechanisms underlying these events [5,6]. More recently, it has been shown that antisense oligonucleotides can be used to decrease endogenous SRA-RNA [7] and that this RNA interacts with other proteins such as Sharp and RNA-binding DEAD-box p72/p68 proteins to modulate steroid
Fig. 1. Schematic representation of SRA-mRNA and gene structure. Human SRA isoforms identified to date differ slightly in their 5' and 3' terminal regions but a common nucleotide alignment sequence can be generated. The only SRA sequence entered in GenBank (AF092038) is fully contained within 10,000 bp of Bac 5 genomic sequence (AC005214). Dark grey boxes correspond to exons numbered appropriately. Numbers indicate positions relative to this latter sequence. Hyp coding SRA: hypothetical coding SRA sequence found using Gene Finder. Positions of SRAU1 and SRAL1 primers are indicated by arrows. Light grey boxes are introns.

Receptor activity [8,9]. Overall, SRA is now considered as a member of the expanding family of functional non-coding RNAs [10].

The first published sequence for SRA (GenBank AF092038) was fully contained in a genomic sequence of chromosome 5 (GenBank AC005214), within five separated exon-like regions (Fig. 1). Within this latter clone we have found, using Gene finder (http://dot.imgen. bcm.tmc.edu:9331/gene-finder/gf.html), the sequence of a hypothetical mRNA which could encode a 236 amino-acid protein that corresponds exactly to the first published SRA sequence, except for an additional 37 nucleotides in the 5' region (Fig. 1). This new 5' region now contains an AUG start codon encoding the first methionine of a novel, putative 236 amino-acid SRA protein in contrast to the previously predicted 162 amino-acids which were unstable. We have investigated the expression of this hypothetical mRNA and its corresponding protein in breast cancer cell lines.

Materials and methods

Human breast tissues and cell lines

Breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10A1, MCF10A1, MCF10AT3B, ZR-75, T47D, T5, MCF7, and HBL100) were grown, harvested, and cell pellets were stored at -70°C, as previously described [11]. Total RNA and DNA were extracted from frozen normal breast tissue sections (obtained from the Manitoba Breast Tissue Bank) and cell pellets using Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions [12]. Total proteins were extracted from frozen cell pellets as previously described [13].

Primers and RT-PCR conditions

Detection and cloning of the hypothetical coding SRA-RNAs. Primers used consisted of SRAU1 primer (5'-TCTTTGGTGCC TTGTGAC-3'; sense: positions 36,132-36,150, GenBank Accession No. AC005214) and SRAL1 primer (5'-AGTCTGGGAAAAGGGG AT-3'; antisense: positions 43,128-43,110, GenBank Accession No. AC005214). One microgram of total RNA was reverse transcribed in a final volume of 25 µl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously described [13,14]. The PCR Optimizer Kit (Invitrogen, Carlsbad, CA) was used to set up optimal RT-PCR amplification conditions. One microliter of RT mixture was amplified in a final volume of 30 µl in the presence of 60 mM Tris-HCl (pH 8.3), 150 mM [NH4]2SO4, 1.5 mM MgCl2, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, 4 ng/µl of each primer, and 1 U Taq DNA polymerase (Gibco-BRL). Each PCR consisted of a 5 min pre-incubation step at 94°C followed by 30 cycles of amplification (94°C, 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C). PCR products were sub-cloned using TOPO TA cloning kit (for sequencing and in vitro translation experiments) and pcDNA3.1/V5- His TOPO TA expression kit (for stable expression) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and sequenced as previously described [14].

Detection of SRA isoform 3. Primers used consisted of SRAU2 primer (5'-GGGCGCTCCATCCTCAGAATG-3'; sense: positions 41,684-41,685, GenBank Accession No. AC005214), SRL2 primer (5'-GCCGCTTCAGATCCCGTTG-3'; antisense: positions 41,813-41,796, GenBank Accession No. AC005214), and SRAL-GTCG primer (5'-C ACATCTCCATCAGTCG-3'; antisense: positions 41,780-41,767 which is specific for SRA isoform 3, GenBank Accession No. AC005214 plus GTCG sequence). Radioactive PCR amplifications were performed in the presence of [α-32P]dCTP and SRAU2/SRAL2 or SRAU2/SRAL-GTCG primers and PCR products were separated on poly-acrylamide gels as previously described [15]. Following electrophoresis, the gels were dried and exposed 30 min to a Molecular ImagerTM-FX Image scanning (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular ImagerTM-FX (Bio-Rad, Hercules, CA). As positive control, we amplified 10 ng of sequenced plasmids previously shown to correspond to SRAod1, SRAod2, and SRAod3.

In vitro synthesis of SRA protein

[35]Methionine-labeled SRA proteins were generated in vitro using Reticulocyte Lysate coupled transcription/translation reactions by the Tnt System (Promega, Madison, WI) according to the manufacturer's instructions using expression plasmids corresponding to SRAod1, SRAod2, or SRAod3 inserts. Lysates were then subjected to SDS-PAGE, after which gels were dried, and [35]Smethionine-labeled protein bands were visualized by exposing overnight to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA) and subsequently scanned using a Molecular Imager-FX (Bio-Rad, Hercules, CA).

Stable transfection and immunofluorescence. MCF7-SRA1 and MCF7-SRAN cells were engineered by stably transfecting MCF-7 breast cancer cells with pcDNA3.1/V5-His TOPO TA vector containing the full SRA1 coding sequence (between primers SRAU1 and SRA-L, see Fig. 2) either in frame with a C-terminal V5-His Tag (SRA1) or in an inverse orientation (SRAN), using Lipofectamine reagent according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD). To insure the retention of the transgene, transfected cells are maintained in the presence of Geneticin (Gibco-BRL, Grand Island, NY, 500 µg/ml). MCF7-SRA1 and MCF7-SRAN cells grown on 4-well slides were fixed in freshly prepared 3.7% paraformaldehyde (30 min at 37°C). Following fixation, slides were blocked in 1% PBS-PBS (overnight, 4°C) and incubated with anti-V5 (Invitrogen, Carlsbad, CA, 1:750 dilution) primary mouse antiorgen (1 h, room temperature). Slides were thoroughly washed in PBS followed by addition of the Cy3-conjugated goat anti-mouse secondary antiorgen (Jackson Immuno-Research, 1 h, 37°C, 1:10,000 dilution). Slides were counterstained with Hoechst (1 mg/ml, 30 min), washed extensively with PBS, mounted in Fluoresave mounting reagent (Calbiochem), and visualized using an E500 Nikon microscope (UV-2A or G-2A filters) with epifluorescence illumination and a DXM 1200 Nikon camera. All images were processed using Act-1 (Nikon) and Adobe Photoshop software.
Fig. 2. Alignment of SRA isoforms and primer positions. Previously (4) cloned SRA sequence (AF923283) is aligned with new SRA isoforms: SRA1 (AF293023), SRA2 (AF293025), and SRA3 (AF293026). Differences between sequences are circled. Positions of two new putative starting ATG codons are indicated (Met 1 and Met 2), together with that of the initially predicted starting codon (Met 3, [4]) and the common stop codon. The positions of SRAU1, SRAU2, SRAL-GTCG, SRL2, and SRA1 primers are also depicted.
Western blot. Protein extracts were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes as previously described [13]. An affinity-purified rabbit polyclonal antibody (anti-SRA) raised against the peptide PGNKERVDDPPQS (see Fig. 3) was obtained from ResGen (Invitrogen, Carlsbad, CA). Immunodetection of SRA protein was performed using anti-SRA antibody as a primary antibody and a goat-anti-rabbit-HRP conjugated antibody as a secondary antibody as previously described [13]. Antibody neutralization experiments were performed by pre-incubating SRA antibody with its corresponding peptide (2 h, room temperature).

Results

Detection of hypothetical coding SRA isoforms in normal breast tissue

To determine if the hypothetical protein encoded by SRA–mRNA was expressed in vivo, primers were designed corresponding to sequences upstream of the putative first AUG codon (SRAU1) and downstream of the putative stop codon (SRAU1). Total RNA was extracted from two normal breast tissue samples. Reverse transcription and PCR amplification were performed as described in Materials and methods. Using these primers, we obtained the predicted 920-bp PCR product (data not shown). Cloning and sequencing of this fragment revealed that it essentially corresponded to the hypothetical SRA coding sequence (Fig. 2). Interestingly, three different SRA-cDNAs were identified: SRA isoform 1 (GenBank AF293024), SRA isoform 2 (GenBank AF293025), and SRA isoform 3 (GenBank AF293026). SRA isoform 1 contained the full coding hypothetical SRA, whereas SRA isoform 2 contained two point mutations at positions 338 (C → T) and 348 (A → C), and SRA isoform 3 contained a point mutation followed by an insertion of three nucleotides at position 520 (G → CGAC). All these sequences contained a potential open reading frame able to encode two 236 aa and one 237 aa protein for SRA isoforms 1, 2, and 3, respectively (Fig. 3).

In vitro translation of three new SRA isoforms: SRA isoforms 1, 2, and 3

Previously cloned SRA–cDNAs, in which only the third ATG codon (encoding Met 3, Figs. 2 and 3) was present, were unable to support detectable protein synthesis in vitro [4]. To determine if the three new SRA–cDNAs isolated in our laboratory could be translated in vitro, expression vectors containing SRA isoform 1, 2, and 3 sequences downstream of a T7 polymerase promoter were used in a TnT coupled Reticulocyte Lysate system as described in Materials and methods. The three different SRA isoforms encoded stable SRA proteins were produced under these conditions (Fig. 4). Surprisingly, the 237 aa protein encoded by SRA isoform 3 was the most highly expressed, while the 236 aa protein encoded by SRA isoform 1 was the least expressed in vitro (Fig. 4).

Fig. 3. Alignment of proteins putatively encoded by SRA isoforms. SRA, SRA1, SRA2, and SRA3: proteins putatively encoded by open reading frames contained in AF092038, AF293024, AF293025, and AF293026, respectively. Positions of two putative first methionine (Met 1 and Met 2), together with initially predicted first methionine (Met 3, [4]), are indicated. Differences between sequences are circled. Fifteen amino acids highlighted by a box show recognition site for antibody generated against SRA.

Fig. 4. In vitro translation of SRA isoform 1, 2, and 3 cDNAs. In vitro transcription/translation reactions were performed using SRA isoforms 1, 2, and 3 corresponding expression plasmids and labeled/unlabeled lysates analyzed as described in Materials and methods. Size in kilodalton, corresponding to the prestained marker, broad range (Premixed format, BioLabs), are shown on the left.
prisingly SRA3 encoded protein, even though one amino acid longer is migrating slightly faster than its SRA1 and SRA2 counterparts. It should also be stressed that two bands were observed for each construction, consistent with the possible use of two different initiating methionine codons (Figs. 2 and 3, Met 1 and Met 2). The observed molecular masses (31 and 30 kDa for SRA isoforms 1–2 and 3, respectively) were slightly higher than those predicted (25.7 and 25.8 kDa for SRA isoforms 1–2 and 3, respectively).

Detection of SRA proteins in vivo by transfection of long SRA isoforms

To check whether the newly isolated long SRA isoforms could be translated in vivo, MCF-7 breast cancer cells were stably transfected with expression vectors encoding SRA1, SRA2, or SRA3 protein in frame with a C-terminal V5-His tag. The presence of SRA-tagged proteins in SRA transfected cells only was confirmed by Western blot performed using anti-V5 antibodies (data not shown). As shown Fig. 5A, SRA protein localizes in both the cytoplasm and the nucleus of SRA transfected cells whereas no signal was seen in control cells (MCF7-SRAN cells) transfected with an antisense SRA sequence (Fig. 5B).

Detection of endogenous coding SRA isoforms in breast epithelial cell lines

To determine if the long coding SRA sequences are expressed in breast cancer cells, total RNA was extracted from different breast cancer cell lines and amplified as described. The predicted PCR product corresponding to the hypothetical coding SRAs was observed in all cell lines (Fig. 6A), confirming these transcripts are expressed in tumorigenic and non-tumorigenic human breast epithelial cells. Blast searches of human EST databases (http://www.ncbi.nlm.nih.gov/blast/) revealed that the specific sequence of SRA isoform 3 (i.e., G → CGAC at position 520) had already been cloned by others (GenBank Accession Nos.: AW954396, AW957456, AW630779, AA305793, AA410852, and AA353911). This confirmed our own data resulting from independent RT-PCR, cloning, and sequencing and underlined that the SRA isoform 3 was not the result of a technical artifact. Therefore, it was of interest to investigate the expression of this isoform in breast cancer cells. PCR primers were designed to specifically amplify a fragment overlapping the putative insertion region (SRAU2, SRAL2) or to specifically anneal with the inserted sequence (SRAL-GTGC) (see Fig. 2 for primer positions). PCR products amplified using the former set of primers (SRAU2, SRAL2) were expected to migrate at an apparent size of 150 and 153 bp for SRA isoform 1–2 and 3 cDNAs, respectively. Using the latter set of primers (SRAU2, SRAL-GTGC), a PCR product 117-bp long was expected only in samples expressing SRA isoform 3 mRNA. Results obtained using SRAU2/SRAL2 and SRAU2/SRAL-GTGC are shown Figs. 6B and C, respectively. Interestingly, some cell lines expressed only the SRA isoform 3 specific fragment (MDA-MB-231, MCF-7) whereas others expressed both SRA isoforms 1–2 and 3 (T47D, T5).

DNA extracted from these cell lines was also amplified using SRAU2 and SRAL2. As shown in Fig. 6D a

Fig. 5. Detection in vivo of SRA proteins transfected in MCF-7 cell line. MCF7-SRA1 (A) and MCF7-SRAN (B) cells were engineered by stably transfecting MCF-7 breast cancer cells with C-terminal V5-tagged SRA1 sequence or SRA antisense sequence (SRAN). V5 epitope was visualized by immunofluorescence (red signal) and nuclei were counterstained using Hoechst (blue signal) as described in Materials and methods.
Fig. 6. Detection of SRA isoforms in breast epithelial cell lines. Total RNA was extracted from breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, HBL-100, ZR-75, T47D, T5, and MCF-7), reverse transcribed, and PCR-amplified using SRAU1/SRAL1 (A), SRAU2/SRAL2 (B), or SRAU2/SRAL-GTGC (C) primers and PCR products were separated on agarose (A, C) or acrylamide (B) gels, as described in Materials and methods. (D) Genomic DNA corresponding to these cell lines was amplified using SRAU1/SRAL2 and PCR products were separated on acrylamide gel as described in Materials and methods.

perfect correlation existed between SRA isoform expression and DNA sequence, suggesting the possible existence of a genetic polymorphism and therefore of at least two alleles of the SRA gene.

**Detection of endogenous SRA proteins in breast cancer cells**

A rabbit polyclonal anti-SRA antibody was generated and Western blots were performed on breast cancer cells (Fig. 7) as described in the Materials and methods. A clean signal, absent when the antibody is pre-incubated with the corresponding peptide, is observed at the expected position (~30 kDa) in different breast cancer cell lines. The specificity of the signal observed is further confirmed by the fastest apparent SRA protein migration (SRA3 protein, see Fig. 4) in MCF-7 and MDA-MB-231 cells, shown by RT-PCR to express only SRA3 isoforms.

**Discussion**

We have identified three new SRA isoforms, longer in their 5’ extremity than those previously described. Database searches revealed that although many partial SRA-like sequences isolated from various normal and tumor tissues have been entered in the human EST sequence database, only a few appear to correspond to these full-length hypothetical protein coding SRA-RNAs. This suggests an overall low expression of these long isoforms or a relative tissue specificity. Interestingly, when investigating SRA-RNA expression by Northern blot Lanz et al. reported the existence of several different sized transcripts expressed in a tissue and cell specific manner. In particular, a 1400 base long SRA-RNA, large enough to contain the minimum 920 bases of our new long SRA isoforms, was strongly expressed in muscle but not in kidney. Such data suggest that the expression of the long hypothetical coding SRA isoforms we described in this study may be tissue specific.

We observed that either one or two SRA isoforms can be expressed in breast epithelial cells. In the situation where two isoforms are expressed at the same time, both alleles are actively expressed. It should be noted that our PCR assays (using SRAU2/SRAL2 and SRAU2/SRAL-GTGC) do not establish whether SRA isoforms 1 and/or 2 are expressed. These assays establish only whether or not SRA isoform 3 is expressed, alone or together with one of the other isoforms (SRA isoform 1 or 2). We found that in breast cancer cells the pattern of SRA isoform expression was directly related to their detection within the genomic DNA. These data suggest the existence of genetic polymorphisms within the SRA gene.

All non-coding SRA isoforms identified by Lanz et al. [4] shared a core sequence starting in exon 2 and stopping in exon 5. This core sequence has been shown to be necessary and sufficient for these RNAs to act as co-activators of steroid receptors. As the longer isoforms we describe here contain this core sequence, it is expected that these latter RNAs could also modify steroid receptor activity as do their shorter previously described counterparts.

Our data are the first to report the detection of an endogeneous SRA protein and to show that naturally expressed human SRA-RNAs can generate a cDNA encoding a detectable SRA protein in vitro and in vivo.
Lanz et al. [4], who reported the identification of three different SRA isoforms, concluded that none of their cloned SRA-cDNA sequences (i.e., predicted to encode a 162 aa protein) could encode a detectable translation product. Interestingly, when engineered to form a fusion protein, e.g., with GST, Gal4 or HSV-thymidine kinase initiation sequences attached to the N-terminal region, the appropriate fusion SRA-like product was detected. Taken together, these data suggest that the 5'terminal of their cloned SRA was missing a functional initiating methionine codon or that the extra 74 N-terminal amino acids predicted in our hypothetical coding SRA-mRNAs are required for the stable expression of the resulting protein. This is reflected by the production of two different sized proteins in vitro and in vivo from each of our isoforms, presumably because of a choice in initiation start sites. Interestingly, during the preparation of this manuscript, Karashiwa et al. [16] reported the translation in vitro of a rat SRA related molecule (SRAP). When comparing SRAP protein sequence with our SRA1 protein (entered into GenBank in 2000), these authors observed a 78% conservation in amino acid sequence identity in the 146 amino-acid C-terminal region of SRA1 protein. The putative SRAP in the rat is much smaller (16 kDa) than the human SRA proteins we describe here. Karashiwa et al. were able to express in vivo a rat SRAP protein when fused with the C-terminal extremity of GST or GFP. However, these authors have still to prove the stable expression in vivo of an SRAP protein from its own naturally occurring initiation AUG codon, as well as an endogenous rat SRAP protein. Of considerable interest is the function described for this SRAP protein. Kawashima et al. reported that SRAP directly interacted with the androgen receptor (AR) and the glucocorticoid receptor (GR) to increase the transcriptional activity of these receptors. As the human SRA proteins share a strong sequence homology with the rat SRAP in their C-terminal domain, we hypothesize that human SRA protein could also interact with and modify the activity of steroid receptors, as do their RNAs. The nuclear localization of SRA protein suggests the possibility of such an interaction. Further experiments are needed to clarify the exact function of these new long SRA isoforms and their encoded proteins. However, if our latter hypothesis is confirmed, SRA might become the first molecule to be active in the same signalling pathway both at the RNA and at the protein levels.

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References