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Identification of Novel Exon-Deleted Progesterone Receptor Variant mRNAs in Human Breast Tissue

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Using an approach based on the co-amplification of wild-type and exon deleted progesterone receptor (PR) variant cDNAs, we identified exon-deleted PR variant mRNAs in both normal and neoplastic human breast tissues. Several naturally occuring variants, whose sequences revealed precise whole exon deletions, may encode putative PR-like proteins which lack some functional domains of the wild-type PR molecule. We suggest that these PR variant proteins could have a pathophysiological role in progestin action, as suggested for estrogen receptor variant proteins.

The progesterone receptor (PR), which belongs to the superfamily of ligand-activated nuclear transcription factors (1), is essential for progestin action in target tissues such as the endometrium and mammary gland. PR is an important prognostic marker in breast cancer as well as a marker of responsiveness to endocrine therapies (2). Its presence in estrogen receptor (ER) positive breast tumors generally indicates a high likehood of responsiveness to endocrine agents (3-4). In contrast, PR absence is often associated with failure to respond to these agents (5). Like all other members of the steroid/thyroid/retinoic acid receptor superfamily (6), PR is divided into structural and functional domains (A-E, Figure 1). Upon binding of ligand, PR dimerizes, undergoes phosphorylation and binds to specific sequences (PRE) located in the 5′ flanking region of PR-responsive genes (7). Further rounds of phosphorylation depending on DNA binding are also involved in trancriptional activation of the PR (8). Depending on the ligand, the isoforms involved (PR-A or PR-B), the target cell type, and the targeted gene, PR-activation will result in increased or decreased gene transcription (8-11).

Two functionally different PR isoforms, PR-A and PR-B (769 and 933 amino acids, respectively), have been previously identified in both normal and neoplastic human tissues (12). These two PR isoforms differ only in that PR-A lacks the NH2-terminal 164 amino acids of PR-B. PR-A and PR-B are translated from two distinct groups of mRNAs transcribed from the same gene under the control of two different promoters (Figure 1, 13). A third PR isoform (PR-C), that would be encoded by mRNAs lacking the translational start sites of PR-B and PR-A mRNAs but whose exact amino acid composition has not yet been established, has also been described (14). Beside the *M*r 90,000 PR-A, *M*r 120,000 PR-B and *M*r 60,000 PR-C proteins, several other PR-related proteins have been observed by Western blot in human breast tumors (15) and in T47D breast cancer cells (14). Characterization of these PR-related proteins and their possible significance in progesterone action still remain unclear. Furthermore, several PR-related mRNAs, ranging in size from 11.4 kb to 2.5 kb, as determined by Northern blot analysis, have been observed in both normal and neoplastic human tissues (16, 17). The origin of all these mRNAs remains unknown, although alternative promoter usage, alternative polyadenylation site selection and absence of

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; WT, wild-type; PCR, polymerase chain reaction.

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splicing have been suggested (17, 18). Although several of these mRNAs could encode PR-A and PR-B, other transcripts could not encode either of these isoforms (17, 18). The significance of the diversity of PR transcripts is therefore unclear. By analogy to the human ER and other members of the steroid receptor superfamily, we hypothesized that the diversity in PR-related transcripts could partly result from differential splicing. Several exon-deleted or truncated ER variant mRNAs have been observed in both normal and neoplastic tissues (19-24). The altered expression of some of these ER variant mRNAs and possibly the putative proteins encoded by these ER variant mRNAs, that lack some of the wild-type (WT) ER functional domains, has been suggested to be involved in the hormone independent phenotype of some breast tumors as well as in breast tumorigenesis (5, 24-28). It was therefore of interest to determine if similar exon-deleted PR variant mRNAs could also be observed in human breast tissues.

MATERIALS AND METHODS

Human breast tissues. Human breast tumor specimens (24 cases) and normal breast tissues obtained from reduction mammoplasty surgical specimens (10 cases), were collected at the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The twenty four primary invasive ductal breast carcinomas were associated with ER levels ranging from 0.5 to 386 fmol/mg protein, as determined by ligand binding assay . Within this group, 11 tumors were progesterone receptor positive (PR$>$15 fmol/mg protein), 12 were borderline positive ($<$15 fmol/mg protein) and 1 was PR negative (PR$=$0 fmol/mg protein), as also determined by ligand binding assay. The breast cancer cell line T-47D-5 was kindly provided by Dr. RL Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells were previously shown to contain a high level of PR mRNA (29) and have therefore been used as positive controls. Total RNA was extracted and reverse transcribed in a final volume of 15 μl as previously described (24).

Polymerase chain reaction (PCR) and identification of PCR-products. The primers used consisted of PRU2 primer (5′-CCAGCCAGAGCCCACAATACA-3′; sense; located in PR exon 2; 2395-2415) and PRL2 primer (5′-GCAGCAATAACT-TCAGACATC-3′; antisense; located in PR exon 8; 3487-3467). The nucleotide positions given correspond to published sequences of the human PR cDNA (13). PCR amplifications were performed and PCR products analyzed as previously described (24). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 10 μl, in the presence of 10 nM [α-^{32}P] dCTP, 4 ng/μl of each primer and 1 unit of Taq DNA polymerase. Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C and 1 minute at 94°C). PCR products were then separated on 4% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (24). PCR products were subcloned and sequenced as previously described (24). Predicted molecule mass and isoelectric point of the putative proteins encoded by the PR variant mRNAs identified in this study were obtained using MacVector™ 4.1.4 software (Kodak Scientific Imaging System, New Haven, CT).

RESULTS

Co-amplification of wild-type and exon-deleted PR mRNAs in breast samples. We used an approach adapted from a recently developed strategy used to study the prevalence of ER variant mRNAs within tumor samples (30). This approach is depicted in Figure 1. cDNAs corresponding to exon-deleted PR variants could be amplified together with the WT-PR mRNA using primers annealing with exon 2 (PRU2) and exon 8 (PRL2) sequences. In order to amplify variant mRNAs possibly related to both PR-A- and PR-B mRNAs, we have confined our approach to the region within exon 2 and exon 8, shared by these two mRNAs. Ten normal breast tissue samples obtained from reduction mammoplasties, and 24 breast tumor samples with a wide range of ER and PR levels, were studied. Total RNA was extracted from each sample, reverse transcribed and PCR performed in the presence of radiolabelled nucleotide. Figure 2 shows typical results obtained. Several different PCR products were observed in both normal and tumor samples. Three bands, that migrated with the apparent sizes of 1093 bp, 966 bp and 794 bp were observed reproducibly (i.e in at least two independent experiments) in most normal and neoplastic breast tissue samples, although the relative abundance of the bands seemed to differ amongst samples. These same bands were also observed in the PR positive T47D-5 human breast cancer cell line. Following subcloning

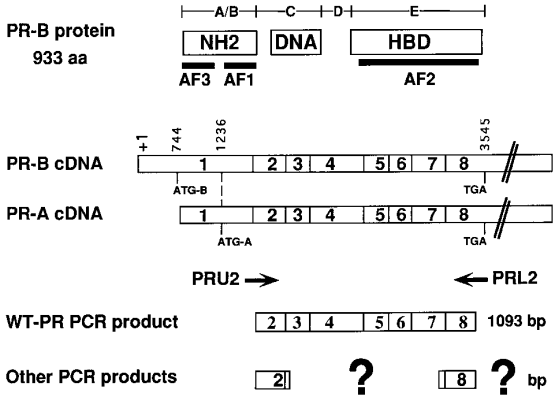

FIG. 1. Schematic representation of PR-B protein, PR-A and PR-B cDNAs, and primers allowing co-amplification of exon-deleted PR variant cDNAs. PR cDNAs contain 8 different exons coding for a protein divided into structural and functional domains (A-E). The NH2-terminal region A/B of the receptor contains two transactivation functions (AF3 and AF1). The DNA-binding domain of the receptor is located in the C region. Region D corresponds to the hinge domain of the protein and region E is involved in hormone binding and contains another transactivation domain (AF2). ATG-B and ATG-A are the translational start sites of PR-B and PR-A, respectively. PRU2 and PRL2 primers allow amplification of a 1093 bp fragment corresponding to WT-PR mRNAs. Co-amplification of all possible exon-deleted variants that contain exon 2 and exon 8 sequences can occur.

and sequencing, these bands were shown to correspond to the WT-PR mRNA, exon-6 deleted and exon-4 deleted PR variant mRNAs, respectively. Sequences of these variants showed a perfect junction between exons (31) surrounding the deletion area as shown in Figure 3. Two other bands, that migrated with the apparent size of 845 bp and 817 bp were also detected in some breast tumor samples, but at an apparent lower frequency than the bands corresponding to WT, exon 4-deleted and exon 6-deleted PR mRNAs. Sequencing of these bands identified a PR variant transcript containing a deletion of both exon 3 and 6, as well as a transcript containing a deletion of both exon 5 and 6 (data not shown). Some other PCR products, which were not reproducibly observed or whose size did not correspond to any putative exon-deleted PR variant mRNA have not yet been characterized. Differences between samples in PR wild-type and variant mRNAs signals are unlikely due to variable input of cDNA, since similar signals were obtained in all samples after amplification of the house-keeping GAPDH cDNA (data not shown). Generally, there was good agreement between ligand binding assay and the wild-type PR reverse transcription (RT) PCR product obtained. Specificity of the RT-PCR approach is demonstrated by the lack of signal in a PR negative tumor (T2), as measured by ligand binding assay. Table 1 summarizes the characteristics (size, predicted molecular mass, predicted isoelectric point) of the putative proteins encoded by the PR variants identified in this study. Because both PR-A and PR-B type variant mRNAs will be identified using our approach, both types of putative protein are presented (we have not analyzed our data with respect to the putative PR-C isoform). Intact functional domains that remain in the resulting protein are indicated for each PR-variant. It should be emphasized that to date, only RT-PCR bands corresponding to the exon 4-deleted and exon 6-deleted variant mRNAs in addition to the wild-type PR mRNA were reproducibly detected in normal breast tissue. Our data suggest that the exon 6-deleted transcript is more frequently detected in neoplastic breast tissues compared to the normal breast tissues examined in this study.

DISCUSSION

We have identified for the first time several exon-deleted PR variant mRNAs present in both normal and neoplastic breast tissues. As previously observed with exon-deleted ER

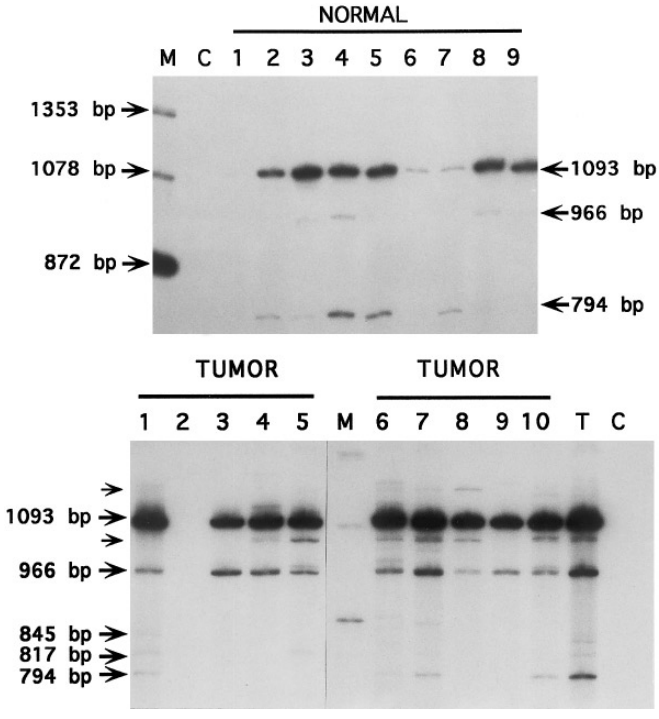

FIG. 2. Co-amplification of WT-ER and deleted variant mRNAs in human breast samples. Total RNA extracted from normal (Normal 1-9) and tumorous (Tumor 1-5 and 6-10) breast tissue samples or from T47D-5 breast cancer cells (T) was reverse transcribed and PCR amplified as described in the ''Material and Methods'' section using PRU2 and PRL2 primers. Radioactive PCR products were separated on a 4% acrylamide gel and visualized by autoradiography. Bands that migrated at 1093 bp, 966 bp, 845 bp, 817 bp and 794 bp were identified as corresponding to WT-PR mRNA and variant mRNAs deleted in exon 6, doubly deleted in exon 3-6, doubly deleted in exon 5-6 and deleted in exon 4, respectively. PCR products indicated by small arrows have not yet been characterized. M: Molecular weight marker (ϕx174 HaeIII digest, Gibco BRL, Grand Island, NY). C, no cDNA added during the PCR reaction.

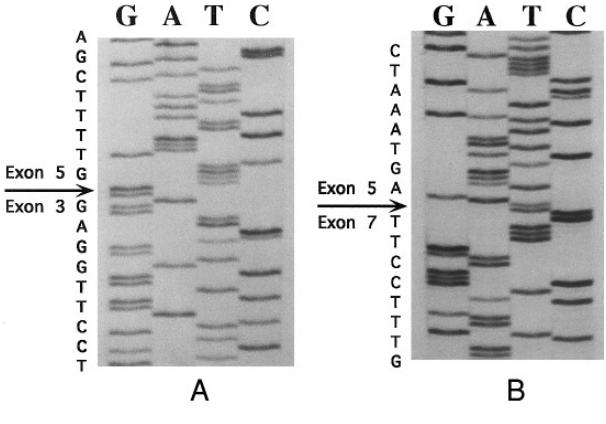

FIG. 3. PR-variant mRNAs sequences. PCR products were subcloned and sequenced as described. A: exon 4-deleted variant sequence, B: exon 6-deleted variant sequence.

TABLE 1
PR Variant mRNAs Identified within Human Breast Tissues and Putative Encoded Proteins

	PR-B mRNA	PR-A mRNA	Functional domains	Samples
Wild-type	933 aa	769 aa		
	Mr 99,035	*Mr* 82,350	A, B, C, D, E	N, T
	pI 6.07	pI 8,50		
Exon 4-deleted variant	831 aa	667 aa	A, B, E	
	Mr 87,788	*Mr* 71,103		N, T
	pI 5.62	pI 6.97	in frame	
Exon 6-deleted variant	797 aa	633 aa	A, B, C, D	
	Mr 83,017	*Mr* 66,332		N, T
	pI 5.55	pI 7.42	truncated, 12 new aa	
Exon 5-6-deleted variant	841 aa	677 aa	A, B, C, D	
	Mr 88,068	*Mr* 71,383		T
	pI 6.06	pI 8.71	in frame	
Exon 3-6-deleted variant	758 aa	594 aa	A, B, D	
	Mr 78,671	*Mr* 61,985		T
	pI 5.03	pI 5.80	truncated, 12 new aa	

Note. For each PR-A or PR-B variant mRNA, the size (in amino acids, aa) of the putative encoded protein, its predicted molecular weight (*Mr*, given in daltons) and its predicted isoelectric point (pI) are given. Functional domains that remain intact are indicated (A-E) as well as the amino acid composition change. Detection in normal (N) or in tumor (T) breast samples is also specified.

variant mRNAs, sequencing these variant mRNAs revealed a perfect junction between exons surrounding the deletion area. This suggests that these naturally occuring variants are generated by alternative splicing of WT-PR primary transcripts. Whether or not all groups of mRNAs (PR-A, PR-B, PR-C) are alternatively spliced remains to be determined. However, any of these variant species, if translated, would encode PR-like proteins which lack some functional domains of the WT-PR proteins. The resulting shorter proteins could contribute to the population of PR-related proteins observed in human breast tumor samples by Graham et al. (15), as suggested by these authors. For example, the putative protein encoded by the exon 4-deleted PR-A variant mRNA is expected to migrate at a apparent molecular mass of 71,103 daltons and could correspond to the PR-related protein (78,000 daltons) observed in 25.7% of the tumors analyzed by Graham et al. (15).

Several different sized PR mRNAs species have previously been described in human and chicken target tissues. Generation of these transcripts is thought to involve several mechanisms: different promoter usage, alternative polyadenylation site selection and a splicing variant have been identified (17, 18). The splicing variant, identified in chicken oviduct (18), seems to consist of a failure to splice the second intron followed by polyadenylation site selection within this intron. The resulting transcript therefore consists of exons 1 and 2 with some intron 2 sequences followed by a polyadenylation signal and a poly-A tail. However, no previous studies have identified complete exon-deletions in PR transcripts. Such deletions would have escaped detection by previous studies using Northern blot and differential hybridization analysis.

By analogy with ER variant mRNAs (20, 21, 32) it is reasonable to hypothesize that the putative encoded PR variant proteins, with structural and functional alterations, may modify WT-PR functions. The presence of PR variant mRNAs in normal tissue therefore suggests that the PR signalling pathway involves more protagonists than PR-A, PR-B or PR-C. Moreover, we showed that the detection of a particular variant using this kind of approach depended on the initial representation of this mRNA within the related-mRNAs population (30). Our results suggest that the differences in PR-variant mRNAs detection between samples may therefore

reflect different relative proportions of these variants within samples. Whether or not differential PR variant mRNA expression is associated with a pathophysiological role in progestin action is under investigation.

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